

Expression of Muscle Genes in Heterokaryons Depends on Gene Dosage

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Abstract. We report that gene dosage, or the ratio of nuclei from two cell types fused to form a heterokaryon, affects the time course of differentiation-specific gene expression. The rate of appearance of the human muscle antigen, 5.1H11, is significantly faster in heterokaryons with equal or near-equal numbers of mouse muscle and human fibroblast nuclei than in heterokaryons with increased numbers of nuclei from either cell type. By 4 d after fusion, a high frequency of gene

expression is evident at all ratios and >75% of heterokaryons express the antigen even when the nonmuscle nuclei greatly outnumber the muscle nuclei. The kinetic differences observed with different nuclear ratios suggest that the concentration of putative *trans*-acting factors significantly influences the rate of muscle gene expression: a threshold concentration is necessary, but an excess may be inhibitory.

An elucidation of the mechanisms that regulate changes in gene expression is of central importance to an understanding of the development of complex multicellular eukaryotes. Cellular differentiation does not appear to be due to loss of genomic material (9, 11, 21). Instead, to generate and maintain the specialized characteristics of cells of differentiated tissues, genes must be activated and repressed in an orderly fashion. There are some indications that the differential regulation of genes is mediated by the interaction of specific *trans*-acting factors with *cis*-acting genomic sequences, such as promoters and enhancers (4). Although the mechanism of action of certain general mediators of transcription has been demonstrated to be concentration dependent (16, 17, 19, 22, 29, 30), relatively little is known regarding the amounts required for tissue-specific gene expression in mammalian cells.

Experiments with somatic cell hybrids suggested that tissue-specific *trans*-acting molecules might play a role in the expression of differentiated functions in mammalian cells. This is now known to be true from studies of the expression of certain cloned genes following transfection. Cell fusion results in the combination of the entire genomes and cytoplasm of two cells of different functional states. This leads either to the repression of the differentiated functions of the expressing cell type or to the activation of silent genes in the non-expressing cell type. The different outcomes obtained with hybrids are correlated with gene dosage, or the relative ratio of the chromosomes contributed by each of the parental cell types. Repression is typically observed with equivalent gene dosage or with increased genetic input from the non-expressing cell type; activation is usually detected only when the genetic input from the expressing cell type is increased (for review see

references 10, 20, 28). However, a limitation of such studies is that the initial fusion products can rarely be analyzed for more than short periods of time after their production; cell division and chromosome loss and rearrangement rapidly ensue. Consequently, a kinetic analysis of the influence of gene dosage on gene expression is not possible.

To examine concentration requirements in the activation and expression of differentiation-specific eukaryotic genes over time, we have used a stable heterokaryon system in which cell division does not occur. Gene dosage can be manipulated in these heterokaryons by altering the ratio of intact nuclei so that the normal location of genes within the genome and the stoichiometry between regulatory and structural genes per nucleus are maintained. The detailed kinetic analysis of the relationship between nuclear ratio and gene expression presented here is possible because the initial fusion product, a heterokaryon, survives for up to 2 wk in culture. We have previously shown that when mouse muscle cells are fused with human fibroblasts to form heterokaryons, human muscle genes are activated. The expression of genes coding for seven diverse functions is detected: a cell surface component (5.1H11), an enzyme essential for energy production (creatine kinase), and five structural proteins of the contractile apparatus (actins and myosin light chains) (1, 2, 6, 7, 12). Furthermore, the frequency and efficiency of gene activation is high; $95 \pm 1\%$ of all heterokaryons express human muscle genes and the relative amounts of the muscle gene products are typical of pure muscle cultures. Examples of gene repression and activation using similar muscle heterokaryons have been reported by others (14, 31, 32).

We show here that regardless of nuclear ratio, muscle gene activation is observed; nuclear ratio, however, significantly

affects the rate of muscle gene expression. Despite the differences in rates, the ultimate efficiency of gene activation reaches a high level of >75% at all nuclear ratios. These results indicate that muscle gene activation does not require increased muscle gene dosage; given sufficient time, a single muscle nucleus can activate muscle genes in heterokaryons containing as many as six fibroblast nuclei. We interpret these results to mean that the expression of muscle genes requires activators and does not result simply from a dilution of repressors. A particularly unexpected finding is that the most rapid accumulation of human muscle-specific proteins is observed in heterokaryons with equivalent muscle to nonmuscle ratios; increased nuclear input from either cell type results in slower kinetics. Thus, the accumulation of muscle gene products is slower when the muscle nuclei in a heterokaryon outnumber the fibroblast nuclei; a relative increase in muscle genes has a negative effect. These results are compatible with the hypothesis that the rate of muscle gene expression in heterokaryons is dependent on the concentration or stoichiometry of muscle *trans*-acting factors: a threshold concentration is required, but increased amounts are inhibitory.

Materials and Methods

Cell Culture and Heterokaryon Production

Mouse muscle cells, C₂C₁₂, were a diploid subclone isolated in our laboratory from the C2 cell line generously provided by Dr. David Yaffe (33) and karyotyped in our laboratory. Human fibroblasts were a diploid strain isolated from fetal lung (MRC-5) (13). Heterokaryons were produced by fusing myotubes and fibroblasts with polyethylene glycol (PEG) (1). Briefly, the cultures were treated with PEG 1000 (50% wt/vol in Dulbecco's modified Eagle's medium), pH 7.4 for 60 s at 37°C, and then rinsed with Dulbecco's modified Eagle's medium three times in succession for 60 s each. To remove unfused cells, the selective agents, cytosine arabinoside (10⁻⁵ M), and ouabain (10⁻⁵ M), were routinely added. Cytosine arabinoside inhibits DNA synthesis and kills dividing cells (8); ouabain inhibits Na⁺-K⁺ ATPase and has a 100-fold greater affinity for the human than the rodent enzyme (23). To distinguish mouse and human nuclei, heterokaryons were stained after fixation with Hoechst 33258 (Riedel-de Haen, Hanover, Germany), 0.12 µg/ml in 0.9% NaCl for 15 min at 37°C as previously described (1). The proportion of heterokaryons with a given nuclear composition (muscle:nonmuscle) was consistent among experiments: 1:>2 (8 ± 1%), 1:2 (17 ± 1%), 1:1 (33 ± 3%), 2:1 (23 ± 2%), >2:1 (19 ± 2%).

Assays for 5.1H11 Expression

The monoclonal antibody, 5.1H11, generously provided by Dr. Frank Walsh (23, 24) recognizes the human muscle-specific cell surface antigen, but not its mouse muscle counterpart (6). Assays for the expression of this antigen were as previously described (7). Briefly, live cells were incubated at 37°C with 5.1H11 antibody either in undiluted hybridoma supernatant or in mouse ascites fluid, washed, incubated with a second horse anti-mouse antibody conjugated to biotin (Vector Laboratories, Inc., Burlingame, CA), washed again, and incubated with avidin conjugated to Texas Red (Zymed Laboratories, San Francisco, CA). Stained cells were fixed sequentially in 1% formalin at 37°C and 100% methanol at -20°C. Specificity of binding was determined by the lack of fluorescence when parallel dishes were exposed either to second antibody only, to mouse gamma globulin (25 µg/ml) partially purified from non-immune ascites fluid, or to undiluted supernatant of a monoclonal antibody which recognizes a neuronal antigen.

Kinetic Analyses and Statistics

The data were pooled into five groups of muscle:nonmuscle nuclear ratios 1:>2; 1:*x* where 1 < *x* ≤ 2; 1:1; *x*:1 where 1 < *x* ≤ 2; and >2:1.

To facilitate comparison of the overall kinetics of gene expression for heterokaryons of different nuclear composition, the *t*₅₀ was determined. The *t*₅₀ is the time required after fusion for half of the heterokaryons to express the antigen, 5.1H11. To determine the *t*₅₀, a curve-fitting program was used to analyze the time course of 5.1H11 expression by heterokaryons of different nuclear composition. The proportion of total heterokaryons expressing the

antigen on day 6 was designated 100% for each pooled ratio and the frequencies obtained on earlier days for these ratios were normalized to the day 6 values. Using Marquardt's least squares analysis, a number of equations were tested for goodness of fit. The one which fit the data best was $y = 100 - ae^{-bx}$, an equation depicting first order kinetics. The significance of the differences between curves for different nuclear ratios was determined by comparing the coefficients *a* and *b* using the Student's *t* test.

Error bars on histograms indicate the standard error of the proportion calculated from the standard binomial equation: $\sqrt{pq/n}$. Where error bars do not overlap, differences are significant at the 0.05 level using a two-sample Student's *t* test.

Results

Nuclear Ratio and Kinetics of Gene Expression

We examined the influence of nuclear ratio on the rate of expression of a human muscle gene. Heterokaryons were produced by fusing mouse muscle cells (C₂C₁₂) with human lung fibroblasts (MRC-5) using polyethylene glycol (PEG). Heterokaryons were identified by the species-specific fluorescent staining pattern of the muscle (mouse) and nonmuscle (human) nuclei they contained using the dye Hoechst 33258 (1) (see also Fig. 1). This permitted a precise determination of the number of each type of nucleus present in individual heterokaryons. The expression of a human muscle cell surface antigen was monitored in the same heterokaryons using a monoclonal antibody 5.1H11 (26, 27) detectable by fluorescence microscopy at a different wavelength. The antibody recognizes an antigen present on human muscle membranes which is both species- and cell type-specific. We have demonstrated previously that the expression of this antigen is induced in the nuclei of lung fibroblasts after fusion with mouse muscle cells to form heterokaryons (6). Since the antigen was never detected in either parental cell type alone, this expression was the result of gene activation.

Heterokaryons were produced at low density in replicate dishes which were scored at seven successive time intervals for the expression of the antigen and for nuclear composition. The frequency of expression of 5.1H11 was determined for up to 6 d after fusion. This was possible since the heterokaryons were stable; nuclear or cell division was not observed even when nonmuscle nuclei were present in excess. The results presented in Fig. 2 are a composite of the data obtained in 19 independent experiments in which a total of 2,684 individual heterokaryons were analyzed. The frequency of heterokaryon production was high; 85 ± 2% of all multinucleated cells contained both mouse and human nuclei and heterokaryons with a broad spectrum of nuclear ratios were obtained. For analyses of kinetics, heterokaryons were pooled into five groups according to nuclear ratio, or the relative number of muscle to nonmuscle nuclei they contained: 1:>2, 1:2, 1:1, 2:1, and >2:1. Consequently, each ratio included a range of nuclear numbers. For example, the 1:2 group included heterokaryons with one muscle and two nonmuscle nuclei, those with two muscle and four nonmuscle nuclei, and those with two muscle and three nonmuscle nuclei (see Materials and Methods). The proportion of heterokaryons of each ratio that expressed the antigen was determined at each time point.

All heterokaryons of a given ratio do not express the antigen at the same time. For example, for nuclear ratio 1:1, 38% of heterokaryons expressed the antigen at 1.5 d after fusion, whereas 84% expressed the antigen at 3 d. This difference in the timing of expression of the antigen on the cell surface

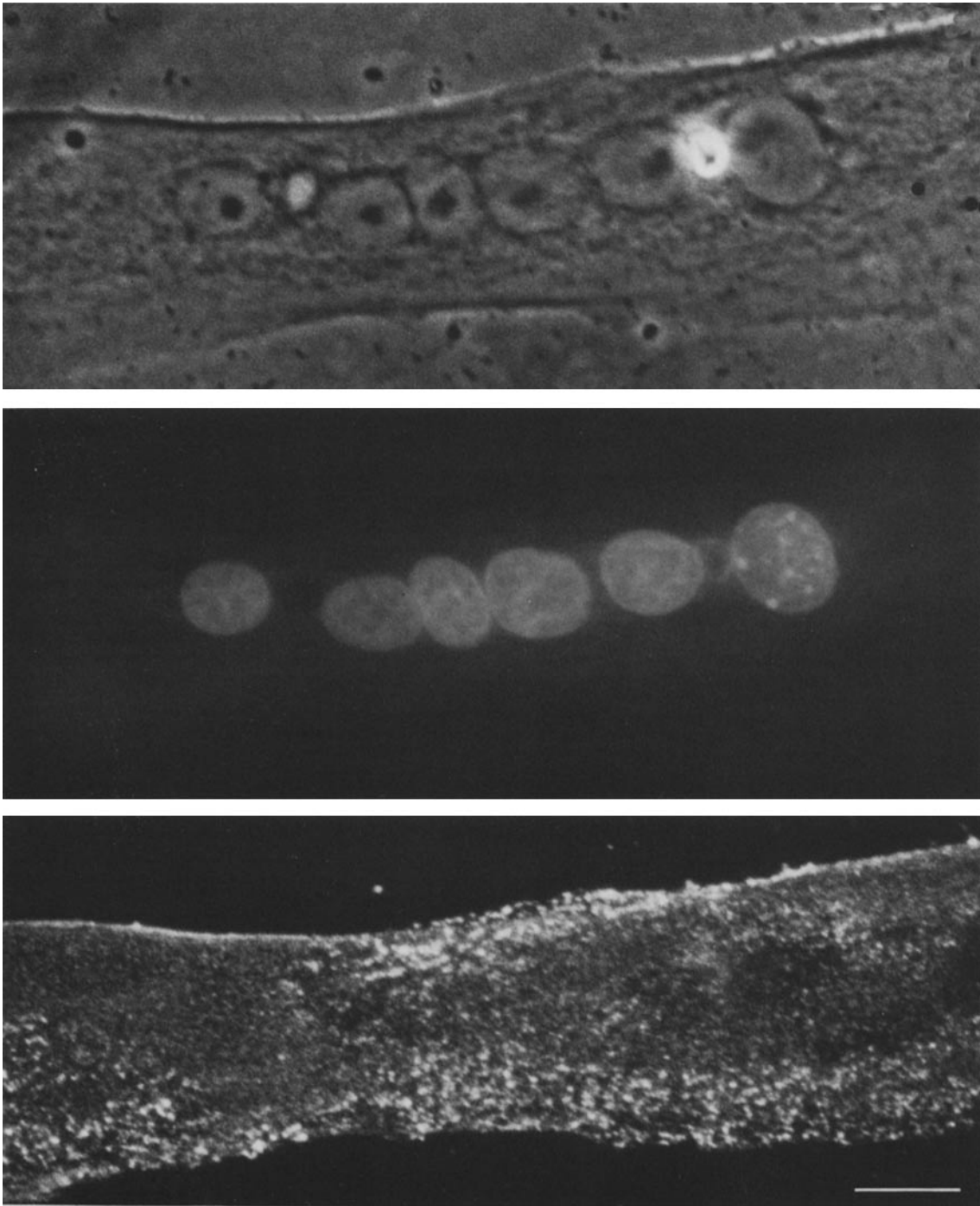


Figure 1. Expression of human muscle gene 5.1H11 in a single heterokaryon. The multinucleated cell shown in phase-contrast microscopy (*top*) is revealed by Hoechst fluorescence (*middle*) to be a heterokaryon containing one punctate muscle nucleus (mouse) and five uniformly stained nonmuscle nuclei (human), or a nuclear ratio of 1:5. Immunofluorescence (*bottom*) using a monoclonal antibody, 5.1H11, followed by biotinylated anti-mouse antibody and Texas Red-avidin reveals the newly synthesized human muscle antigen distributed along the length of the cell. Bar, 20 μm .

could be due to heterogeneity within the muscle cell population at the time of fusion. The C_2C_{12} cells used in these experiments, like most muscle cell lines and primary muscle cells, differentiate in a stochastic manner (18). If the muscle cells constituted a spectrum of differentiation stages which differed in the amount of regulatory molecules they contained, the observed asynchronous expression of the antigen for each nuclear ratio would be expected. Alternatively, the difference

in timing could be due to heterogeneity within the fibroblast population. These cells had a doubling time of 25 h and were in different phases of the cell cycle at the time of fusion.

From the histograms in Fig. 2, it was evident that nuclear ratio influenced the kinetics with which heterokaryons expressed the human muscle antigen. As early as 1.5 d after fusion, a difference in the proportion of heterokaryons exhibiting activation was observed; nearly 40% of heterokaryons of

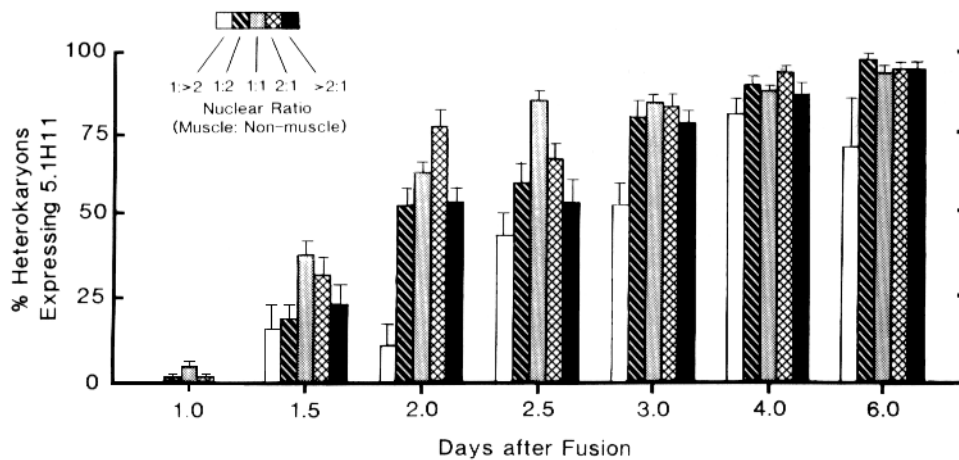


Figure 2. Time-course of 5.1H11 expression in heterokaryons of different nuclear ratios. Individual heterokaryons were analyzed for nuclear composition and the expression of 5.1H11 between 1 and 6 d after PEG fusion. For purposes of comparison, the data were pooled into the five indicated groups of muscle:nonmuscle nuclear ratios. The expression of 5.1H11 was first detected after a lag of ~1 d. Each bar of the histogram represents the data derived from between 31 and 236 heterokaryons, with a mean of 89 ± 9 . Error bars indicate the standard error of the proportion calculated from the standard binomial equation. Where error bars do not overlap, differences are significant at the 0.05 level using a two-sample Student's *t* test.

nuclear ratio 1:1 expressed the antigen as compared to ~20% of those of muscle to nonmuscle ratio 1:>2 or >2:1. Significant differences in the frequency of expression of the antigen persisted at 3 d. By 4 and 6 d after fusion, a high frequency of >75% was observed for heterokaryons of all ratios. These results were highly reproducible; they were obtained in several independent experiments which were performed at different times and used different passages of cells. Thus, at a nuclear ratio of 1:1 and even when muscle nuclei were greatly outnumbered by fibroblast nuclei, the fibroblast gene coding for the muscle antigen was activated. However, the ultimate efficiency was consistently reduced when the proportion of fibroblasts was increased (1:>2): 75% as compared to >95% for all other nuclear combinations. These results demonstrate that although nuclear ratio does not determine whether a heterokaryon will express the antigen, it does affect the rate of antigen appearance. The fastest kinetics are observed with equivalent, or near-equivalent nuclear input from the two cell types. The slowest kinetics are observed when the relative contribution of nuclei of one type, muscle or nonmuscle, is increased.

The reduction in the rate of expression of the antigen when either nonmuscle or muscle nuclei are present in excess is more clearly demonstrated in Fig. 3. The best fit lines for the data presented in Fig. 2 are shown. A lag period of ~1 d preceded the detection of the 5.1H11 antigen in heterokaryons regardless of nuclear ratio (Fig. 3, *a* and *b*). This lag presumably reflects the time required to synthesize and accumulate sufficient antigen on the cell surface to be detectable by immunofluorescence. Consequently, the lag includes the time required for activation and transcription of the gene as well as translation and processing of its product via the cell secretory apparatus. Although the day 1 results in the histogram in Fig. 2 suggest differences in the lag among heterokaryons of different nuclear ratios, these differences are not definitive, since the lag was not studied with precision: time intervals of <12 h were not analyzed. Following the lag, the proportion of heterokaryons expressing the antigen gradually increased

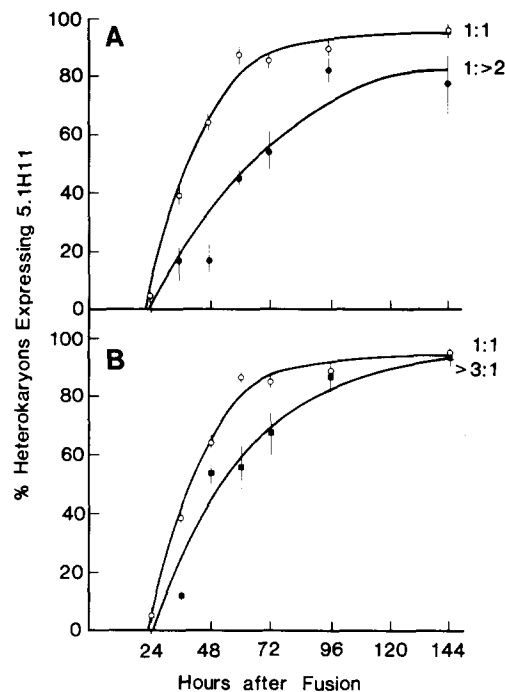


Figure 3. Effect of nuclear ratio on the kinetics of 5.1H11 expression. The mean \pm standard error of the proportion (see Materials and Methods) are shown for each time point for (A) increased nonmuscle (1:>2) and (B) increased muscle (>3:1). The values are compared with those for a nuclear ratio of 1:1. Increased proportions of nuclei of either cell type results in a decreased rate of accumulation of 5.1H11. Where error bars do not overlap, differences are significant at the 0.05 level using a two-sample Student's *t* test.

over a 5-d period. The slopes of the curves for different pooled ratios revealed that the relative input of nonmuscle and muscle nuclei in heterokaryons significantly influenced the rate of antigen appearance; with either increased nonmuscle or increased muscle nuclei, the kinetics of antigen expression were slower.

Heterokaryon Size and Kinetics of Gene Expression

We examined the possibility that the differences in the kinetics of gene expression observed with different nuclear ratios were due to differences in total nuclear number and consequently heterokaryon size. The size of a heterokaryon could theoretically influence the potential to detect the antigen, since more antigen might be required for visualization in a greater surface area. The pooled nuclear ratios analyzed in Figs. 2 and 3 included heterokaryons with a broad range in the total number of nuclei they contained. To examine the possibility that heterokaryon size might influence the analysis of the kinetics of gene expression, heterokaryons in groups containing equivalent total numbers of nuclei in different proportions of muscle and nonmuscle were compared. As shown in Table I, we grouped all heterokaryons with four nuclei as follows: (A) 2 muscle + 2 nonmuscle, (B) 3 muscle + 1 nonmuscle, and (C) 1 muscle + 3 nonmuscle. The kinetic curves and t_{50} values were determined for each group. Although the nucleus/surface area ratio appeared to be similar for all three groups, the group of heterokaryons with equivalent numbers of muscle and nonmuscle nuclei (2 + 2) exhibited significantly faster kinetics of 5.1H11 expression ($P < 0.05$).

A similar result was obtained for heterokaryons grouped according to the ratio of nuclei (Table I). These heterokaryons contained between 2 and 20 nuclei, and consequently, a range of cell sizes. For example, 74% of the heterokaryons with equivalent muscle and nonmuscle nuclei, i.e., of 1:1 ratio, were binucleate. A comparison of the t_{50} values for these heterokaryons with primarily two nuclei and those of Group A with four nuclei revealed no significant difference (38 and 40 h, respectively). We conclude that the t_{50} values differ due to relative nuclear input, not absolute nuclear number or heterokaryon size. Moreover, we have determined that the more muscle nuclei a heterokaryon contains, the less diffuse and more localized is the 5.1H11 antigen in the vicinity of the human nucleus responsible for its production (Pavlat, G. K., and H. M. Blau, manuscript in preparation). Thus, 5.1H11 is readily detectable even in large multinucleated heterokaryons. We conclude that the differences in the rates of accumulation of muscle gene products reflect differences due to nuclear ratio, rather than limited detection due to cell size.

Nuclear Position and Kinetics of Gene Expression

We examined whether the position of the human nonmuscle nucleus relative to the muscle nuclei within a heterokaryon influenced either the rate or the ultimate potential to express the human muscle antigen, 5.1H11. For this purpose, only

heterokaryons containing a single nonmuscle nucleus were analyzed to ensure that the expression of 5.1H11 could only have resulted from the activation of that nucleus. A total of 512 heterokaryons with a single nonmuscle nucleus were separated into two groups: those bordered unilaterally and those bordered bilaterally by muscle nuclei. As shown in Fig. 4, the frequency of gene expression in these groups of heterokaryons was analyzed as a function of time after heterokaryon production. The results indicate that the position of the nonmuscle nucleus relative to the muscle nuclei in a heterokaryon significantly affects the frequency of 5.1H11 expression at early time points. In fact, except for the 1.5-h time point, the rate of 5.1H11 accumulation is significantly reduced for bilaterally bordered nuclei relative to unilaterally bordered nuclei. This is not due to the fact that the products of unilaterally bordered nuclei are more readily detected due to limited diffusion and less rapid dilution over the surface of the membrane. First, unilaterally bordered nuclei were frequently positioned in the center of the myotube, rather than at a distal end. Second, as described above, when two or more muscle nuclei are present in a heterokaryon, the 5.1H11 gene

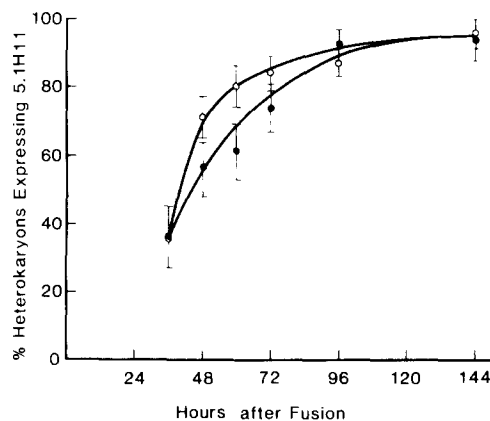


Figure 4. Effect of nonmuscle nuclear position on expression of 5.1H11. Heterokaryons with only one nonmuscle nucleus and different numbers of muscle nuclei were grouped according to nuclear position: nonmuscle bordered bilaterally or unilaterally by muscle nuclei. The expression of 5.1H11 was analyzed on different days after fusion. The number of heterokaryons analyzed was 307 unilaterally bordered and 205 bilaterally bordered. The rate of accumulation of 5.1H11 for bilaterally bordered nuclei (●) is slower than that of unilaterally bordered nuclei (○) at early time points. The mean values \pm standard error of the proportion (see Materials and Methods) are shown at each time point. Where error bars do not overlap, differences are significant at the 0.05 level using a two-sample Student's t test.

Table I. Influence of Nuclear Number and Nuclear Ratio on Kinetics of Gene Expression

	Absolute number of nuclei (Total: 4 nuclei)			Ratio of nuclei (Total: 2 to 20 nuclei)		
	Muscle + nonmuscle	Heterokaryons scored	t_{50} *	Muscle:non-muscle	Heterokaryons scored	t_{50} *
A. Equivalent	2 + 2	154	40	1:1	953	38
B. Excess muscle	3 + 1	127	48	3:1	206	47
C. Excess nonmuscle	1 + 3	109	52	1:3	149	55

* The t_{50} values (time in hours required for 50% of heterokaryons of a given nuclear composition to express 5.1H11) were calculated from computer-derived curves (see Materials and Methods). The kinetics of expression of 5.1H11 did not differ significantly between heterokaryons grouped according to absolute number and ratio of nuclei. However, for each group the values in A differed significantly from those in B and C ($P < 0.05$).

product becomes localized and more readily detectable irrespective of nuclear position.

Discussion

In the studies presented here, we report that gene dosage does not affect the high ultimate potential for gene activation (>75%), but markedly affects the kinetics of expression of a eukaryotic gene characteristic of muscle. This kind of kinetic analysis of differentiation-specific gene expression as a function of gene dosage or nuclear ratio has not been previously demonstrated in mammalian cells. The present study was made possible by the development of single cell assays for nuclear identification and gene expression and their application to an analysis of stable fusion products in which the entire chromosome complement and cytoplasm of each cell type remains present for up to 2 wk. The findings are unexpected. The rate of expression is relatively fast in heterokaryons with equal or near-equal numbers of nuclei (1:1 or 2:1) and slower when the relative number of either nonmuscle or muscle nuclei is increased (1:>2 and >2:1). The results have proved consistent for heterokaryons containing three different strains of human fibroblasts derived from fetal lung and from fetal and adult skin (Pavlath, G. K., C.-P. Chiu, and H. M. Blau, manuscript in preparation). The kinetics are not affected by heterokaryon size, but are affected by the position of the nonmuscle nucleus relative to muscle nuclei within heterokaryons. A limitation of this analysis is that the gene product studied was a cell surface antigen. Accumulation of this antigen could be affected by transcription, translation, and subsequent processing steps. However, our results may well reflect a gene dosage relationship at the transcriptional level; in other studies of gene expression in mass cultures of heterokaryons, the kinetics of accumulation of the cell surface antigen 5.1H11 and of transcripts of the α -cardiac actin gene were similar (Pavlath, G. K., C.-P. Chiu, and H. M. Blau, manuscript in preparation), our results are compatible with the following hypothesis: muscle gene expression is finely controlled by the concentration of *trans*-acting regulatory molecules.

Human muscle gene expression is detected in heterokaryons in which the nonmuscle nuclei greatly outnumber the muscle nuclei. Even in heterokaryons in which the ratio of mouse muscle to human fibroblast nuclei is 1:6, the expression of the human muscle antigen, 5.1H11, is observed. However, the rate of accumulation of gene products in these heterokaryons is consistently slower than that in heterokaryons containing equal numbers of both types of nuclei. This kinetic difference suggests that in heterokaryons with an increased number of nonmuscle nuclei, *trans*-acting regulatory molecules are limiting, because they must be shared. Increased time is required either for the limited number of molecules to find their appropriate binding sites or for the synthesis and accumulation of the requisite number of molecules. These molecules must be activators or de-repressors. Accordingly, a previously proposed model for differential gene expression in the development of phenotypes which is based solely on an irreversible repression of genes (5) cannot account for our findings. We conclude that one muscle nucleus can produce sufficient amounts of regulatory molecules to activate the 5.1H11 gene(s) in one or more nonmuscle nucleus and that the time required for gene expression depends on the concen-

tration of these molecules.

Most surprising is the finding that when muscle nuclei outnumber fibroblast nuclei, the rate of 5.1H11 accumulation is consistently slower than when both are present in equal numbers. This is contrary to expectation, since the greater the proportion of muscle nuclei in a heterokaryon, the more genes coding for *trans*-acting regulatory molecules. This should lead to an increase in the concentration of molecules per cell and a consequent increase in the rate of accumulation of gene products. Alternatively, it is possible that the amount of regulatory factor per nucleus is reduced in heterokaryons containing an increased number of muscle nuclei due to a decrease in factor production during differentiation. As a result, more time would be required for a limited number of molecules to find their target sites. It is also possible that the results with increased muscle nuclei are due to a requirement for two different *trans*-acting regulatory factors, one contributed by each of the two fused cell types. Accordingly, the nonmuscle cell might provide a factor that is necessary for its nucleus to respond to the muscle-activating factor. This would explain why the most rapid kinetics are observed when the stoichiometry or nuclear input from the two cell types is equivalent and balanced. Finally, it is theoretically possible that an increased concentration of regulatory factors could result in a slower accumulation of muscle gene products. This possibility is supported by the data presented in Fig. 4, in which it is shown that the rate of 5.1H11 expression at early time points is slower when nonmuscle nuclei are bordered bilaterally by muscle nuclei than when they are unilaterally bordered. Accordingly, nonmuscle nuclei that are flanked on both sides by muscle nuclei might be exposed to higher concentrations of regulatory factors that are inhibitory. An inhibition of transcription would occur if the factors bound to multiple sites with different affinities and ultimate effects on gene expression. These different possibilities will soon be directly testable. Given the evidence described below, we find this last hypothesis most appealing.

In other systems, *trans*-acting regulatory molecules affect gene expression in a dose-dependent manner and in some cases have opposite effects at different concentrations. The T-antigen of SV40 and the repressor of bacteriophage lambda accumulate to a threshold concentration and bind to specific DNA sites with different effects (16, 17, 19, 24, 25). The sequence of binding and ultimate effect on gene expression is the consequence of cooperative interactions as well as differential affinities of the molecules for DNA sites. Of particular interest are lambda repressor and the E1A products of adenovirus-2, which at different concentrations act either as positive or negative regulators of gene transcription (3, 16, 19). In eukaryotes, concentration-dependent effects of regulatory factors on gene expression have also been reported. Gene dosage affects the synthesis of proteins required for phosphate utilization in *Neurospora* heterokaryons (15). In rat hepatoma cells, multiple glucocorticoid hormone-receptor complexes regulate the rate of murine mammary tumor virus transcription *in vivo* by binding to several distinct enhancer sequences, possibly with different consequences (Yamamoto, K., personal communication). The change from oocyte to somatic gene expression during *Xenopus* development is due to a combination of differential affinity for these genes for a 40-kD transcription factor and of a marked decrease in factor concentration (29, 30).

Our analyses of the effects of gene dosage on muscle antigen expression in heterokaryons suggest that the expression of muscle genes is also modulated by the concentration of *trans*-acting factors. A threshold concentration is required but at high concentrations the factors may be inhibitory. These results suggest that the differential control of gene expression underlying cell specialization in eukaryotes, like the examples cited above, may be regulated by a combination of changes in factor concentration and binding affinity to related but distinct DNA sequences with different functions.

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