

## Regulation of a Human Cardiac Actin Gene Introduced into Rat L6 Myoblasts Suggests a Defect in Their Myogenic Program

ROBERT HICKEY,<sup>1†</sup> ARTHUR SKOULTCHI,<sup>1\*</sup> PETER GUNNING,<sup>2</sup> AND LARRY KEDES<sup>2</sup>

Department of Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461,<sup>1</sup> and The MEDIGEN Project, Department of Medicine, Stanford Medical School and Veterans Administration Medical Center, Palo Alto, California 94304<sup>2</sup>

Received 24 February 1986/Accepted 16 May 1986

**The rat myogenic cell line L6E9 induces skeletal but not cardiac  $\alpha$ -actin mRNA upon fusion to form myotubes. However, when a human cardiac  $\alpha$ -actin gene was introduced into L6E9 myoblasts, differentiation of the cells led to the accumulation of human gene transcripts in parallel with those derived from the endogenous skeletal  $\alpha$ -actin gene. This result demonstrates that factors which direct rat myogenesis can regulate a muscle gene from another species and that the L6E9 cells may have a defect in their ability to activate endogenous cardiac actin gene expression.**

The fusion of myoblasts to form myotubes is accompanied by a rapid induction of the contractile proteins which constitute the skeletal muscle sarcomere (3). At the same time, a number of genes encoding the proteins of the myoblast cytoskeleton are substantially down-regulated (3). This switching of cellular structural components is best characterized in terms of the actins. During early myotube formation, the synthesis of the nonmuscle  $\beta$ - and  $\gamma$ -actins is repressed, while the synthesis of muscle  $\alpha$ -actin is induced (3, 20, 22).

Recent work has demonstrated that the induction of muscle  $\alpha$ -actin is more complex than originally thought. During skeletal muscle differentiation in chickens (16), rats (8), mice (1, 10), and humans (11), both skeletal and cardiac  $\alpha$ -actins are induced. Furthermore, cardiac actin has been shown to be the predominant  $\alpha$ -actin transcript during early skeletal muscle development in chicken primary cultures (16), in the mouse myogenic C2 cell line (1), and in human myoblasts (11). With a lower vertebrate, *Xenopus levis*, Mohun et al. (12) demonstrated that the  $\alpha$ -cardiac and  $\alpha$ -skeletal actin mRNAs are expressed at the earliest stages of muscle cell differentiation and throughout embryogenesis. Thus, coexpression of  $\alpha$ -skeletal and  $\alpha$ -cardiac actin genes, a feature in all these systems, is likely to be common to all vertebrate myogenic programs.

Two sets of preliminary data are in seeming conflict in interpretations of sarcomeric actin gene expression in one particular line of myogenic rat cells. Buckingham and co-workers (2) presented preliminary data that, in contrast to the usual myogenic programs, the rat myogenic cell line L6 induces skeletal, but not cardiac,  $\alpha$ -actin upon fusion to form myotubes. On the other hand, our preliminary data (6) suggest that the human cardiac actin gene is capable of being expressed after it is introduced into the L6E9 subclone of rat L6 myoblasts. One explanation for these conflicting results (2) is that the L6 myoblast cell line might represent cells from an early stage of muscle development, a hypothetical stage in which  $\alpha$ -skeletal mRNA, but not  $\alpha$ -cardiac mRNA, is expressed. According to this interpretation, the chicken, mouse, and human culture systems described above would

represent cells active later in development, at a stage when  $\alpha$ -cardiac mRNA is also expressed. Alternatively, the failure of L6 cells to express  $\alpha$ -cardiac actin mRNA might be related to a defect in the myogenic program of this transformed cell line. Since L6 cells are the most widely used cell line in studies on the cellular and molecular biology of myogenesis, we attempted to discriminate between these explanations.

We investigated skeletal and cardiac  $\alpha$ -actin expression in the L6E9 subclone (14) of rat L6 myoblasts (26). RNA was isolated (5) from L6E9 proliferating myoblasts and from myotubes at 6, 8, and 10 days after fusion was induced by plating single-cell suspensions of  $5 \times 10^5$  cells per 100-mm dish into Dulbecco modified Eagle medium containing 2% fetal calf serum and 2.5% horse serum. Size-fractionated RNA was hybridized with probes specific for either the 3' untranslated region of the skeletal or cardiac  $\alpha$ -actin mRNA as previously described (4). Cardiac actin mRNA was not present in either myoblasts or myotubes (Fig. 1A). The RNA blot (Fig. 1A) was then hybridized to the skeletal actin-specific probe. A low level of skeletal  $\alpha$ -actin mRNA was present in myoblast cultures, and after fusion, the level of the mRNA dramatically increased (Fig. 1B). By comparison with known RNA standards, we concluded that cardiac actin mRNA was present at much less than 1% of the level of skeletal  $\alpha$ -actin mRNA in myotubes (18). Since the blots (Fig. 1A and B) were from the same sample, the absence of a hybridization signal in the blot shown in Fig. 1A could not have been due to a technical problem with either the RNA extraction or blotting procedures. Furthermore, the specificity and sensitivity of the radiolabeled  $\alpha$ -cardiac actin DNA probe used in the experiment (Fig. 1A) was demonstrated by the ability of this probe to detect  $\alpha$ -cardiac actin transcripts and their gene (Fig. 1C and Fig. 2).

One simple explanation of the failure of cardiac actin to be expressed is that the gene is deleted or rearranged in this cell line. To evaluate this possibility, we digested total rat thymus and L6E9 genomic DNAs with the restriction endonuclease *EcoRI* and, after agarose gel electrophoresis and blotting onto a nitrocellulose filter, hybridized the blot to the cardiac actin-specific 3' untranslated region probe. The patterns of hybridization of both DNA samples were identical (Fig. 1C). We concluded, therefore, that the cardiac actin gene is present in the L6E9 cell line and that its failure to be

\* Corresponding author.

† Present address: Worcester Foundation for Biology, Cell Biology Group, Shrewsbury, MA 01545.

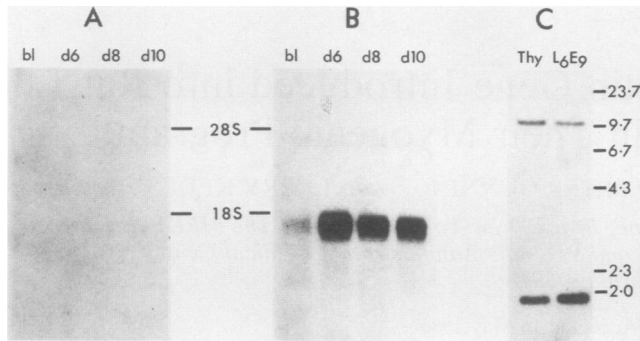


FIG. 1. (A) Rat cardiac  $\alpha$ -actin is not expressed during L6E9 differentiation. Total RNA was isolated from L6E9 myoblasts (bl) and myotubes at 6 (d6), 8 (d8), and 10 (d10) days postfusion (1, 18). Samples (10  $\mu$ g) of each RNA were electrophoresed on a gel (2.2 M formaldehyde, 1% agarose) and transferred to a nitrocellulose filter (4). The filter was hybridized to the cardiac  $\alpha$ -actin-specific probe pHMcA-3'UT-DB (4), washed, and autoradiographed for 3 weeks. The migration of 28S and 18S rRNA is indicated. (B) Induction of skeletal  $\alpha$ -actin mRNA during L6E9 differentiation. The blot in 1A was rehybridized with the skeletal  $\alpha$ -actin-specific probe pHMcA-3'UT-Fnu (4), washed, and autoradiographed for 1 day. (C) Detection of cardiac actin gene in L6E9 cells. Total DNA was isolated from rat thymus and L6E9 cells, digested with *Eco*RI, size fractionated on a 0.6% agarose gel, and transferred to a nitrocellulose filter (18). The filter was hybridized to the cardiac  $\alpha$ -actin-specific probe pHMcA-3'UT-DB (4), washed, and autoradiographed. The migration positions of coelectrophoresed size standards are shown in kilobases.

expressed is not due to gene deletion or gross rearrangement.

One approach to defining why the L6E9 cells did not activate transcription of the cardiac actin gene upon myoblast fusion is to introduce a known functional cardiac actin gene into these cells and evaluate the accumulation of the specific transcripts during fusion. We previously showed that a cloned copy of the human cardiac actin gene can be expressed in mouse L cells and produces intact cardiac  $\alpha$ -actin mRNA and protein (5). Accordingly, in a typical experiment we cotransfected  $2 \times 10^7$  rat L6E9 cells with either 12.5 or 25  $\mu$ g of pHRL83-R1 (5) containing the human

cardiac  $\alpha$ -actin gene and either 1 or 0.5  $\mu$ g, respectively, of pSV-*gpt* (13) by the calcium phosphate cotransfection procedure (25). Clones resistant to 25  $\mu$ g of mycophenolic acid per ml, 250  $\mu$ g of xanthine per ml, and HAT (13) in Dulbecco modified Eagle medium containing 20% fetal calf serum were picked about 2 weeks after transfection and expanded to obtain DNA. Clones containing intact copies of the cotransfected human cardiac  $\alpha$ -actin gene were identified by Southern blotting (data not shown). Total RNA was prepared from dividing myoblasts and myotubes fused in Dulbecco modified Eagle medium containing 3% horse serum plus selection agents at various times during the fusion process. The RNA was size fractionated and hybridized to a subclone of the human cardiac actin 3' untranslated region which hybridizes only to the human mRNA (4).

Two types of human cardiac actin transcripts were detected. In clone NB, a 6- and a 1.6-kilobase (kb) transcript were seen in both the myoblasts and myotubes (Fig. 2B) and the amounts of the two transcripts were very similar before and after differentiation. The same transcript pattern was seen previously in mouse L cells transfected with this gene (5). The 6-kb transcript proved to be an unprocessed nuclear transcript, whereas the 1.6-kb mRNA corresponded to the mature cardiac  $\alpha$ -actin mRNA (5).

In contrast, in clones JA and HA, the mRNA was present at significantly higher levels in the myotubes than in the myoblasts and at levels about twofold higher in the myotubes of clone NA (Fig. 2A, C, and D). Clones JA and HA accumulated only the 1.6-kb mRNA, whereas clone NA also accumulated the 6-kb precursor. This result demonstrated that, in three of four clones tested, the rat L6E9 cells were capable of increasing the accumulation of human cardiac  $\alpha$ -actin transcripts during fusion to form myotubes.

To demonstrate the specificity of the induction of the human cardiac actin gene, we measured the level of *gpt* transcripts in each of these RNAs. Serial dilutions of the RNAs were applied to nitrocellulose paper and hybridized to a radiolabeled *gpt* probe. The expression of *gpt* RNA in the HA clone actually decreased by about 50% upon fusion of the myoblasts into myotubes (Fig. 3A). In the JA clone, the myotubes and myoblasts expressed identical levels on days 8 and 10 (Fig. 3B). The *gpt* transcripts were also identical in the RNA samples of clone NA (data not shown). Thus, we

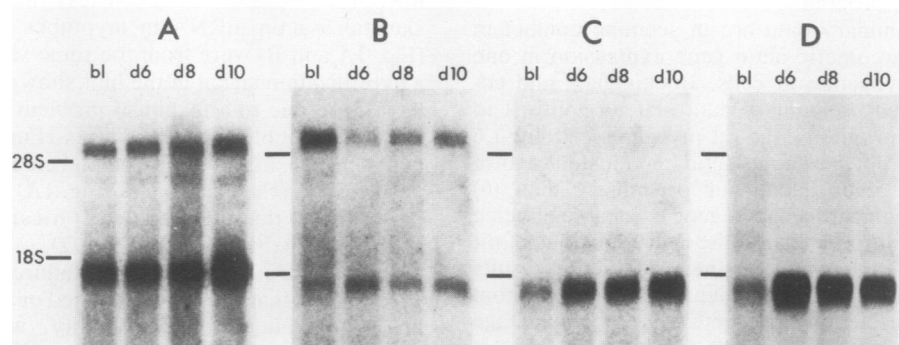


FIG. 2. Expression of the human cardiac actin gene in rat L6E9 cells. Total RNA was isolated from myoblasts (bl) and at 6 (d6), 8 (d8), and 10 (d10) days postfusion from myotubes generated from each of four L6E9-transfected clones containing the human cardiac gene. Samples (10  $\mu$ g) of each RNA were electrophoresed on a gel (2.2 M formaldehyde, 1% agarose) and transferred to a nitrocellulose filter (4). The filter was hybridized to the cardiac  $\alpha$ -actin-specific probe pHMcA-3'UT-DR (4) and washed with  $0.1 \times$  SSC ( $1 \times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate at 65°C. These washing conditions prevent hybridization of the probe to the rodent cardiac actin mRNA (4). The sizes of the cardiac actin transcripts were determined from the mobilities of the 28S and 18S rRNAs (18). Cell lines: A, NA; B, NB; C, JA; D, HA.

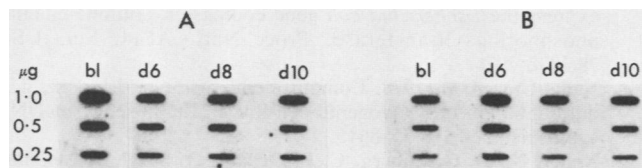


FIG. 3. Expression of the *gpt* gene in rat L6E9 cells. Serial dilutions of total RNA isolated from myoblasts (bl) and myotubes at 6 (d6), 8 (d8), and 10 (d10) days postfusion of the clones. HA (A) and JA (B) were applied to a nitrocellulose filter by using a slot-blot manifold (Schleicher & Schuell, Inc.). The filter was hybridized to the *Bam*HI-*Hind*III fragment of pSV2-*gpt* (13) containing the coding region of the *gpt* gene. After being washed in  $0.5 \times$  SSC-0.1% sodium dodecyl sulfate at 65°C, the filters were autoradiographed. The quantity of RNA in each slot is indicated in micrograms.

could conclude that the induction of cardiac  $\alpha$ -actin transcripts in these three cell clones was specific for the transfected cardiac actin gene.

The induction of human cardiac  $\alpha$ -actin mRNA in three of the four expressing clones of the transfected rat cells clearly demonstrates that a transferred muscle-specific gene can be regulated appropriately in a myogenic system of another species. Melloul et al. (9) showed that the rat skeletal  $\alpha$ -actin gene reintroduced into cells from the same species, the rat L8 myogenic cell line, is correctly regulated. However, Seiler-Tuyns et al. (21) found that the mouse C2 myogenic cell line does not regulate either of two heterospecific genes, the chicken skeletal or cardiac actin genes. This latter result contrasts with the heterospecific regulation of the human cardiac actin gene in the L6E9 cell line shown here. Recently Nudel et al. (15) demonstrated that a chicken skeletal actin gene is appropriately regulated in the rat L<sub>8</sub> myogenic cell line. There are a number of potential interpretations of these results, including selective cross-species incompatibility and intrinsic differences among myogenic cell lines. To resolve this, it may be necessary to perform parallel experiments using both chicken and human cardiac actin genes.

The data presented here demonstrate that the rat L6E9 myogenic line is capable of regulating an exogenous cardiac  $\alpha$ -actin gene. A legitimate question is whether the up-regulation of the cardiac actin gene is appropriate or normal for all myogenic cells or whether L6E9 cells, mutagenized during primary rat muscle culture, represent a heretofore undiscovered subpopulation of myogenic cells that induce skeletal but not cardiac actin. We believe that the latter possibility (2) is less likely and that L6E9 cells are aberrant and not representative of the myogenic programs of the frog (12), chicken (16), mouse (1, 10), rat (8), and human (11), which involve the early induction of both skeletal and cardiac actin. Thus, it appears that the widely used L6 cell line may have a defect in its myogenic program. Furthermore, the defect resulting in failure to induce cardiac actin mRNA is not due to deletion or gross rearrangement of the rat cardiac actin gene.

What is the basis of this defect in L6 cardiac actin gene expression? The answer depends on the mechanism normally used by muscle cells to induce expression of the cardiac actin gene. Studies in chickens (16), mice (1, 10), rats (8), and humans (11, 18) demonstrated that, although cardiac actin expression is restricted to sarcomeric muscles, the level of accumulation of cardiac actin mRNA differs over 2 orders of magnitude among various muscles and during development. This variable degree of expression suggests

that myogenesis involves both an activation of the gene into a transcription-permissive state and a second phase involving modulation of transcript accumulation (11). These considerations suggest two major possibilities for the basis of the L6E9 cardiac actin gene expression defect.

First, the L6E9 cardiac actin gene may carry a mutation which either precludes its activation into a transcription-permissive state or prevents transcription of the activated locus. This is certainly testable and would involve isolating the L6E9 cardiac actin gene and reintroducing it into these cells. Weintraub (24) showed that cloned genes transfected into L cells assume an altered chromatin configuration, i.e., a cloned chicken  $\beta$ -globin gene transfected into these cells exhibits a 5' hypersensitive DNase site, whereas an inactive, endogenous globin gene does not exhibit DNase sensitivity. Therefore, it can be assumed that a transfected L6E9 cardiac  $\alpha$ -actin gene would also be in a transcription-active state and perhaps capable of being properly regulated during myoblast differentiation in much the same way that we observed for the transfected human cardiac  $\alpha$ -actin gene. That this is reasonable is further suggested by the enhanced expression, in response to glucocorticoid stimulation, of a human growth hormone gene (19) and a rat  $\alpha$ -2 macroglobulin gene (7) transfected into mouse L cells, while their endogenous counterparts remain unresponsive to glucocorticoid stimulation.

Second, the L6E9 cells may fail to make a component necessary for the activation of the rat cardiac  $\alpha$ -actin gene. Since these cells activate other myogenic genes, such as myosin heavy-chain and skeletal actin, the putative absent component would be relatively gene specific. By randomly integrating the human cardiac actin gene into L6E9 cells, however, we may have bypassed the need for activation of the cardiac gene by such a gene-specific factor, since the transfected gene is already in a transcription-permissive state (Fig. 2A to D, lane 1). It is interesting that the L6E9 myotubes do not synthesize several other structural components, myosin light chains 1<sub>F</sub> and 3<sub>F</sub> (2) and one of two myosin heavy-chain components (17), which are induced (2, 23) upon fusion in other myogenic cell lines. Furthermore, some L6 lines are peculiar from several additional standpoints, pointed out to us by Mark Pearson (personal communication). First, some L6 derivatives make only the M isozyme of creatine kinase, although the original L6 line made both the M and B forms (26). Second, electron microscopy of these cells reveals no myofilaments with long-range periodicity, but only short, truncated filaments. Third, this morphological abnormality may also be responsible for the failure, in general, of L6 myotubes to contract spontaneously. Taken together, these observations raise the possibility that there may actually be a defect in a common factor which regulates a subset of the myogenic program of this widely used L6 cell subclone. Further analysis of the expression in L6E9 cells of a number of other muscle-specific genes will provide further insight into the molecular basis of this defect.

The capacity of the human cardiac  $\alpha$ -actin gene to respond in a physiological way by up-regulation after myogenic fusion of rat cells supports the notion that the gene is responding to heterospecific factors. Substantial regions of conserved 5' flanking and 3' untranslated region sequences are shared by human and rat  $\alpha$ -cardiac actin genes (4, 11). Such sequences are likely locales for *cis*-acting regulatory regions that interact with the myogenic factors. Current studies are focused on identifying these structural features (11).



L6E9 cells were a gift from B. Nadal-Ginard.

This work was supported by Public Health Service grants CA16368 to A.S. and HD17031 to L.K. from the National Institutes of Health and by a grant from the Veterans Administration and the Muscular Dystrophy Association to L.K.

#### LITERATURE CITED

- Bains, W., P. Ponte, H. Blau, and L. Kedes. 1984. Cardiac actin is the major actin gene product in skeletal muscle cell differentiation in vitro. *Mol. Cell. Biol.* **4**:1449-1453.
- Buckingham, M., S. Alonso, P. Barton, G. Bugaisky, A. Cohen, P. Daubas, I. Garner, A. Minty, B. Robert, and A. Wevdert. 1984. *Molecular biology of development*, p. 275-292. Alan R. Liss, New York.
- Buckingham, M. E., and A. J. Minty. 1983. Contractile protein genes, p. 365-395. *In* N. Maclean, S. P. Gregory, and R. A. Flavell (ed.), *Eucaryotic genes: their structure, activity and regulation*. Butterworths, London.
- Gunning, P., T. Mohun, S.-Y. Ng, P. Ponte, and L. Kedes. 1984. Evolution of the human sarcomeric actin genes: evidence for unit of selection within the 3' untranslated regions of the mRNAs. *J. Mol. Evol.* **20**:202-214.
- Gunning, P., P. Ponte, L. Kedes, R. Hickey, and A. Skoultchi. 1984. Expression of human cardiac actin in mouse L cells: a sarcomeric actin can be associated with a nonmuscle cytoskeleton. *Cell* **36**:709-715.
- Kedes, L. H. 1985. Human actin genes, p. 309-332. *In* R. C. Strohman and S. Wolf (ed.), *Gene expression in muscle*. Publishing Corp., New York.
- Kurtz, D. T. 1981. Hormone inducibility of rat  $\alpha_{2\mu}$ -globulin genes in transfected mouse cells. *Nature (London)* **291**:629-631.
- Mayer, Y., H. Czosnek, P. E. Zelson, D. Yaffe, and U. Nudel. 1984. Expression of the genes coding for the skeletal muscle and cardiac actin in the heart. *Nucleic Acids Res.* **12**:1087-1100.
- Melloul, D., D. B. Aloni, J. Calvo, D. Yaffe, and U. Nudel. 1984. Developmentally regulated expression of a chimeric gene containing muscle actin DNA sequences in transfected myogenic cells. *EMBO J.* **3**:983-990.
- Minty, A. J., S. Alonso, M. Caravatti, and M. E. Buckingham. 1982. A fetal skeletal muscle actin mRNA in the mouse and its identity with cardiac actin mRNA. *Cell* **30**:185-192.
- Minty, A. J., H. Blau, and L. Kedes. 1986. Two-level regulation of cardiac actin gene transcription: muscle-specific modulating factors can accumulate before gene activation. *Mol. Cell. Biol.* **6**:2137-2148.
- Mohun, T. J., S. Brennan, N. Dathan, S. Fairman, and J. B. Gurdon. 1984. Cell type specific activation of actin genes in the early amphibian embryo. *Nature (London)* **311**:716-721.
- Mulligan, R. C., and P. Berg. 1981. Selection of animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase. *Proc. Natl. Acad. Sci. USA* **78**:2072-2076.
- Nadal-Ginard, B. 1978. Commitment, fusion and biochemical differentiation of a myogenic cell line in the absence of DNA synthesis. *Cell* **15**:855-864.
- Nudel, U., D. Greenberg, C. P. Ordahl, O. Saxel, S. Neuman, and D. Yaffe. 1985. Developmentally regulated expression of a chicken muscle-specific gene in stably transfected rat myogenic cells. *Proc. Natl. Acad. Sci. USA* **82**:3106-3109.
- Paterson, B. M., and J. D. Eldridge. 1984. Alpha cardiac actin is the major sarcomeric isoform expressed in embryonic avian skeletal muscle. *Science* **224**:1436-1438.
- Periasamy, M., R. M. Wydro, M. Strehler-Page, E. E. Strehler, and B. Nadal-Ginard. 1985. Characterization of cDNA and genomic sequences corresponding to an embryonic myosin heavy chain. *J. Biol. Chem.* **260**:15856-15862.
- Ponte, P., P. Gunning, H. Blau, and L. Kedes. 1983. Human actin genes are single copy for  $\alpha$ -skeletal and  $\alpha$ -cardiac actin but multicopy for  $\beta$ - and  $\gamma$ -cytoskeletal genes: 3' untranslated regions are isotype specific but are conserved in evolution. *Mol. Cell. Biol.* **3**:1783-1791.
- Robins, D. M., I. Paek, P. H. Seeburg, and R. Axel. 1982. Regulated expression of human growth hormone genes in mouse cells. *Cell* **29**:623-631.
- Schwartz, R. J., and K. N. Rothblum. 1981. Gene switching in myogenesis. Differential expression of the chicken actin multigene family. *Biochemistry* **20**:4122-4129.
- Seiler-Tuyns, A., J. D. Eldridge, and B. M. Paterson. 1984. Expression and regulation of chicken actin genes introduced into mouse myogenic and nonmyogenic cells. *Proc. Natl. Acad. Sci. USA* **81**:2980-2984.
- Shani, M., D. Zevin-Sonkin, O. Saxol, Y. Carmon, D. Katcoff, U. Nudel, and D. Yaffe. 1981. The correlation between the synthesis of skeletal muscle actin myosin heavy chain, and myosin light chain and the accumulation of corresponding mRNA sequences during myogenesis. *Dev. Biol.* **86**:483-492.
- Umeda, P. K., C. J. Kavinsky, A. M. Sinha, H. Hsu, S. Jakovcic, and M. Rabinowitz. 1983. Cloned mRNA sequences for two types of embryonic myosin heavy chains from chick skeletal muscle. *J. Biol. Chem.* **258**:5206-5214.
- Weintraub, H. 1983. A dominant role for DNA secondary structure in forming hypersensitive structures in chromatin. *Cell* **32**:1191-1203.
- Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin. 1979. DNA mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. *Proc. Natl. Acad. Sci. USA* **76**:1373-1376.
- Yaffe, D. 1968. Retention of differentiation potentialities during prolonged cultivation of myogenic cells. *Proc. Natl. Acad. Sci. USA* **61**:477-483.