Muscle cell components dictate hepatocyte gene expression and the distribution of the Golgi apparatus in heterokaryons

Steven C. Miller,^{1,2} Grace K. Pavlath,^{1,3} Bruce T. Blakely,¹ and Helen M. Blau¹

¹Department of Pharmacology, Stanford University School of Medicine, Stanford, California 94305-5332 USA

Major changes in cytoarchitecture and gene expression were induced in short-term heterokaryons. When human hepatocytes were fused with mouse muscle cells, the hepatocyte Golgi apparatus changed from its usual polar location to a uniformly circumnuclear location typical of striated muscle. Human liver albumin ceased to be expressed, and expression of the human muscle cell-surface antigen 5.1H11 was induced without DNA replication or cell division. Coexpression of liver and muscle proteins was rarely observed. These novel findings provide insight into the regulation of gene expression and the targeting and localization of organelles with a central role in cell polarity, intracellular transport, and secretion.

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To understand the regulation of tissue-specific genes in differentiated cells, a number of approaches are being employed. The transfection of DNA into mammalian cultured cells has been particularly useful in the analysis of gene structure and function. Such studies have helped to define the *cis*-acting DNA sequences that regulate tissue-specific gene expression. These sequences can, in turn, be used in some cases to purify the *trans*-acting factors that act directly to alter their expression (Kadonaga and Tjian 1986). However, a transfected gene differs from its endogenous counterpart in its responsiveness to regulatory factors (Robins et al. 1982; Weintraub 1985). This has been demonstrated most recently in experiments in which stably transfected prolactin genes, but not their endogenous counterparts, were activated in heterokaryons (Lufkin and Bancroft 1987). An understanding of the mechanisms underlying the regulation of endogenous genes should provide insight into the derivation and maintenance of differentiated cells during development.

Of central importance to the function of the differentiated cell is the location and interaction of organelles that direct intracellular traffic. In most animal cells, the Golgi apparatus and microtubule organizing center (MTOC) are colocalized toward one side of the nucleus

at the centrioles of the centrosome (Rogalski and Singer 1984). This probably provides the polarity necessary for mitosis and motility (Singer and Kupfer 1986). In contrast, in differentiated striated muscle cells where this type of polarity is not required, the centrioles appear to be nonfunctional and the Golgi and MTOC have a different location, the vicinity of the nuclear membrane (Tassin et al. 1985a,b; Kronebusch and Singer 1987). Thus, in muscle, the factor(s) that targets or localizes the Golgi and MTOC is dissociated from the centrioles. In addition, the nature of the interaction between the MTOC and Golgi is altered (Louvard et al. 1982; Rogalski and Singer 1984; Tassin et al. 1985b).

Heterokaryons provide one approach to studying the regulation of endogenous genes and the targeting of organelles during differentiation (Blau et al. 1983, 1985). Because the nuclei in heterokaryons, or short-term non-dividing somatic cell hybrids, remain separate and intact, the interactions between two genomes derived from cells of different functional states can be examined within a common cytoplasm. Our laboratory has used heterokaryons previously to demonstrate that nine different endogenous muscle genes can be activated in a wide range of nonmuscle cell types in response to *trans*-acting factors present in muscle cells (Blau et al. 1983, 1985; Chiu and Blau 1984, 1985; Hardeman et al 1986; Pavlath and Blau 1986). Our findings have been corroborated and extended by others using similar muscle heterokaryons (Wright 1984a,b; Clegg and Hauschka 1987) and heterokaryons produced with other cell types (Baron

²Current address: Department of Medicine, Division of Oncology, Stanford University Medical Center, Stanford, California 94305 USA. ³Current address: Department of Molecular and Cellular Biology 308 BSW, University of Arizona, Tucson, Arizona 85721 USA.

and Maniatis 1986; Enoch et al. 1986). These observations demonstrate that the heterokaryon state provides an experimental system that is well suited to the study of cellular factors that mediate the selective expression or repression of endogenous genes which accompanies cell specialization.

In the present study, for the first time we addressed questions regarding the fate of the nonmuscle cell type after fusion with a muscle cell. In the course of cell specialization during the development of the organism, cells make a choice and the expression of different differentiated states is generally mutually exclusive. We determined whether this was also the case in heterokaryons by examining whether two differentiated phenotypes were coexpressed in the same cell. Toward this end, we monitored the expression of both muscle and liver gene products and the distribution of the Golgi apparatus, which mediates their intracellular transport. Our results demonstrate that muscle components alter hepatocyte gene expression and induce major changes in cytoarchitecture involving the relocation of organelles of central importance to cell polarity, intracellular transport, and secretion.

Results

The hepatocyte Golgi distribution changes to a muscle Golgi distribution

The Golgi apparatus has a characteristic distribution that differs between differentiated striated muscle cells

and other animal cell types (Tassin et al. 1985a,b; Kronbusch and Singer 1987). In hepatocytes, like undifferentiated myoblasts, the Golgi is asymmetrically localized toward one pole of the cell (Fig. 1A), whereas in differentiated skeletal muscle cells the Golgi is compactly circumnuclear and located in close proximity to the nuclear envelope (Tassin et al. 1985b). Heterokaryons were formed by fusing human hepatocytes of the Hep G2 line and mouse muscle C2C12 cells. Individual cells were analyzed to determine whether hepatocyte nuclei contained in a heterokaryon retained a Golgi distribution that was typical of hepatocytes or changed to one typical of striated muscle. For this purpose we used an antibody to a Golgi enzyme, galactosyl transferase, which is localized in the *trans* cisternae of the Golgi stack and readily visualized by immunofluorescence (Berger et al. 1981; Berger and Hesford 1985; Lucocq et al. 1987).

Two types of circumnuclear Golgi distribution were detected in the 115 heterokaryons analyzed 6 days after fusion. A compact circumnuclear Golgi, similar to that observed in differentiated skeletal muscle cells, was found in the vicinity of all nuclei in heterokaryons that contained more muscle than hepatocyte nuclei (Fig. 2A). This compact circumnuclear Golgi distribution was not due to constraints in organelle movement imposed by the elongated shape of the differentiated muscle cell. It was also observed in large, amorphous heterokaryons that contained greater numbers of muscle than hepatocyte nuclei (Fig. 2B). The second type of Golgi distribution that was observed in heterokaryons was also circumnuclear but diffuse in appearance (Fig. 2C). This diffuse circumnuclear distribution was typical of

Figure 1. The hepatocyte Golgi apparatus is polar. In greater than 98% of individual hepatocytes, the Golgi is oriented at one side of the nucleus (A). In hepatocytes fused to themselves as homokaryons, the Golgi typically aggregates and retains a polar distribution at one end of the multinucleated cell (B). The same cells are shown stained with Hoechst 33258 to show nuclei (*left*) and by immunofluorescence with an antibody to galactosyl transferase to show the position of the Golgi apparatus (*right*). Magnification, 530 $\times$.

Figure 2. The hepatocyte Golgi apparatus changes from a polar to a circumnuclear distribution after fusion with muscle to form heterokaryons. Heterokaryons with an increased proportion of muscle nuclei have a compact circumnuclear Golgi typical of striated muscle, regardless of whether the cell shape is elongated (A) or amorphous (B). Heterokaryons with an equal or increased proportion of nonmuscle nuclei have a diffuse circumnuclear Golgi (C). The same cells are shown stained with Hoechst 33258 to distinguish punctate mouse muscle and uniform human hepatocyte nuclei (*left*) and by immunofluorescence with an antibody to galactosyl transferase to show the position of the Golgi apparatus (*right*). The heterokaryon in A has a total of two mouse and one human nuclei; B is part of a heterokaryon with eight mouse and seven human nuclei; and C is a heterokaryon with one mouse and four human nuclei. Magnification, 425 $\times$.

heterokaryons that contained a greater number of hepatocyte nuclei, or an equivalent number of nuclei of each cell type.

Both types of circumnuclear Golgi distribution observed in heterokaryons contrasted sharply with the polar distribution observed in Hep G2 cells alone (Fig. 1). The change from a hepatocyte-like to a muscle-like Golgi distribution could not be induced by growth arrest or the formation of a multinucleated Hep G2 cell. Hep G2 cells were fused to themselves with polyethylene glycol (PEG) and maintained for 6 days in the presence of the DNA synthesis inhibitor cytosine arabinoside (ara-c), a treatment that eliminates dividing cells. The Golgi

apparatus in the resulting homokaryons was polar, forming a single aggregate at one end of the cell. In contrast, the presence of a single muscle nucleus resulted in a major disruption of the characteristic polar Golgi aggregate derived from multiple fused hepatocytes. This is shown most clearly by the strikingly different Golgi distributions in homokaryons (Fig. 1B) and in a heterokaryon containing four hepatocyte nuclei and only one muscle nucleus (Fig. 2C).

Influence of gene dosage on the Golgi distribution

The type of circumnuclear Golgi distribution observed

in heterokaryons was influenced by the relative numbers of muscle and hepatocyte nuclei, or the gene dosage contributed by each cell type. As shown in the histogram in Figure 3, a compact circumnuclear Golgi distribution was found in $68 \pm 8\%$ of heterokaryons in which muscle nuclei predominated. In contrast, only $22 \pm 5\%$ of heterokaryons that contained equivalent numbers of muscle and hepatocyte nuclei, or in which there was an increased proportion of hepatocyte nuclei, had a compact circumnuclear Golgi, whereas $70 \pm 5\%$ had a diffuse circumnuclear Golgi. A polar Golgi typical of hepatocytes alone was rarely observed. Less than 10% of all heterokaryons had a polar Golgi. These results suggest that muscle cell components dictate in a dosage-dependent manner the distribution of a secretory organelle in heterokaryons containing muscle and hepatocyte nuclei.

Activation of human muscle 5.1H11 and extinction of human albumin

We examined the expression of the human liver protein albumin and the human muscle cell-surface antigen 5.1H11 in 2687 individual heterokaryons. Examples of heterokaryons that expressed either albumin or 5.1H11 6 days after fusion are shown in the same field in Figure 4 (A–D). The expression of albumin and 5.1H11 was monitored simultaneously in heterokaryons by double immunofluorescence microscopy using species-specific antibodies. In the heterokaryon on the right side of the field, only 5.1H11 is detected, and its distribution is typical of a cell-surface antigen (Fig. 4C). Because 5.1H11 is never detected in either cell type alone or in homokaryons, its expression in heterokaryons appears to result from gene activation by *trans*-acting regulators (Chiu and Blau 1984, 1985). In the heterokaryon on the left side of the field, only albumin is expressed, and it is localized in the vicinity of the Golgi apparatus (Fig. 4D). Albumin is a secreted protein that exits the cell within 30 min after its synthesis (Feldhoff et al. 1977). To detect albumin in our experiments, heterokaryons were exposed for several hours prior to fixation to the ionophore monensin, which blocks exocytosis in the Golgi apparatus (Tartakoff 1983). Therefore, the albumin detected in the experiments presented here was newly synthesized. Accordingly, the heterokaryon on the left side of the micrograph (Fig. 4D) is an example of one in which the translation of mRNA encoding liver albumin continued to occur for 6 days after fusion with muscle.

An example of a heterokaryon that expressed both human muscle 5.1H11 and human albumin is shown in Figure 4 (E–H). This was a very rare event; only 28 heterokaryons coexpressed both the muscle and liver proteins, a frequency of approximately 1%. Of these, only four contained a single human nucleus; the others, like the one shown in Figure 4 (E–H), had multiple human nuclei. Thus, only in four cases, or 0.2% of all heterokaryons analyzed, did the two types of proteins definitively derive from the same nucleus.

We examined the expression of the human muscle and liver proteins in heterokaryons over time. Duplicate

Figure 3. The Golgi distribution is influenced by gene dosage in heterokaryons. The frequency of polar (□), diffuse circumnuclear (■), and compact circumnuclear (▨) distributions of the Golgi apparatus was correlated with the ratio of muscle to hepatocyte nuclei. A total of 115 heterokaryons was analyzed. The data are expressed as the mean $\pm$ the standard error of the proportion.

dishes of heterokaryons, control Hep G2, and C2C12 homokaryons were assayed at 0.25, 2, 4, 6, 8, and 11 days after fusion for both 5.1H11 and for albumin. As shown in Figure 5, the activation of the human muscle antigen 5.1H11 was first detectable on day 2, and the frequency with which it was expressed increased thereafter, reaching a maximum of $36 \pm 2\%$ of all heterokaryons by day 6. In contrast, the extinction of human albumin in heterokaryons was rapid. Only $65 \pm 3\%$ of all heterokaryons contained detectable amounts of albumin 6 hr after fusion, the first time point that could be assayed. The marked reduction in the number of heterokaryons that produced albumin was not a direct result of the fusion procedure or the culture conditions, because greater than 95% of homokaryon Hep G2 cells stably synthesized albumin for the duration of the time course. At any given time point, the frequency of heterokaryons that coexpressed both liver and muscle proteins was low. Albumin expression in heterokaryons continued to decline steadily. By day 11 after fusion, 99% of the heterokaryons analyzed no longer produced albumin. Thus, virtually all hepatocytes in heterokaryons extinguished the synthesis of human liver albumin, and in one third of the heterokaryons the human muscle gene for 5.1H11 was activated.

Influence of gene dosage on 5.1H11 and albumin expression

The kinetics of muscle 5.1H11 expression by hepatocyte nuclei were correlated with the proportion of muscle nuclei in heterokaryons. Heterokaryons were pooled

Figure 4. (A–D) Two heterokaryons are shown in the same field that expressed either the liver protein albumin (*left*) or the muscle cell-surface antigen 5.1H11 (*right*) 6 days after fusion. (E–H) A single heterokaryon that coexpressed both human muscle 5.1H11 and liver albumin. (A,E) Heterokaryons in phase-contrast microscopy; (B,F) the same heterokaryons with Hoechst fluorescence to distinguish human (uniform) or mouse (punctate) nuclei; (C,G) Texas Red immunofluorescence to detect the muscle cell-surface antigen, 5.1H11; (D,H) fluorescein immunofluorescence to detect liver albumin. The position of the Golgi apparatus, determined by the distribution of albumin, is not as compactly circumnuclear as that in Fig. 2. This is due, in part, to the presence of albumin in the rough endoplasmic reticulum and secretory vesicles and to the effects of monensin, which was used only to visualize albumin. Monensin inhibits secretion and causes dramatic swelling of the Golgi in both muscle and nonmuscle cells (Tassin et al. 1985b). Magnification, 300 $\times$.

Figure 5. Time course of muscle 5.1H11 and liver albumin expression in heterokaryons. The frequency of expression in heterokaryons of 5.1H11 (—●—), albumin (—■—) or both 5.1H11 and albumin (—●—) is shown. Control hepatocytes fused to themselves as homokaryons continue to express albumin (—△—). More than 200 heterokaryons were analyzed per dish; duplicate dishes were analyzed at each time point. The data are expressed as the mean $\pm$ the standard error of the proportion.

into five groups according to nuclear ratio, or the relative number of muscle (mouse) to hepatocyte (human) nuclei they contained. The proportion of heterokaryons of a given nuclear ratio that expressed either albumin or 5.1H11 at different time points after fusion is shown in Figures 6 and 7. The fastest kinetics were observed when the number of muscle nuclei exceeded the number of hepatocyte nuclei, and the slowest kinetics were observed when the converse was true. In contrast, in heterokaryons with fibroblasts, the fastest kinetics of 5.1H11 expression were observed with equivalent nuclear input (Pavlath and Blau 1986).

In addition, as described previously (Blau et al. 1985), the ultimate frequency of 5.1H11 activation in heterokaryons showed a striking dependence on nuclear ratio. At most, $57 \pm 4\%$ of heterokaryons containing a greater proportion of muscle nuclei activated 5.1H11. Heterokaryons with equivalent nuclear input had a peak frequency of $37 \pm 3\%$, and those containing a greater proportion of hepatocyte nuclei never exceeded a frequency of 5.1H11 activation of $11 \pm 3\%$. In contrast, in heterokaryons with fibroblasts, early differences in the frequency of gene activation due to gene dosage disappeared with time, and eventually 95% of heterokaryons expressed 5.1H11, irrespective of nuclear ratio (Pavlath and Blau 1986).

The kinetics of albumin extinction were also dependent on gene dosage. At early time points, heterokaryons with an increased proportion of hepatocyte nuclei continued to express albumin at a higher frequency than those with an increased proportion of muscle nuclei. However, by later time points, almost no albumin was detectable in heterokaryons, regardless of nuclear ratio. Even when hepatocyte nuclei outnumbered muscle nuclei, albumin ceased to be synthesized. These results suggest that the muscle phenotype is dominant and that the extinction of albumin in heterokaryons is due to one or more muscle-derived factors.

DNA replication is not required for 5.1H11 activation, albumin extinction, or Golgi redistribution

We determined at the single-cell level whether DNA replication was required for the activation of 5.1H11, the extinction of albumin, or the change in Golgi distribution in heterokaryons. We had shown previously that DNA synthesis could be inhibited by 98% in heterokaryons produced with fibroblasts without affecting the frequency of 5.1H11 activation (Chiu and Blau 1984). The slower kinetics of expression of 5.1H11 in heterokaryons containing hepatocytes suggested that the mechanism of activation might differ from that in fibroblasts (Blau et al. 1985). Possibly, changes in chromatin structure associated with DNA replication occurred during this period of time. To address this point at the single-cell level, hepatocytes were exposed to the DNA synthesis inhibitor, ara-c, for 24 hr prior to and continuously after fusion with muscle cells. Six days after fusion, the cells were assayed and compared with controls.

A total of 957 individual heterokaryons were analyzed for DNA synthesis by autoradiography and for the expression of 5.1H11 and albumin by double immunofluorescence. As shown in Table 1, the presence or absence of ara-c did not significantly alter the frequency of expression of either protein. It did, however, have a significant effect on the level of DNA synthesis. In contrast to controls, none of the hepatocyte heterokaryons that activated 5.1H11 and less than 10% of the heterokaryons that extinguished albumin had any detectable DNA synthesis in the presence of ara-c. Not a single hepatocyte nucleus inside a heterokaryon underwent a complete round of DNA replication in the presence of ara-c. ara-c was also present in the experiments in which the Golgi changed from a polar to a circumnuclear location. These data suggest that DNA replication is not a prerequisite for 5.1H11 activation, albumin extinction, or the change in Golgi distribution that occurs in heterokaryons.

Figure 6. Time course of effect of gene dosage on albumin or 5.1H11 expression in heterokaryons. A total of 2687 individual heterokaryons were grouped according to nuclear composition, or the number of mouse and human nuclei they contained. Five different groups were established that included the following ratios of mouse (muscle) to human (hepatocyte) nuclei: 1 : > 2 ($\square$); 1 : 2 ($1 : x$ where $1 < x \leq 2$) (diagonal lines); 1 : 1 (dots); 2 : 1 ($x : 1$, where $1 < x \leq 2$) (cross-hatch); and >2 : 1 ($\blacksquare$). The proportion of heterokaryons with different nuclear compositions gave a Gaussian distribution with the 1 : 1 ratio most frequent. The data are expressed as the mean $\pm$ the standard error of the proportion.

Discussion

In this study we address questions regarding the fate of the nonmuscle cell in heterokaryons. Previous experiments had demonstrated that upon fusion with muscle cells a pattern of gene expression typical of muscle could be induced in a range of mesodermal, ectodermal, and endodermal cell types, including fibroblasts, keratinocytes, adrenal cells, neural cells, and hepatocytes, that normally never express muscle genes (Blau et al. 1983, 1985; Wright 1984a,b; Hardeman et al. 1986). In this report, the possibility that muscle factors alter the expression of the other phenotype—in this case, the hepatocyte—was examined. We determined the influence of muscle cell components on hepatic gene expression and cytoarchitecture, in particular, the disposition of the Golgi apparatus, an organelle with a central role in cell polarity, intracellular transport, and secretion.

The change in Golgi location signifies a major change in cytoarchitecture

After fusion and heterokaryon formation, the Golgi changed from its typical polar location at one side of the hepatocyte nucleus to a uniform circumnuclear location characteristic of striated muscle cells. The change in Golgi location was not caused by growth arrest or the multinucleated state. In homokaryons of Hep G2 cells, the Golgi derived from several nuclei were found clustered together at one end of the cell in a single aggregate (Fig. 1B), similar to the aggregates of centrosomes described by others in homokaryons of either hamster kidney BHK21 cells or HeLa cells (Wang et al. 1979; Tassin et al. 1985a). Instead, the circumnuclear location of the Golgi appears to be a specific property of differentiated striated muscle cells, as it is also found in mononucleated cardiac but not smooth muscle cells (Kronebusch and Singer 1987).

The change from a polar to a circumnuclear Golgi has broad implications for intracellular organization. The Golgi plays a central role in directing macromolecular traffic within the cell and in determining cell polarity. It

is essential to the intracellular transport and processing of membrane components such as muscle 5.1H11 and secretory proteins such as liver albumin. In dividing cells, like myoblasts or hepatocytes, the Golgi apparatus and the MTOC from which microtubules originate are colocalized toward one side of the nucleus at the centro-

Figure 7. Influence of nuclear composition on the kinetics of albumin extinction and 5.1H11 expression. The same heterokaryons analyzed in Fig. 6 were combined into five groups. (Top two groups): (—○—) increased or equivalent proportion of muscle nuclei ($\geq 1 : 1$); (---●---) increased proportion of hepatocyte nuclei ($1 : > 1$). (Bottom three groups): (—○—) increased proportion of muscle nuclei ($> 1 : 1$); (---□---) equivalent nuclear input ($1 : 1$); (---●---) increased proportion of hepatocyte nuclei ($1 : > 1$). The data are expressed as the mean $\pm$ the standard error of the proportion.

Table 1. Activation of 5.1H11 and extinction of albumin in heterokaryons occurs in the absence of DNA replication

Ara C	Total heterokaryons scored	Heterokaryons that activated 5.1H11 (%)	Heterokaryons that extinguished albumin (%)	Heterokaryons that activated 5.1H11 and have labeled human nuclei (%)		Heterokaryons that extinguished albumin and have labeled human nuclei (%)	
				>10 grains	>50 grains	>10 grains	>50 grains
–	461	23 ± 2	92 ± 1	23 ± 4	6 ± 2	40 ± 2	16 ± 2
+	496	19 ± 2	90 ± 1	0	0	7 ± 1	0

Heterokaryons were scored 6 days after fusion. A human hepatocyte nucleus that replicated one-fifth of its genomic DNA could be detected by our methods (Chiu and Blau 1984). A nucleus was scored as labeled if it had 10 or more silver grains over it, a level threefold above background. A nucleus with more than 50 grains over it was scored as heavily labeled and considered likely to have undergone a complete round of replication. The results were expressed as the mean ± standard error of the proportion.

some, composed of a pair of centrioles and pericentriolar material (Tassin et al. 1985a; Petit et al. 1986; Singer and Kupfer 1986). In contrast, in differentiated striated muscle cells, the Golgi apparatus and MTOC are no longer centered on the centrioles but circle the nucleus at a uniform distance of 0.2 μ m (Tassin et al. 1985b). Indeed, in differentiated skeletal muscle cells, unlike most animal cells, the centrioles appear to be reduced in number, dispersed, and probably nonfunctional (Tassin et al. 1985a). The finding that the centrioles can be dissociated from the MTOC and Golgi suggests that their primary role is to establish the polarity necessary for elaborating astral microtubules for mitosis and cell movement (Singer and Kupfer 1986). Like muscle, cells that do not require such polarity have a different target for these organelles, the vicinity of the nuclear membrane. The relationship between the Golgi and the MTOC is also altered in differentiated muscle. A nocodazole-induced depolymerization of microtubules leads to a reversible dispersion of the polar but has no effect on the circumnuclear Golgi apparatus (Louvard et al. 1982; Rogalski and Singer 1984; Tassin et al. 1985b).

The heterokaryon studies described here demonstrate that by fusing disparate cell types, the function and interrelationship of cell organelles can be altered dramatically. This change occurs within a few days in the absence of cell division or DNA replication. These results raise the intriguing possibility that there may be one or more muscle components or *trans*-acting factors capable of targeting the MTOC and Golgi to the nuclear periphery, independent of the centrioles. By liposome or red blood cell-mediated fusion, osmotic lysis of pinocytic vesicles, or cellular microinjection (Capecchi 1980; Szoka et al. 1981; Okada and Rechsteiner 1982; Godfrey et al. 1983), it should now be possible to introduce muscle macromolecules into the responsive hepatocytes described here and test this hypothesis directly.

Muscle proteins are expressed and liver proteins are extinguished

Our results suggest that in heterokaryons, as in the developing organism, the expression of one phenotype precludes the expression of the other. The coexpression of the human liver product albumin and the novel induced human muscle cell-surface antigen 5.1H11 in the same cell occurred with a frequency of 1% in the 2687

heterokaryons analyzed. Thus, it appears that the simultaneous expression of liver and muscle products within a heterokaryon is the exception to the rule and may never occur at the transcriptional level in a single nucleus.

Gene dosage influenced the time course of extinction and activation in heterokaryons, suggesting that the balance of positive and negative factors contributed by muscle and liver cell types was critical. Gene dosage also influenced the ultimate frequency of activation in heterokaryons with hepatocytes, in contrast to previous findings with fibroblasts (Pavlati and Blau 1986). Extinction, on the other hand, was ultimately observed in virtually all heterokaryons, even those in which hepatocyte nuclei outnumbered muscle nuclei, suggesting that the continuous production of factors only by muscle was sufficient. Although the extinction of albumin is likely to be mediated, in part, by post-transcriptional mechanisms (Clayton et al. 1985a,b; Bachmair et al. 1986), transcriptional mechanisms presumably play an important role. That gene loci isolated from fibroblasts are capable of stably producing *trans*-acting repressors of liver functions has been demonstrated by both Killary and Fournier (1984) and Petit et al. (1986). To date, this has been achieved by chromosome transfer, not by transfection of naked DNA, possibly because an intact fibroblast chromatin structure is required. Presumably, a similar *trans*-dominant locus in muscle cells could cause the extinction of albumin seen in our experiments, despite the presence of positive regulatory factors contributed by hepatocytes (Courtois et al. 1987).

Studies that examine the requirements for the expression of endogenous genes, like those presented here, are central to understanding differentiation. Although much has been learned regarding tissue-specific gene expression from the transfection of well-characterized DNA sequences, it is clear that the heritable changes associated with the stable integration of transfected genes and the heritable changes that accompany determination during development differ. For example, in heterokaryons produced by fusing pituitary cells and fibroblasts stably transfected with a silent prolactin gene promoter construct, the transfected but not the endogenous prolactin gene was activated (Lufkin and Bancroft 1987). The need to study the regulation of endogenous genes is underscored by these and other findings (Robins et al. 1982; Weintraub 1985). The heterokaryons described

here provide a means for inducing changes in endogenous gene expression that can be analyzed at a molecular level. The elucidation of the different requirements for the extinction and activation of endogenous genes in a specialized cell type such as the hepatocyte should provide insight into the derivation and maintenance of that phenotype in the course of human development.

Methods

Cells and culture conditions

The mouse muscle cell line C2C12 has been described previously [Yaffe and Saxel 1977; Blau et al. 1983]. Hepatocytes (Hep G2), a nontumorigenic human hepatoma cell line, were a generous gift of Dr. Barbara Knowles [Aden et al. 1979]. These cells stably express albumin in addition to 16 other liver-specific proteins [Knowles et al. 1980]. Hep G2 cells were grown in minimal Eagle's medium (MEM) with 10% fetal calf serum but plated in C2C12 fusion medium (FM) containing Dulbecco's modified Eagle's medium (DMEM) with 2% horse serum for homokaryon and heterokaryon studies.

Production of heterokaryons

Heterokaryons and homokaryons were produced as described previously [Blau et al. 1983; Chiu and Blau 1984]. Mouse muscle C2C12 cells were fused with Hep G2 hepatocytes using PEG 1000 (BDH). Except in studies of DNA synthesis (see below), ara-c, 10^{-5} M was routinely added 24 hr after fusion together with ouabain, 10^{-5} M to eliminate unfused and proliferating cells.

Immunofluorescence assay of Golgi apparatus

To study the distribution of the Golgi apparatus, an affinity-purified rabbit polyclonal antibody (O_{12}) to the human Golgi enzyme galactosyl transferase was used, which was the generous gift of Dr. Eric Berger [Berger et al. 1981; Berger and Hesford 1985]. Monensin was not used in any experiments in which the Golgi distribution was analyzed with antibody O_{12} . All procedures were carried out at room temperature unless otherwise noted. Homokaryon and heterokaryon cells were fixed in 1.5% formaldehyde in phosphate-buffered saline (PBS) for 15 min, rinsed once with PBS, and incubated in 50 mM ammonium chloride in PBS for 10 min, followed by one rinse with PBS. The cells were then permeabilized with 0.1% saponin in PBS for 1 min and washed three times with PBS. Fixed cells were incubated with the antibody to galactosyl transferase at a 1 : 5 dilution in 0.5% bovine serum albumin (BSA) in PBS for 30 min. The cells were washed twice rapidly and then twice for 5 min each with 0.5% BSA and 0.5% Tween 20 in PBS. The cells were then incubated with a fluorescein-conjugated anti-rabbit IgG (Cappel Laboratories, Cooper Diagnostics, Inc., Malvern, Pennsylvania), diluted 1 : 200 in 0.5% BSA in PBS for 30 min, washed as before, and stained with Hoechst 33258 [Riedel-de Haën, Hannover, Germany]. As a control for antibody specificity, replicate dishes were stained with antibody to galactosyl transferase that had been mixed previously with purified human galactosyl transferase [Dr. E. Berger] for 2 days at 4°C. To reduce photobleaching of the fluorescein signal, the antioxidant, *n*-propyl gallate (Sigma Chemical Co., St. Louis, Missouri) was added at 2% (wt/vol) in 30% 0.1 M Tris, pH 9.0, in glycerol, as described by Giloh and Sedat [1982].

Immunofluorescence assays for albumin and 5.1H11

To detect albumin, cells were incubated at 37°C in medium containing 10^{-6} M monensin (Sigma) prior to fixation. Monensin is a carboxylic ionophore that blocks secretion in the Golgi by altering the Na^+-K^+ balance essential to this step of the pathway [Tartakoff 1983]. As a result, albumin accumulates inside the cell. In the absence of monensin, albumin was difficult to detect because it is secreted within 30 min of its synthesis [Feldhoff et al. 1977]. At each time point (0.25, 2, 4, 6, 8, and 11 days after fusion), replicate dishes of fused cells were labeled live with monoclonal antibodies for the detection of the human muscle cell-surface antigen 5.1H11. Monoclonal antibody 5.1H11 was generously provided by Dr. Frank Walsh [Walsh and Ritter 1981] and used as described previously [Chiu and Blau 1985]. The cells were then fixed with 1% formaldehyde in PBS for 20 min at 37°C. The cells were permeabilized with methanol ($-20^{\circ}C$) for 20 min. Monensin was present immediately before and throughout the staining procedure until the cells were fixed, for a total time of approximately 4 hr.

Following fixation and permeabilization, cells were stained for albumin with polyclonal goat anti-human albumin IgG from Cappel Laboratories (Cooper Diagnostics). To remove antibodies that cross-reacted with mouse albumin, the antibody was affinity purified on a column of mouse albumin coupled to Sepharose. The affinity-purified antibody was shown to be human-specific by its immunofluorescent staining of human hepatoma cells (Hep G2) and lack of staining of mouse hepatoma cells (Hepa1c1c7, a gift of J. Whitlock). Both cell types were shown to contain albumin by labeling with a non-species-specific anti-albumin antibody (data not shown). The fixed cells were incubated with the anti-human albumin antibody in 1% BSA in PBS for 30 min at 37°C. The dishes were washed twice rapidly and then twice for 5 min with 1% BSA and 0.5% Tween 20 in PBS. The cells were then incubated with a 1 : 100 dilution of a fluorescein-conjugated rabbit anti-goat IgG antibody (Zymed Laboratories, Inc., South San Francisco, California) in 1% BSA in PBS for 30 min at 37°C. The dishes were washed as described above and stained with Hoechst 33258 as described previously [Blau et al. 1983]. In the presence of Hoechst 33258, the nuclei can be distinguished: mouse nuclei appear punctate and human nuclei appear homogeneously stained.

Assay for DNA synthesis

Hepatocytes were labeled with [^3H]thymidine at 1.0 μ Ci/ml (83 Ci/mmole, NEN, DuPont Co., Wilmington, Delaware) for the first 48 hr after fusion. To inhibit DNA synthesis, cultures were exposed to 10^{-5} M ara-c for 24 hr prior to fusion and continuously thereafter until day 6 after fusion when the cells were fixed. Control cultures were not exposed to ara-c until 24 hr after fusion; at this point, addition of the inhibitor served to prevent proliferation and cell overgrowth. We have shown previously that a 1-hr exposure to ara-c at 10^{-5} M reduces incorporation of [^3H]thymidine into TCA-precipitable material to 1% of control levels [Chiu and Blau 1984]. Duplicate dishes of cells that had been labeled with antibodies for detection of 5.1H11 and albumin were processed for autoradiography, as described previously [Chiu and Blau 1984].

Statistics

In all cases, the error indicates 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.

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