Finally, the genetic map will provide information important to the task of spanning large regions of the chromosome by physical methods now being developed (*14*, *15*). The defined loci will provide essential reference points for studies that seek to "walk" along the X chromosome.

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Plasticity of the Differentiated State

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Choy-Pik Chiu, Laura Silberstein, Steven G. Webster
Steven C. Miller, Cecelia Webster

Tissue-specific phenotypes result from a sequence of developmental stages. Totipotent cells in the early embryo give rise to stem cells specific to three distinct layers—endoderm, ectoderm, and mesoderm. Although the lineage, or progression from stem cell to tissue-specific phenotype is not always fixed (*1*), once a cell is determined, it is generally destined for specialization along a specific pathway, such as erythropoiesis or myogenesis. The option to generate other phenotypes no longer exists for the determined vertebrate cell, and its progeny stably inherit its limited potential. The determined cell will give rise to other phenotypes only under unusual experimental conditions, such as at sites of regeneration in amphibian limbs where transdifferentiation has occurred or after treatment with a drug such as 5-azacytidine; even then, only derivatives of the same embryonic lineage are obtained (*2*). At some point in development, the determined cell expresses its phenotype, and the genes necessary for its role in the function of a particular tissue are transcribed.

To obtain tissue-specific phenotypes, a sequence of regulatory mechanisms must exist that determine when in a cell's history specific genes are transcribed. The genetic composition of eukaryotic cells is generally stable and heritable. Chromosomes are not lost in the course of cell specialization. This is evident since entire frogs can be generated from the transplantation of nuclei of specialized intestine cells into enucleated oocytes and since a diversity of normal tissue-specific cell types can be generated from malignant tumor cells introduced into early mouse embryos (*3*). The current model for the differential expression of genes characteristic of tissues at different points in development requires regulation by DNA sequences on the same chromosome (*cis*-acting) and on different chromosomes (*trans*-acting). *Cis*-acting DNA sequences that impart tissue-specific regulation have been identified from the study of the expression of cloned genes after transfection into cultured cells (*4*). The diffusible products of *trans*-acting genes are assumed to be negative or positive regulators of the *cis*-acting gene sequences. Although some general mediators of gene transcription and gene-specific binding proteins have been characterized in eukaryotes (*5*), with the exception of the factors that bind the *Drosophila* alcohol dehydrogenase gene (*6*), no tissue-specific *trans*-acting regulators have yet been isolated. An understanding of how the expression of tissue-specific genes is activated is not only of fundamental biological interest but also of practical importance in implementing genetic engineering and possibly gene therapy.

Muscle provides a model system for studies of the mechanisms controlling the appearance of tissue-specific functions. For a number of species, developmentally distinct stages are readily recognized by their morphological and biochemical properties, and conversion from one stage to another can be mimicked under the controlled conditions of tissue culture (*7*) (Fig. 1). First, a mesodermal stem cell gives rise to a myoblast, destined for myogenesis. The determined myoblast is capable of recognizing and spontaneously fusing with other myoblasts leading to the production of a differentiated myotube. The multinucleated myotube no longer divides or synthesizes DNA but produces muscle proteins in large quantity. These include constituents of the contractile apparatus and specialized cell-surface components essential to neuromuscular transmission. Eventually the differentiated muscle cell exhibits characteristic striations and rhythmic contractions. A further step in this pathway is maturation: the contractile apparatus in muscle at different stages of development contains distinct isoforms of muscle proteins such as myosin and actin, encoded by different members of multigene families (*8*, *9*).

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Heterokaryons: A Model System for Studying Cell Specialization

To study the mechanisms regulating cell specialization, we developed a system in which nonmuscle cells can be induced to express muscle genes predictably and stably. We combine entire muscle cells with cells of other phenotypes and from other species through the use of polyethylene glycol. In these fused cells, or heterokaryons, expression of previously silent muscle genes is activated in response to tissue-specific factors present in muscle (*10–12*). The nuclear composition of the heterokaryons and the activation of genes can be determined by taking advantage of species-specific differences: the nonmuscle cells used are always human and the muscle cells are always mouse. Furthermore, the fusion product is stable; in contrast with typical interspecific hybrids (synkaryons), cell division does not occur and the parental cell nuclei remain intact and retain a full complement of chromosomes. Finally, changes in gene expression can be assayed immediately after fusion and monitored for relatively long periods thereafter.

We have shown that muscle genes are activated in nonmuscle nuclei upon fusion with muscle. This activation must be mediated by diffusible, *trans*-acting molecules that are transported to nuclei through the cytoplasm, since the nuclei of the two cell types remain separate and distinct. These results show that gene expression by nuclei of highly specialized cells is remarkably plastic. Thus, the muscle genes in cells with very different roles (skin, cartilage, lung, and liver) are receptive to activation by muscle regulatory factors. The differences in the requirements for gene activation in these cell types provide insight into the molecular mechanisms that lead to the generation and maintenance of phenotypes.

Gene Activation in Heterokaryons

The mouse muscle cell line used to produce heterokaryons is a diploid subclone, C_2C_{12} (*13*). To test the influence of histogenetic state and of in vivo aging on muscle gene expression, heterokaryons were produced with mouse muscle and eight different human nonmuscle cell types, including keratinocytes (ectoderm), chondrocytes and fibroblasts (mesoderm), and hepatocytes (endoderm) (*14*). In addition, the four strains of fibroblasts used were either from different tissues (amniotic fluid, skin, and lung) or from different developmental

Summary. Heterokaryons provide a model system in which to examine how tissue-specific phenotypes arise and are maintained. When muscle cells are fused with nonmuscle cells, muscle gene expression is activated in the nonmuscle cell type. Gene expression was studied either at a single cell level with monoclonal antibodies or in mass cultures at a biochemical and molecular level. In all of the nonmuscle cell types tested, including representatives of different embryonic lineages, phenotypes, and developmental stages, muscle gene expression was induced. Differences among cell types in the kinetics, frequency, and gene dosage requirements for gene expression provide clues to the underlying regulatory mechanisms. These results show that the expression of genes in the nuclei of differentiated cells is remarkably plastic and susceptible to modulation by the cytoplasm. The isolation of the genes encoding the tissue-specific *trans*-acting regulators responsible for muscle gene activation should now be possible.

Table 1. Cell types in which human muscle genes were activated in heterokaryons.

Biological function	Muscle gene product	Assay	Cell lineage tested	Phenotype tested
Enzyme	Creatine kinase	Electrophoresis and enzyme activity	Mesoderm	Amniotic fibroblast
	Human MM			Fetal skin fibroblast
	Human MB			Adult skin fibroblast
	Mouse-human hybrid MM			Lung fibroblast
				HeLa (malignant)
				Chondrocyte
			Ectoderm	Keratinocyte
			Endoderm	Hepatocyte
Contractile apparatus	Myosin light chains	Electrophoresis and monoclonal antibodies	Mesoderm	Amniotic fibroblast
	Fetal			
	1s			
	2s			
	2f			
	Actin mRNA's	cDNA probes		
	α-cardiac		Mesoderm	Fetal skin fibroblast
				Adult skin fibroblast
			Ectoderm	Keratinocyte
	α-skeletal		Mesoderm	Fetal skin fibroblast
				Adult skin fibroblast
			Ectoderm	Keratinocyte
Membrane components	Cell surface antigens	Monoclonal antibodies		
	24.1D5		Mesoderm	Fetal skin fibroblast
				Lung fibroblast
	5.1H11		Mesoderm	Fetal skin fibroblast
				Adult skin fibroblast
				Lung fibroblast
				HeLa (malignant)
			Ectoderm	Keratinocyte
			Endoderm	Hepatocyte

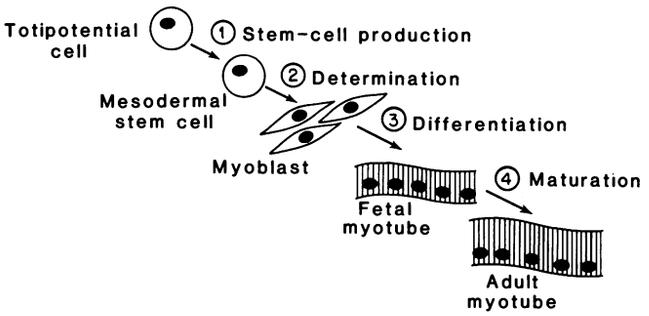

Fig. 1. Points of regulation in muscle cell specialization.

stages (fetal skin and adult skin). One malignant cell type, HeLa, was also tested. To assess the ability of these nonmuscle cell types to be reprogrammed to express muscle functions in heterokaryons, it was important to verify their identity and determine that they continued to function as specialized cells in tissue culture. Accordingly, at the time of fusion with muscle cells, tissue-specific products were identified in cultures of chondrocytes, keratinocytes, and hepatocytes by immunofluorescence with antibodies to type II collagen, keratin, and albumin, respectively (*15*). Although phenotypic markers are not well characterized for fibroblasts, we determined that the two strains of skin fibroblasts used in the studies of developmental stage, from a fetus (14 weeks) and from an adult (66 years), were distinct cell types: they differed in their proliferative properties, cell size, and binding of the mitogens, insulin, and insulin-like growth factor 1 (*16, 17*).

We took advantage of species differences to assay the induction of expression of muscle genes in nonmuscle cells. A heterokaryon is readily identified by its mixed nuclear composition: mouse nuclei appear punctate and human nuclei are uniformly stained with the fluorescent dye Hoechst 33258 (Fig. 2). The novel activation of a muscle gene is evident by detection of a product specific to human muscle on the heterokaryon cell surface, a product which neither cell type alone produces. This gene product, 5.1H11, is an antigen present in small amounts on human myoblasts and in large amounts on myotubes (*18*). Unfused nonmuscle cells grown on the same dish with heterokaryons did not express muscle functions. Thus, assays of gene products could be performed at the level of a single cell in heterokaryons of defined nuclear composition. Gene activation was also analyzed in mass cultures at biochemical and molecular levels.

In each case tested, human muscle genes were activated (Table 1). Human myosin light chains—1s, 2s, 2f, and fetal—and human isozymes of creatine kinase (CK)—MB, MM, and a functional mouse-human hybrid MM—were distinguished from their mouse muscle counterparts by their mobility upon gel electrophoresis; their identities were confirmed either by reaction with monoclonal antibodies on Western blots or by in situ assays of enzyme activity (*10, 19*). The human muscle-specific transcripts of two sarcomeric actin genes, α-cardiac and α-skeletal actin, were detected by Northern blot and S1 nuclease analysis with species- and isotype-specific complementary DNA (cDNA) and genomic DNA probes (*20*). Expression of the human muscle-specific cell surface antigens, 24.1D5 and 5.1H11, was induced and could be monitored on single heterokaryons with monoclonal antibodies (*11, 17, 21, 22*). Thus, the genes that were activated encoded diverse products: enzymes critical to energy production, structural components of the contractile apparatus, and cell surface components. The relative amounts and sequence of expression of these different muscle gene products in heterokaryons paralleled myogenesis in pure cultures of human muscle cells.

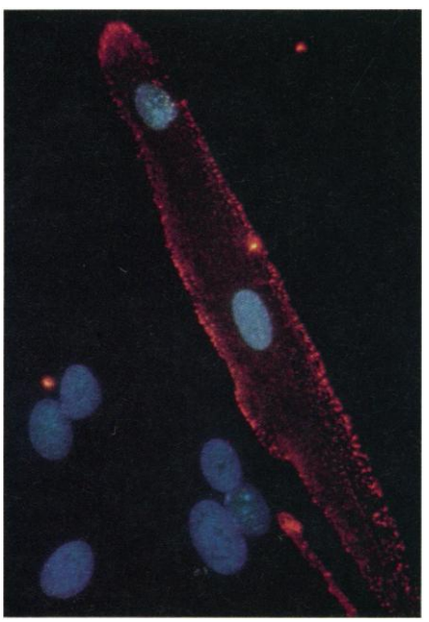

Fig. 2. Activation of human muscle gene for 5.1H11 in heterokaryons. Live cells were incubated with monoclonal antibody to 5.1H11 followed by biotin conjugated to an antimouse antibody and avidin conjugated to Texas red. Heterokaryons are shown in fluorescence microscopy at two different wavelengths. The binucleate heterokaryon containing one punctate mouse muscle nucleus and one uniformly stained human hepatocyte nucleus expresses the antigen, which is uniformly distributed on the cell surface. The trinucleate heterokaryon (lower center) has not activated the gene for 5.1H11.

The Kinetics of Gene Expression Differ

We examined the time course and frequency of muscle gene expression in different cell types contained in heterokaryons. The proportion of individual heterokaryons expressing the cell surface antigen 5.1H11 was determined between 1 and 15 days after fusion in 19 independent experiments in which more than 7000 individual heterokaryons were analyzed (Fig. 3). The three distinct patterns observed differed in the time course of gene expression, primarily because of differences in the lag period before 5.1H11 could be detected. In addition, the ultimate frequency of gene expression differed among cell types and approximated 95, 60, and 25 percent for fibroblasts, keratinocytes, and hepatocytes, respectively. Thus, tissue derivation and possibly embryonic origin have marked effects: fibroblasts, which are from the same embryonic lineage as muscle (mesoderm), exhibit faster kinetics and a higher ultimate frequency of 5.1H11 expression than keratinocytes (ectoderm) and hepatocytes (endoderm). In contrast, the kinetic curves for skin fibroblasts from two developmental stages were indistinguishable (*16, 17*).

To test whether the differences in the kinetics of 5.1H11 accumulation were due to phenotypic differences in the translation and subsequent processing of a cell surface protein, the expression of another muscle gene was examined at the transcriptional level. The relative levels of the messenger RNA's (mRNA's) for the muscle-specific α-cardiac and α-skeletal actins were studied in mass cultures of heterokaryons. We used species- and isotype-specific cDNA probes that recognize the transcripts of the human α-cardiac and α-skeletal actin genes, respectively, but not those of mouse or of other actin genes (*23*). Both α-cardiac and α-skeletal actin expression was evident in heterokaryons, and the time course and relative levels of the two transcripts differed (Fig. 4). These differences in sarcomeric actin expression were similar to those observed in pure cultures of human muscle cells (*20*). With this assay we compared the kinet-

ics of accumulation of actin transcripts in heterokaryons produced with keratinocytes and with fibroblasts. The results paralleled those obtained for 5.1H11 expression. α-Cardiac actin transcripts were not detectable in keratinocyte heterokaryons on days 1 and 2 after fusion, when the levels in fibroblast heterokaryons were marked. By day 5, transcripts were detectable in keratinocyte heterokaryons as well (*16*). Since the differences in kinetics observed at the protein level were also observed at the mRNA level, they probably reflect differences among cell types in steps necessary for the activation of gene expression.

Mechanisms for Gene Activation

To examine potential mechanisms required for gene activation, we determined whether DNA replication was necessary to reprogram cells from different stages of development and of different phenotypes to express muscle genes (*11, 16, 17*). We exposed nonmuscle cells to the DNA synthesis inhibitor cytosine arabinoside (Ara-C) before and after fusion. The expression of human muscle CK was assayed in extracts of whole cells, and the expression of 5.1H11 was monitored on individual heterokaryons. The human M-CK gene was activated in all of the seven nonmalignant cell types tested, and three novel CK isozymes containing this subunit were detected: human MB, human MM, and mouse-human hybrid MM (Fig. 5). In all cases, similar amounts of the human isozymes were present regardless of whether DNA synthesis was inhibited. Furthermore, inhibition of DNA synthesis also had no effect on the frequency of 5.1H11 expression at the single-cell level. These results suggest that alterations in chromatin configuration requiring DNA replication are not necessary for the muscle genes in these cell types to be accessible to and respond to regulatory factors present in differentiated muscle cells.

In contrast to these results, CK and 5.1H11 muscle gene expression were never observed when we formed heterokaryons with HeLa cells, a malignant, aneuploid cell type (*12*). We examined whether prior treatment with 5-azacytidine (5Ac), a drug thought to reduce gene methylation (*24*), could alter the responsiveness of HeLa cells to muscle gene regulators in heterokaryons. Indeed, the expression of both 5.1H11 and CK isozymes containing human subunits was detected in heterokaryons formed with 5Ac-treated HeLa cells, but not in control heterokaryons containing untreated HeLa cells (Fig. 5) or in 5Ac-treated HeLa cells fused to themselves (*12*). Therefore gene activation in HeLa cells appears to require a mechanistic step not required by the other cell types tested: first a change induced by 5Ac is necessary and then interaction with muscle gene regulators.

Gene Dosage Influences Gene Expression

Further differences among cell types in the activation of muscle genes were apparent when we examined the effects of gene dosage, or nuclear ratio, on the frequency of muscle gene expression

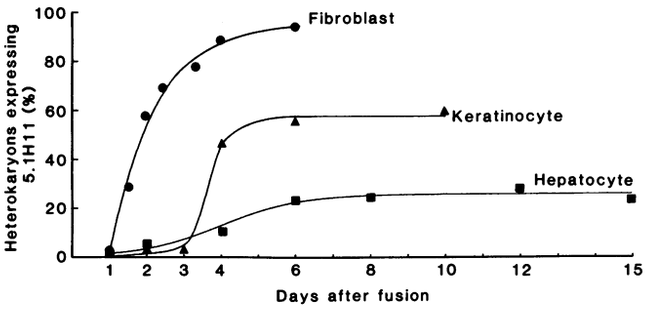

Fig. 3. Kinetics of 5.1H11 expression in heterokaryons containing different cell types. Individual heterokaryons containing nuclei from muscle and from either lung fibroblasts (●), keratinocytes (▲), or hepatocytes (■) were analyzed in replicate cultures for nuclear composition and the expression of 5.1H11 between 1 and 15 days after polyethylene glycol fusion. Curves are computer-derived best fit lines of the data. The size of the symbols includes ±1 standard error of the proportion calculated from the standard binomial equation.

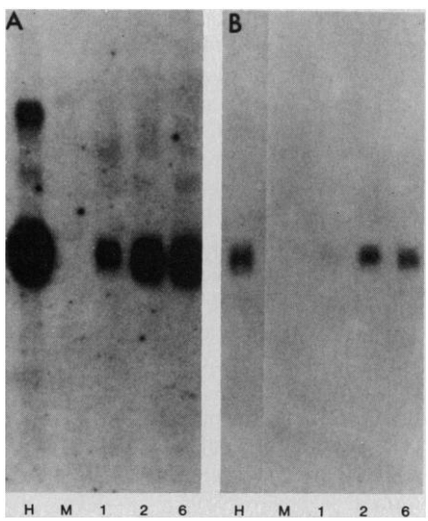

Fig. 4. Activation of human muscle-specific actin mRNA's in heterokaryons. Total RNA's were isolated from differentiated human muscle cells (H), differentiated mouse muscle cells (M), and mouse muscle–human lung fibroblast heterokaryon cultures on days 1, 2, and 6 after polyethylene glycol–mediated fusion. RNA's (5 μg from human muscle cells and 10 μg from each of the other samples) were electrophoresed on agarose gels, transferred to nitrocellulose, and hybridized with the human-specific cDNA probes to either α-cardiac actin (A) or α-skeletal actin (B). Autoradiograms were exposed to XAR-5 film at −80°C for 6 days.

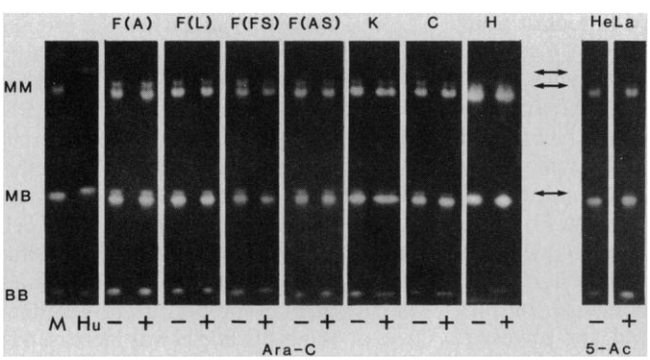

Fig. 5. Activation of human creatine kinase in different nonmuscle cells. Whole-cell extracts were electrophoresed on 5 percent nondenaturing polyacrylamide gels, and the CK isozymes were detected with ultraviolet illumination and a coupled enzyme reaction, yielding the reduced form of $NADP^+$ as its end-product. CK isozymes are shown for mouse (M) muscle cells and human (Hu) muscle cells. Heterokaryons formed between mouse muscle cells and fibroblasts from amniotic fluid F(A), lung F(L), fetal skin F(FS), and adult skin F(AS), keratinocytes (K), chondrocytes (C), and hepatocytes (H) are shown. Cultures marked + were exposed to cytosine arabinoside (Ara-C) for 24 hours before and 24 hours after fusion to ensure inhibition of DNA synthesis. Cultures marked − were not exposed to Ara-C during this period. Heterokaryons formed between mouse muscle cells and HeLa cells were either untreated (−) or exposed to 5 μ*M* of 5-azacytidine (5-Ac) for 3 days followed by 1 day of drug removal (+). Equivalent enzyme activities were loaded in each lane. Arrows indicate CK isozymes containing human M subunits. Abbreviations: BB is the nonmuscle isozyme, M-subunit synthesis is initiated early in differentiation, and the dimers MB and MM are characteristic of differentiated muscle.

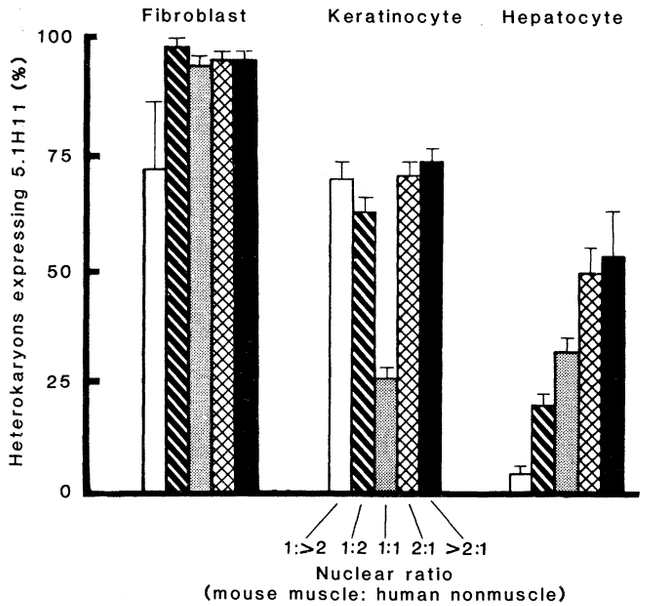

Fig. 6. Effect of nuclear ratio on 5.1H11 expression in heterokaryons containing different cell types. Individual heterokaryons containing nuclei from muscle and from either lung fibroblasts, keratinocytes, or hepatocytes were analyzed for nuclear composition and the expression of 5.1H11 6 days after fusion. Data were grouped into five ratios of muscle : nonmuscle nuclei. Error bars indicate the standard error of the proportion calculated from the standard binomial equation.

(*25*). Nuclear composition and the expression of the human muscle cell surface antigen 5.1H11 were monitored in the same heterokaryons with fluorescence microscopy at two different wavelengths (Fig. 2). The results for heterokaryons of different nuclear composition were pooled into five groups according to nuclear ratio, or the relative number of muscle to nonmuscle nuclei they contained (Fig. 6). The proportion in each group that expressed the antigen on a given day was determined. Examples for the 6-day time point are shown, beyond which the pattern of gene expression did not change.

The effect of gene dosage on 5.1H11 expression differed markedly for the three cell types. For fibroblasts, a high proportion of heterokaryons (95 percent) expressed 5.1H11 at all nuclear ratios, except in the increased fibroblast group (1: > 2), in which it was ~70 percent. In keratinocyte heterokaryons, gene expression in the 1:1 nuclear ratio group never exceeded 30 percent, whereas increased proportions of either muscle or keratinocyte nuclei resulted in the maximum expression of 70 percent. For hepatocyte heterokaryons, the frequency of gene expression was greatest (50 percent) when the relative number of muscle nuclei was increased and lowest (5 percent) when the proportion of hepatocyte nuclei was increased.

Although the interaction between two disparate cell types combined by fusion is likely to be complex, some noteworthy relationships are apparent from the studies of gene dosage (*16, 25*). (i) Even when the nonmuscle nuclei of each cell type outnumbered the muscle nuclei, muscle gene expression was induced, albeit with different frequencies. (ii) The kinetics of gene expression in fibroblast heterokaryons with increased proportions of nonmuscle nuclei were slower than when the nuclear ratio was 1:1. These results could be due to a requirement for gene activation of a threshold concentration of positive regulators: increased time would be necessary for the progressive accumulation of factors that must be shared among fibroblast nuclei. The rate with a 1:1 nuclear ration exceeded that with increased muscle nuclei for fibroblast heterokaryons, which suggests that increased input from muscle was not optimal; instead a balance of factors contributed by each cell type might be required.

In hepatocyte heterokaryons, the frequency of activation of muscle gene expression was lowest when the proportion of hepatocyte nuclei was increased and highest when the proportion of muscle nuclei was increased. That these frequencies did not change with time suggests that gene expression was determined by the continued balance between components contributed by each cell type. Keratinocytes were more frequently activated to express muscle genes when either the proportion of keratinocyte or of muscle nuclei was increased in heterokaryons. The increased frequency of gene expression in heterokaryons with excess keratinocyte nuclei could be due to the existence of subpopulations of cells in keratinocyte cultures that differed in their ability to respond to muscle regulatory factors. Accordingly, the chance of obtaining a keratinocyte in which activation could occur would increase with the number of keratinocyte nuclei. Stratification within colonies of keratinocytes was evident in our cultures, and although the upper, more mature cells reportedly do not replate well (*26*), possibly some were incorporated into heterokaryons.

Expression of the Nonmuscle Phenotype

To test whether the phenotype of the nonmuscle cell persists in heterokaryons, we monitored the expression of albumin in individual hepatocyte heterokaryons. Six days after fusion, when the muscle product 5.1H11 was expressed at maximum frequency, albumin was still present in a large proportion of hepatocyte heterokaryons. By 15 days after fusion 5.1H11 was still expressed at maximal frequency, but the proportion of albumin-containing heterokaryons was reduced. The persistence of albumin at 6 days was not due to the stability of previously synthesized proteins, since albumin is secreted within a few hours of its production. To detect albumin in heterokaryons, we had to inhibit secretion with monensin 2.5 hours before fixation (*16, 17*). Furthermore, the decline in albumin-containing heterokaryons with time was not due merely to extinction of the liver phenotype in the course of long-term culture, since parallel cultures of hepatocytes fused to themselves with polyethylene glycol (PEG) contained albumin on day 15. From these experiments we cannot determine whether the expression of the genes of one phenotype precludes the expression of the genes of the other. This determination would require assays of active transcription for genes characteristic of both nonmuscle and muscle phenotypes in single nuclei. Despite this limitation, our results indicate that the combination of specialized cells in a common cytoplasm does not result in the immediate degradation of differentiation-specific proteins. Instead, we conclude that the nonmuscle phenotype persists for a time in hepatocyte heterokaryons, but the muscle phenotype dominates.

Regulatory Circuits Between Nuclei in Heterokaryons

To examine whether the nonmuscle cell influences gene expression in the muscle cell, we examined the accumulation of mouse and human mRNA's for α-cardiac actin, a major component of the muscle contractile apparatus. We took advantage of the fact that primary human

muscle cells and cultured mouse C_2C_{12} cells exhibited distinct patterns of α-cardiac actin transcript accumulation with time after myotube formation (*20, 27–29*). The amount of α-cardiac actin mRNA in each sample was normalized to the amount present in a human heart standard analyzed at the same time. In the mouse muscle C_2C_{12} cultures, α-cardiac actin transcripts accumulated rapidly, transiently peaked at a level comparable to 50 percent of the α-cardiac actin mRNA expressed in human heart within 24 hours, and declined (Table 2). In contrast, α-cardiac actin transcripts accumulated steadily in human myotubes over a 6-day period and exceeded the peak reached in the C_2C_{12} cell line 16-fold, a level comparable to 800 percent that observed in human heart. In heterokaryon cultures, the time course of human transcript accumulation and the amount of transcript per nonmuscle nucleus were similar to those observed in pure human, not mouse, muscle cultures. Of greatest surprise was the result obtained for mouse α-cardiac actin mRNA in heterokaryons: instead of continuing to decline, as in pure mouse muscle cultures, the amount of mouse transcripts increased after heterokaryon formation.

These findings suggest that the human α-cardiac actin gene responds to mouse muscle cytoplasmic factors by producing transcripts with the time course and at levels typical of human, not mouse, muscle cultures. These differences in transcript accumulation could arise in part from differences in the *cis*-acting regulatory regions of the mouse and human α-cardiac actin genes. In addition, the results are compatible with the hypothesis that *trans*-acting mouse muscle factors activate human muscle regulatory genes encoding factors that, in turn, act on mouse muscle genes or stabilize mouse α-cardiac actin transcripts. Finally, the "activated" phenotype seems to dominate, since the usual decline in the amount of mouse α-cardiac actin transcripts is overridden by the presence of the human nuclei.

Table 2. Relative α-cardiac actin mRNA in pure muscle and in heterokaryon cultures. PEG treatment occurred after the mouse muscle myotubes had been in fusion medium for 3 days. Thus, to compare the mouse time scale in fusion medium (left) with that after PEG treatment (right), add 3 days.

Days in fusion medium	α-Cardiac actin mRNA in human heart (%)		Days after PEG treatment	α-Cardiac actin mRNA in human heart (%)	
	Mouse muscle C_2C_{12}	Human primary muscle		Mouse in heterokaryons	Human in heterokaryons
0	20	1	0	—	0
1	50	100	1	10	50
2	25	200	2	30	200
6	20	800	6	40	700

Muscle Gene Regulators at Distinct Stages of Development

Like the experiments described above, the majority of studies of gene expression in specialized cells have utilized the differentiated stage of development (Fig. 1). This is true primarily because for all cell types, this stage is generally the best characterized and most readily obtained in tissue culture. The experiments described below show that other stages of muscle development—determination and maturation—are now amenable to analysis.

To test whether muscle cells at the determined stage of development elaborate distinct muscle gene regulators, we produced heterokaryons with fibroblasts and muscle cells at the myoblast (determined) and myotube (differentiated) stages (*22*). Convenient markers for this study were provided by two cell-surface antigens: 5.1H11 and 24.1D5. The 5.1H11 antigen is expressed on cultured myoblasts to some extent, but markedly increases in amount on myotubes. The 24.1D5 antigen is detected only on myoblasts and is no longer present once myoblasts fuse to form multinucleated differentiated myotubes. The human forms of both muscle cell–surface components were recognized with species-specific monoclonal antibodies (*17, 20*). The 24.1D5 antigen was detected only in myoblast heterokaryons (22 percent) (Table 3).

In contrast, 5.1H11 was expressed in both myoblast heterokaryons (19 percent) and myotube heterokaryons (92 percent). Thus, the expression of these two antigens in heterokaryons paralleled their expression in human myogenesis. By day 5, a maximum frequency of expression was reached in both cases and further increases were not observed with time. The lower frequency of gene expression in myoblast than in myotube heterokaryons may be due to myoblast nuclei that were in different phases of the cell cycle, whereas most of the myotube nuclei were presumably withdrawn from the cell cycle. These results suggest that muscle cells at myoblast and myotube stages of development differ in the concentration or type of at least two regulatory factors.

The study of the maturation stage of muscle development is facilitated by the myosin heavy chains. These proteins are major components of the contractile apparatus that are encoded by different genes in fetal, neonatal, and adult muscle tissues (*8*). Studies of maturation have been hindered because these transitions

Table 3. Evidence for two temporally distinct muscle gene regulators. Fetal skin fibroblasts were used except in myotube experiment 3, in which lung fibroblasts were used; S.E.M., standard error of the mean.

Experiment	Heterokaryons expressing			
	24.1D5		5.1H11	
	Percentage exhibiting detectable antigen	Number scored	Percentage exhibiting detectable antigen	Number scored
	Fusion partner: myoblast			
1	34	228	8	229
2	13	131	19	59
3	20	113	30	93
Mean ± S.E.M.	22 ± 6	472	19 ± 6	
Total				381
	Fusion partner: myotube			
1	1	556	90	407
2	0	800	98	166
3	0	600	88	376
Mean ± S.E.M.	0	1956	92 ± 3	
Total				949

are not readily obtained in vitro; primarily fetal forms of muscle proteins are expressed in cultured myotubes. We have determined that C_2C_{12} cells express developmentally distinct myosins. These myosin isoforms were detected with monoclonal antibodies generated to myosin purified from human muscle at different developmental stages (*30*). C_2C_{12} cells are stained with two distinct antibodies that recognize myosin isozymes present at fetal and neonatal stages of development (Fig. 7). That the intensity of staining differed among myotubes suggests that the relative concentrations of fetal and neonatal myosins differ. A progressive increase in the relative amount of neonatal myosin was observed with time in culture. The results were not due to the use of the C_2C_{12} cell line, since they have been corroborated with human primary muscle cultures (*31*). Consequently, neural contact and complex substrates do not seem to be required for certain steps in muscle maturation. Since some of our antibodies to myosin are human-specific, it should now be possible to examine the activation of myosin isozymes in heterokaryons and the regulatory mechanisms underlying the maturation stages of muscle development.

Discussion

Several theories have been proposed to account for the development of cell diversity: changes in the genome that accompany quantal mitoses (*32*), sequential repression of genes (*33*) or modulation of gene expression by activators and repressors (*34*) with additional complex regulatory effects resulting from the relative concentration and affinity of these factors for specific sequences within genes (*4–6*). Some major questions are raised by these theories: How fixed and irreversible is the differentiated state or tissue-specific phenotype of a cell? What is required to maintain the differentiated state? What underlying molecular mechanisms regulate the transition from the determined to the differentiated to the mature cell phenotype? Do these mechanisms differ for different specialized cell types?

Our approach to these questions was inspired by reports by Weiss and others of experiments with somatic cell hybrids, in which the expression of genes atypical of a cell's normal phenotype could be induced (*35, 36*). A limitation in the comparative analysis of gene activation in different cell types is that highly aneuploid transformed cells were usually used. In addition, hybrid cells were frequently obtained after proliferation in mitogen-rich media, which led to variable retention and rearrangement of genetic material (*35, 37*), both of which are likely to have influenced gene expression (*38*). As a result, certain phenotypes were thought to be incapable of being activated, because of either their specialized state or their developmental stage (*37, 39*).

The activation of muscle gene expression in all of the eight nonmuscle cell types we tested is likely to be due to several factors. To facilitate the study of normal regulatory mechanisms, most nonmuscle cells were diploid primary cells. To overcome the problems of gene loss and rearrangement, we used a heterokaryon system that remained stable for the 2-week life-span of the cells and permitted a systematic analysis of gene expression over time. We cannot rule out the possibility that muscle is particularly well suited for internuclear communication and existence as a heterokaryon, since it is naturally a multinucleated cell in its differentiated state. However, the use of culture conditions which promoted muscle differentiation, not cell division, was probably critical to the success of our experiments. Proliferation and differentiation are antagonistic, mutually exclusive states in muscle cells (*40*). Furthermore, specific culture conditions that differ markedly among cell types are required for the optimal expression of phenotypes in vitro (*41*). Many investigators using somatic cell genetics to analyze the expression of differentiated functions used a nonspecific mitogen-rich culture medium. We propose that with culture conditions that favor the differentiation of the phenotype of interest, gene activation in stable heterokaryons could be achieved with cell types other than muscle.

The ability to activate muscle genes in heterokaryons is not a peculiarity of the C_2C_{12} cell line. We have recently been able to induce human muscle gene expression with another mouse muscle line, the myogenic derivative of a fibroblastic 10T1/2 cell treated with 5-azacytidine (*13*). However, the frequency of gene activation in fibroblast heterokaryons formed with 10T1/2 is half that observed with C_2C_{12} cells. We selected the C_2C_{12} clone for its potential to give rise to extensive differentiated contractile myotubes within a short period, and in this respect it exceeds all other myogenic lines with which we have had experience. Thus, C_2C_{12} may be an optimal

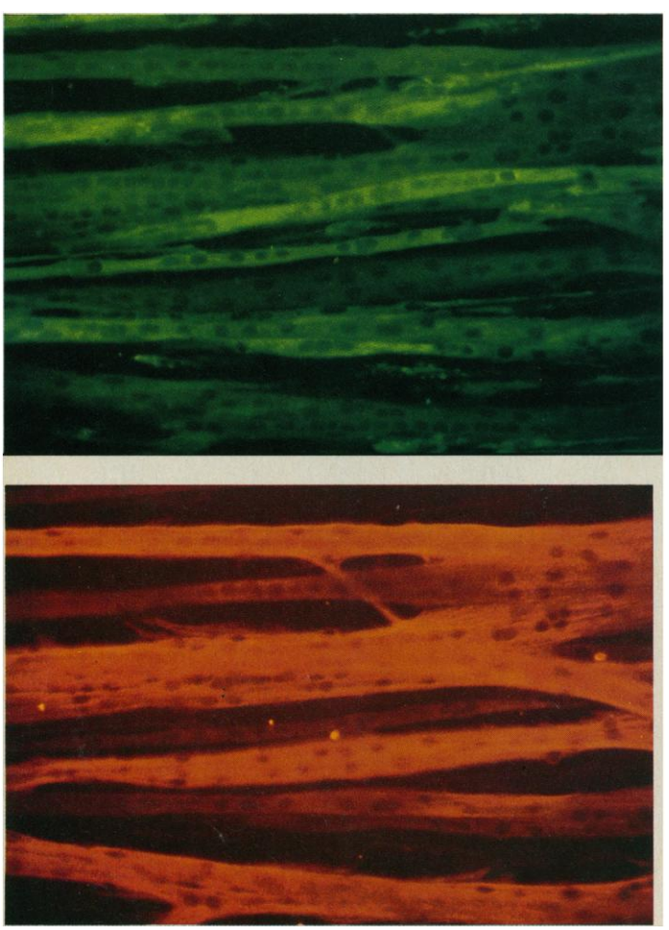

Fig. 7. Expression of fetal and neonatal myosin isozymes in C_2C_{12} muscle cultures. (Top) Reaction of cells with an immunoglobulin G (IgG) monoclonal antibody (F1.193) that recognizes fetal myosin, visualized with fluorescein-conjugated antiserum to mouse IgG. (Bottom) Reaction of the same cells with an immunoglobulin M (IgM) monoclonal antibody (N3.36) that recognizes neonatal myosin, visualized with rhodamine-conjugated antiserum to mouse IgM. The cells were fixed with 1 percent formalin 8 days after being transferred to differentiation medium (2 percent horse serum in Dulbecco's modified Eagle's medium).

source of muscle gene regulatory factors. Another myogenic cell line that may be enriched for regulators has been obtained by Wright, who selected for muscle cells resistant to an inhibitor of differentiation, bromodeoxyuridine (*42*). The selection of cells that undergo rapid differentiation should also prove useful for characterizing tissue-specific regulators in other phenotypes.

The plasticity in the function of the nucleus of specialized cells is remarkable. In each nonmalignant cell type tested, the differential state could be altered in the absence of DNA replication or cell division and the cells induced to express human muscle functions after exposure to muscle cytoplasm. Representatives of different embryonic lineages, phenotypes, and developmental stages were tested, including four strains of fibroblasts and chondrocytes (mesoderm), keratinocytes (ectoderm), hepatocytes (endoderm), and malignant HeLa cells. Differences among cell types were observed in the frequency, kinetics, and gene dosage requirements for the expression of human muscle genes. Cells that were more closely related to muscle—mesodermal derivatives—consistently expressed muscle genes sooner and at a greater frequency than the cells of ectodermal or endodermal origin.

Our experiments suggest that the modulation of gene expression which accompanies eukaryotic cell differentiation is mediated by a number of positive and negative regulatory factors in a concentration-dependent manner. When cells are mixed by fusion, the end result is likely to be due to complex interactions; the following simple interpretation of our experiments may nonetheless provide a useful conceptual framework. The delayed but high frequency of expression of muscle genes in heterokaryons with one muscle and multiple nonmuscle nuclei suggests that muscle regulators must reach a threshold concentration for gene activation to occur. The observation that muscle gene expression in fibroblast heterokaryons is fastest with a 1:1 ratio suggests that a balance of factors contributed by both cell types is optimal for that cell type. On the other hand, the frequency of gene expression in hepatocytes differs in heterokaryons containing different proportions of muscle and nonmuscle nuclei, and the differences persist with time, suggesting that hepatocytes produce negative regulators that must be titrated by the positive regulators in muscle cells. Muscle cells at different stages of development seem to synthesize different positive regulators. Finally, the reprogramming of the nonmuscle nucleus in a heterokaryon results in the synthesis by that nucleus of muscle regulatory factors. Thus, from an analysis of the combined effects on gene expression of two cell types fused to form heterokaryons, inferences regarding the regulators that generate and maintain these phenotypes can be drawn.

Gene expression in cells specialized for very different functions is not fixed and irreversible. Although under normal circumstances the pattern of gene expression of specialized cells is stable and heritable, it can be altered if the regulatory circuits between nucleus and cytoplasm are disrupted. Thus, changes in gene expression during development depend not only on the nucleus, but also on the cytoplasm, which plays an essential role as signal transducer. Our results extend the observations that nuclear gene expression is subject to modulation by the cytoplasm (*43*) to a system that may be particularly amenable to molecular analysis.

Future Prospects

Heterokaryons have utility as experimental models for investigating the regulation of gene expression in differentiated cells. The cells that are reprogrammed in heterokaryons should provide useful test systems for studying the mechanisms of action of the relevant molecules. Differences among cell types in the lag period before gene expression, the time course of accumulation of gene products, and the frequencies of gene expression at different nuclear ratios suggest different mechanistic steps. These could result from differences in gene conformation due to DNA modifications or binding proteins in addition to differences in the requirement for the type, concentration, or stoichiometry of *trans*-acting factors. We and others have begun to characterize the *cis*-acting regulatory sequences of muscle-specific genes (*44*). The isolation of the genes for *trans*-acting muscle-regulatory molecules at determined, differentiated, and mature stages of muscle development should now also be possible. Approaches that may prove useful for isolating mRNA's and genes for uncharacterized factors include microinjection of cytoplasmic components, use of subtractive hybridization to enrich for mRNA's for stage-specific regulators, and transfection of either cDNA's made to the enriched mRNA's or of genomic DNA into cells in conjunction with selective assays for gene expression (*45*). Elucidation of the molecular mechanisms underlying the reprogramming of nonmuscle cell types should lead to a better understanding of the generation and maintenance of these phenotypes and of different myogenic stages.

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