Control of Myogenic Differentiation by Cellular Oncogenes

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Abstract

The establishment of a differentiated phenotype in skeletal muscle cells requires withdrawal from the cell cycle and termination of DNA synthesis. Myogenesis can be inhibited by serum components, purified mitogens, and transforming growth factors, but the intracellular signaling pathways utilized by these molecules are unknown. Recent studies have confirmed a role for proteins encoded by cellular proto-oncogenes in transduction of growth factor effects that lead to cell proliferation. To test the contrasting hypothesis that cellular oncogenes might also regulate tissue-specific gene expression in developing muscle cells, myoblasts have been modified by incorporation of the cognate viral oncogenes, the corresponding normal or oncogenic cellular homologs, and chimeric oncogenes, whose expression can be induced reversibly. Regulation of the endogenous cellular oncogenes also has been examined in detail. Down-regulation of c-*myc* is not obligatory for myogenesis; rather, inhibitory effects of *myc* on muscle differentiation are contingent on sustained proliferation. In contrast, activated *src* and *ras* genes block myocyte differentiation directly, through a mechanism that is independent of DNA synthesis and is rapidly reversible, resembling the effects of inhibitory growth factors. The coordinate regulation of diverse tissue-specific gene products including muscle creatine kinase, nicotinic acetylcholine receptors, sarcomeric proteins, and voltage-gated ion channels, raises the hypothesis that inhibitors such as transforming growth factor-β and *ras* proteins might exert their effects through a trans-acting transcriptional signal shared by multiple muscle-specific genes.

Index Entries: Oncogene; *src; ras; myc;* growth factors; myogenesis; skeletal muscle cells; differentiation; muscle creatine kinase; ion channels.

Introduction

In developing skeletal muscle cells, the relationship between proliferative growth and expression of tissue-specific gene products is seemingly reciprocal and mutually exclusive (Okazaki and Holtzer, 1966; Nadal-Ginard, 1978; Linkhart et al., 1980). This dichotomy of cell function has led to several contrasting lines of research that attempt to explicate the molecular mechanisms through which growth signals might delay, prevent, or even reverse the differentiated phenotype in muscle. Recently, the search for cellular machinery responsible for the transmembrane signaling of growth factor effects has implicated a set of so-called cellular proto-oncogenes, whose mutagenesis by a retrovirus, base change, or chromosomal translocation both can augment cells' ability to proliferate and also disrupt the normal differentiation program (Weiss et al., 1982; Land et al., 1983b; Varmus,1984; Bishop, 1985, 1987). Moreover, the possibility that undamaged proto-oncogenes might themselves be critical for cell growth and differentiation was heralded by the finding that certain cellular oncogenes encode familiar growth factors, their receptors, or receptor-coupling proteins (Duesberg, 1983; Hunter, 1984).

This review will consider evidence for the regulation of muscle differentiation by cellular oncogenes. Important tools that have added to our understanding of myogenesis, include experimental models of skeletal muscle which differentiate appropriately in vitro (Yaffe, 1986; Schubert et al., 1974; Yaffe and Saxel, 1977), the identification of specific components of mitogenic medium, which themselves do not cause cell division yet can abolish muscle-specific gene expression at less than nanomolar concentrations (Gospardorowicz et al., 1976; Olson et al., 1986), and methods to uncouple the biochemical differentiation of myoblasts from their subsequent fusion and "irreversible" activation of muscle-specific genes (Emerson and Becker, 1975; Nguyen et al., 1983). "Differentiation," therefore, is used here to denote the induction of muscle-specific gene products rather than the formation of myotubes. That cellular oncogenes are likely to be involved in the establishment or maintenance of the differentiated phenotype in skeletal muscle cells has been favored by observations of proto-oncogene expression during normal or defective myogenesis (Sejersen et al., 1985; Leibovitch et al., 1986), and has been tested more directly by introducting into myoblasts the eponymous viral oncogenes (Holtzer et al., 1975; Fiszman and Fuchs, 1975; Moss et al., 1979; Falcone et al., 1985; reviewed in Alema and Tato, 1987), their "activated" and normal cellular homologs (Schneider et al., 1987; Olson et al., 1987a; Caffrey et al., 1987a; Payne et al., 1987), and conditionally expressed oncogenes whose phenotypic effects are contingent on temperature or specific induction of an upstream promoter (Fiszman and Fuchs, 1975; Falcone et al., 1985; Gossett and Olson, 1988).

Myogenesis

Morphological and Biochemical Correlates of Myogenic Differentiation

Terminal differentiation of skeletal muscle involves a complex series of molecular events, as proliferating undifferentiated myoblasts cease dividing and fuse to form multinucleate myotubes (Fig. 1; Merlie et al., 1977; Schubert, 1984; Pearson and Epstein, 1982; Emerson et al., 1986). Fusion is accompanied by down-regulation of gene products associated with proliferation and by coordinate activation of a battery of muscle-specific genes. Tissue-specific proteins that accumulate during myogenesis include α-cardiac and α-skeletal actin, myosin

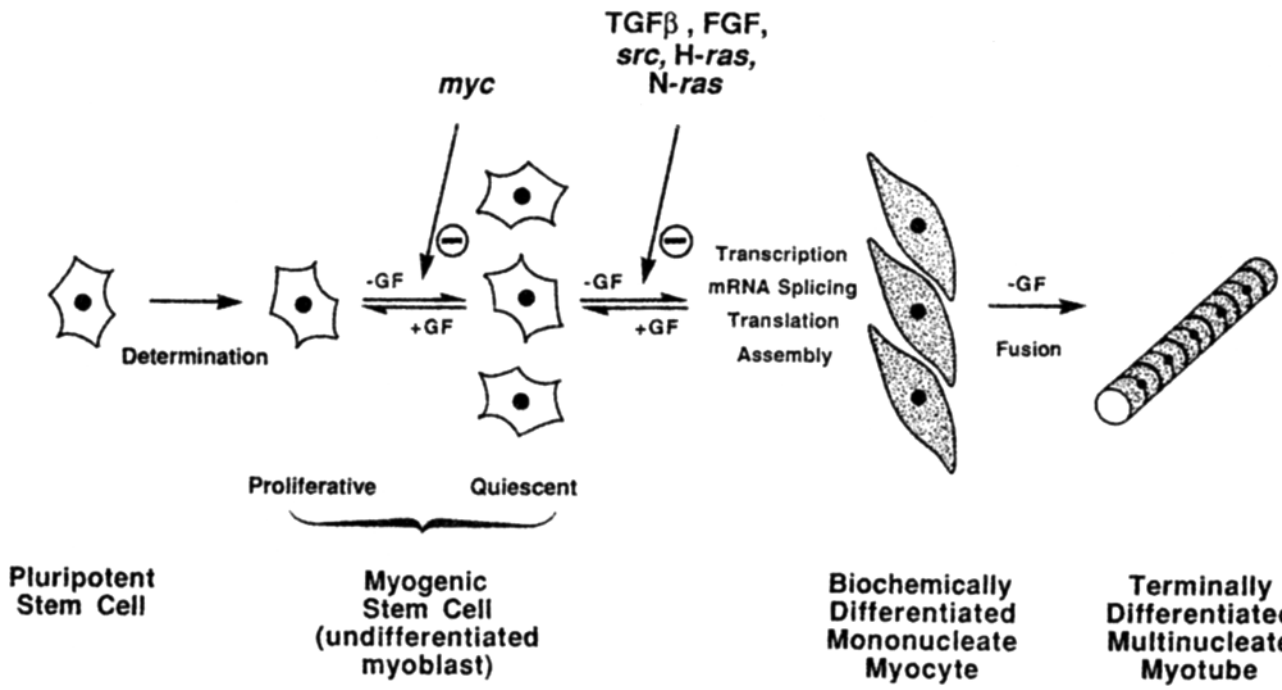

Fig. 1. Differentiation of skeletal muscle cells and its control by growth factors or cellular oncogenes. Entry of primitive mesodermal cells into a myogenic pathway ("determination") does not itself result in expression of muscle-specific genes, whose initial transcription in mononucleate muscle cells requires growth arrest. The effects of *c-myc* and v-*myc* on muscle differentiation vary in relation to the potential of modified cells to sustain proliferation under conditions that normally block proliferative growth. In contrast, mutant *H-ras* and *N-ras* genes, like inhibitory growth factors (GF) such as FGF and TGFβ, prevent the induction of muscle-specific genes even in quiescent myoblasts. Neither inhibitory growth factors nor activation of an inducible mutant ras gene can suppress the myogenic phenotype after fusion and "terminal" differentiation of a multinucleate myotube.

heavy and light chains, tropomyosin, troponin, desmin, the muscle isoenzyme of creatine kinase (MCK), acetylcholine esterase, the nicotinic acetylcholine (ACh) receptor, and voltage-gated Na^+ and Ca^{2+} channels (Devlin and Emerson, 1978; Gard and Lazarides, 1980; Shani et al., 1981; Caravatti et al., 1982; Garfinkel et al., 1982; Hastings and Emerson, 1982; Medford et al., 1983; Olson et al., 1983a,b, 1984; Caffrey et al., 1987a,b). This array of developmentally regulated gene products makes skeletal myoblasts an attractive model system to investigate mechanisms that control gene expression during differentiation. Moreover, the events associated with muscle differentiation in vivo can be faithfully reproduced and modulated in primary myoblast cultures and established muscle cell lines, making myogenesis accessible to molecular analysis (Yaffe, 1968; Konigsberg, 1971; Schubert et al., 1974; Yaffe and Saxel, 1977).

Myoblast fusion requires extracellular calcium and is inhibited by media that are calcium-free, yet in the presence of EGTA and the absence of mitogens, mononucleate avian and C2 mouse myoblasts can induce muscle-specific genes (Emerson and Becker, 1975; Moss and Strohman, 1976; Vertel and Fischman, 1976; Hu et al., 1987). Analogously, the mouse muscle cell line, BC_3H1, cannot fuse, form complex

transverse tubules, or assemble striated myofibrils, but can undergo biochemical differentiation after contact inhibition or mitogen withdrawal causes exit from the cell cycle (Schubert et al., 1974; Olson et al., 1983a,b; Spizz et al., 1986). The finding that BC_3H1 cells express voltage-gated calcium channels whose biophysical properties resemble those found in T-tubules of intact skeletal muscle (Caffrey et al., 1987a,b) agrees with the presence of MCK and AChR in this cell line, and supports its use as a model to investigate early events during the onset of the differentiated phenotype. Myoblast fusion is not a prerequisite for muscle-specific gene induction (whereas biochemical differentiation may be obligatory for fusion), and the necessary signal to trigger myoblast differentiation is cessation of DNA synthesis. However, as illustrated by results in rat L6E9 cells, the additional possibility exists of translational control, uncoupling transcript accumulation from muscle-specific protein synthesis (Endo and Nadal-Ginard, 1987). Following myoblast fusion, muscle nuclei become irreversibly committed to the post-mitotic state and activation of muscle-specific genes becomes persistent ("terminal" differentiation: Nadal-Ginard, 1978; Nguyen et al., 1983). In the absence of fusion, muscle-specific genes remain suceptible to inhibitory growth factors, indicating that fusion or an intimately associated event is responsible for their irreversible activation (Nguyen et al., 1983). Whether differentiated myoblasts prevented from fusing can reenter the cell cycle is ambiguous. Under nonfusing conditions, mouse MM14 myoblasts lose the capacity to reenter S phase within 5 h of mitogen withdrawal (Linkhart et al., 1980), whereas unfused mouse C2 and rat L6E9 myoblasts retain the capacity to reinitiate DNA synthesis after exiting the cell cycle (Nadal-Ginard, 1978; Hu et al., 1987), as do differentiated BC_3H1 cells (Lathrop et al., 1985b; Spizz et al., 1986).

The Role of Polypeptide Growth Factors in Myogenesis

By maintaining myoblasts in a proliferative state, fetal calf serum has been known to inhibit myogenic differentiation, either directly or indirectly. Specific components of serum that suppress differentiation include the acidic and basic forms of fibroblast growth factor (FGF), which prevent muscle-specific gene expression in the absence of other growth factors (Gospodarowicz et al., 1976; Lathrop et al., 1985a; Spizz et al., 1986; Clegg et al., 1987). Competence and progression factors such as PDGF, EGF, insulin, insulin-like growth factor, or bombesin do not interfere with myoblast differentiation in the BC_3H1 and C2 cell lines (Olson et al., unpublished results). In at least some myogenic systems, FGFs exhibit no mitogenic activity, indicting that the ability of FGF to inhibit myogenesis does not require cell proliferation, but may be more direct (Lathrop et al., 1985a; Spizz et al., 1986, 1987; Clegg et al., 1987).

Recently, type β-transforming growth factor was identified as an inhibitor of myoblast differentiation, even more potent than FGF (Olson et al., 1986; Massagué et al., 1986; Florini et al., 1986). TGFβ is a 25 kdalton polypeptide homodimer that is abundant in platelets and also is present in a wide range of adult and embryonic tissues, as well as in transformed cells (Sporn et al., 1986). The actions of TGFβ are highly cell-type specific and include both positive and negative effects on proliferation and differentiation (Roberts et al., 1985; Masui et al., 1986). TGFβ has been demonstrated to inhibit the biochemical and morphological manifestations of differentiation in primary cultures of rat, quail, and chick myoblasts as well as in the C2, L6, and BC_3H1 myoblast cell lines (Olson et al., 1986; Massagué et al., 1986; Florini et al., 1986). Recently, TGFβ also was shown to block reversibly the formation of functional

"transient," "fast," and "slow" voltage-gated Ca^{2+} channels in C2 myocytes and to prevent synthesis of the associated dihydropyridine receptor protein (Caffrey et al., 1987b). Because the continual presence of TGFβ is required to inhibit differentiation and removal of this growth factor rapidly induces muscle proteins, the intracellular signals generated by TGFβ may be transient and require continual occupancy of its receptor. TGFβ neither stimulated nor inhibited myoblast proliferation and, thus, its suppressive effects do not involve DNA synthesis. As discussed below, the intracellular signaling pathways utilized by these growth factors that regulate myogenesis remain elusive.

The ability of FGF and TGFβ to specifically repress myogenic differentiation in vitro suggests that these growth factors may play important roles in early embryonic development in vivo. The timing of myoblast fusion or maintenance of satellite cells, for example, may be determined by local concentrations of one or both of these growth factors in the embryo. Such a role might be suggested by the homologies shared by TGFβ with Müllerian inhibiting substance and a *Drosophila* gene product implicated in pattern formation (Cate et al., 1985; Padgett et al., 1987), and by similarities between FGF and the oncogene *int*-2 (Marx, 1987). At present, however, these postulated functions for FGF, TGFβ, and certain myogenic growth factors remain speculative (Kardami et al., 1985).

Types of Control of Muscle-Specific Gene Expression

The molecular machinery that controls the accumulation of muscle-specific gene products during myogenesis includes not only transcriptional regulation (Pearson and Epstein, 1984; Emerson et al., 1986), but also mechanisms for translational control (Endo and Nadal-Ginard, 1987; Taubman et al., 1987). Furthermore, the generation of protein isoform diversity may often involve alternative mRNA splicing, a process that may require the induction of developmentally regulated, muscle-specific factors acting in *trans* or alternative promoter utilization (Medford et al., 1984; Nadal-Ginard et al., 1986; Breitbart and Nadal-Ginard, 1987). That transcription of unlinked muscle-specific genes is activated coordinately during myogenesis suggests the existence of common *cis*-acting elements that might confer regulation by *trans*-acting factors. Indeed, 5' flanking regions of the skeletal α-actin, troponin-I, myosin light chain-2, nicotinic ACh receptor, and MCK genes each confer developmental and tissue-specific regulation to a downstream reporter gene (Nudel et al., 1985; Konieczny and Emerson, 1985, 1987; Shani, 1985; Bergsma et al., 1986; Minty and Kedes, 1986; Jaynes et al., 1986; Klarsfeld et al., 1987; Sternberg et al., 1988). Nucleotide sequence analysis has revealed stretches of highly conserved sequences in upstream regions of different muscle-specific genes from divergent species, such as the CArG motif shared by cardiac and skeletal actin (but also β actin), α-cardiac myosin heavy chain, cardiac and skeletal myosin light chain-2, and cardiac troponin-T (Bergsma et al., 1986; Minty and Kedes, 1986; cf. Jaynes et al., 1986). Such homology suggests a strong evolutionary constraint on these sequences, which may play an important role in muscle-specific regulation. In principle, these sequences may interact with either positive or negative *trans*-acting factors (cf. Walsh and Schimmel, 1987), however, the possibly dominant functional role of positive transcription factors is favored both by the deletion studies cited above and by the activation of muscle-specific genes in nonmuscle nuclei of heterokaryons that incorporate muscle cells (Blau et al., 1983). As yet, no muscle-specific transcription factors have been isolated, nor is it known whether a common factor might coregulate multiple muscle-specific genes, an attractive possibility supported by evidence that the same positive *trans*-acting factors bind both

CArG sequences in the human α-cardiac actin gene (Miwa and Kedes, 1987). Although the temporal expression of muscle-specific genes in developing myocytes might at first suggest a uniform transcriptional mechanism, this is unlikely to be the case. For example, appropriate expression of *mck* is conferred by interaction of a proximal element with a distal enhancer sequence (Sternberg et al., 1988; Jaynes et al., 1988), and a contrasting muscle-specific enhancer lies downstream of the myosin light chain 1/3 gene (Donoghue et al., 1987). Furthermore, developmental and tissue-specific expression of the α-actin genes may require only information within a few hundred nucleotides upstream of the transcription start site, whose distance- and orientation-dependence are not enhancer-like (Miwa and Kedes, 1987). In contrast, developmental regulation of the troponin-I gene involves complex interactions between 5' flanking sequences that confer maximal transcription and an intragenic element within the first intron that is necessary for differentiation-specific expression (Konieczny and Emerson, 1987). Finally, actin expression in developing skeletal muscle involves sequential induction of α-cardiac and α-skeletal actin genes (Minty et al., 1982; Bains et al., 1984), and a series of myosin heavy chain genes also become activated consecutively (Whalen et al., 1981; Periasamy et al., 1984). These seeming disparities might be explained in part by postulated models that account for the consecutive regulation of multiple genes through differential affinity for a small number of transcription factors.

Several growth-associated molecules are down-regulated during myogenesis, including ornithine decarboxylase, thymidine kinase, RNA polymerase, β-and γ-cytoplasmic actin, vimentin, and α_2(I)procollagen, fibronectin, and several cell surface growth factor receptors (Lim and Hauschka, 1984; Olson and Spizz, 1986; Lazarides and Capetanaki, 1986; Ignotz et al., 1987; Hu et al., 1987; Ewton et al., 1988). Whereas myotubes express muscle-specific gene products persistently and would appear refractory to growth factors, many myoblast-associated gene products can be reinduced by mitogens even after terminal differentiation (Ignotz et al., 1987; Hu et al., 1987; Olson and Capetanaki, unpublished results). Thus, growth factor signaling pathways for induction of these genes remain operative in myotubes that cannot replicate DNA. Mechanisms for the insensitivity of muscle-specific genes to suppression by growth factors after fusion remain elusive but are central to understanding the complex controls governing myogenesis. FGF and EGF receptors in MM14 myoblasts are down-regulated within 3–5 h in mitogen-free medium (Lim and Hauschka, 1984), as was also shown for the TGFβ receptor in L6 and C2 myoblasts (Hu et al., 1987; and Ewton et al., 1988). Although these events could account for myotubes' loss of inhibition by growth factors, several observations cannot be explained by this model. For example, serum can inhibit muscle-specific gene expression only in myoblasts but can induce growth-associated genes even in myotubes (Ignotz et al., 1987; Hu et al., 1987; Olson and Capetanaki, unpublished results). Thus, myotubes must retain a subset of growth factor receptors as well as functional signal transduction pathways. The serum factors that induce myoblast-associated genes may be distinct from those that suppress the differentiated phenotype. Alternatively, muscle-specific genes may become refractory to mitogenic regulation at a step distal to growth factor receptor binding. Moreover, the post-mitotic phenotype is dominant in heterokaryons formed between post-mitotic myocytes and proliferating G1 myoblasts, suggesting that a block to intracellular mitotic signals might precede the subsequent loss of certain growth factor receptors (Clegg and Hauschka, 1987).

Down-regulation of FGF and TGFβ receptors occurs in association with myoblast fusion and does not accompany differentiation of monocleate C2 myoblasts in mitogen-free medium containing EGTA (Hu et al., 1987; Ewton

et al., 1988). Persistent expression of these receptors also was found in nonfusing BC_3H1 cells: Both systems are reversibly differentiated and respond to these peptides by down-regulating muscle-specific genes. Down-regulation of growth factor receptors thus appears to be linked to irreversible exit from the cell cycle rather than entry into a differentiated state (cf. Lim and Hauschka, 1984). Because of other myoblast-associated gene products down-regulate during myocyte differentiation under nonfusing conditions, FGF and TGFβ receptors are subject to a distinct form of regulation that may be coupled to terminal differentiation.

Mechanisms for Growth Factor-Mediated Regulation of Myogenesis

Little is known of the intracellular signaling cascades utilized by serum mitogens, FGF, or TGFβ to inhibit myogenesis. Because neither FGF nor TGFβ is mitogenic for myoblasts in the absence of other serum factors, their ability to suppress differentiation is not secondary to cell proliferation (Lathrop et al., 1985a,b; Olson et al., 1986; Clegg et al., 1987). As discussed for TGFβ, the FGF signal is transient and requires continual occupancy of its cell surface receptor. Although suppression of muscle-specific genes by FGF is independent of transit into S phase or beyond, the mechanism may involve traversal to a region of G_1 that is incompatible with the differentiated phenotype (Lathrop et al., 1985b).

To examine whether growth factors might inhibit myogenesis as a primary response without intervening gene induction, the ability of serum to down-regulate *mck* mRNA in differentiated myocytes was tested in the presence of cycloheximide (Spizz et al., 1986). Under these conditions, serum cannot inhibit *mck* mRNA expression, suggesting that ongoing protein synthesis is necessary for repression. Whether such proteins might represent gene products induced by serum vs labile constituents of the signal transduction pathway, remains to be resolved.

Cellular Oncogenes: An Overview

Cellular oncogenes residing in avian and mammalian cells were noted first as unsuspected genomic sequences, homologous with the acutely transforming genes that certain RNA tumor viruses acquire by transduction (Weiss et al., 1982; Land et al., 1983b; Varmus, 1984; Bishop, 1985, 1987). As the antecedents of virus-borne oncogenes, these eukaryotic predecessors were designated "proto-" or "cellular" oncogenes, which can become amplified or mutationally activated to induce neoplastic transformation. Recent evidence indicates that proteins encoded by cellular oncogenes might constitute a regulatory cascade in the physiological pathway that propagates growth signals from cell membrane to nucleus (Fig. 2). Thus, cellular oncogenes can encode growth factors, growth factor receptors, and proteins that couple receptor occupancy to cell growth. For example, the c-*sis* gene encodes one subunit of the peptide mitogen, platelet-derived growth factor (Doolittle et al., 1983; Waterfield, 1983). Not only do platelets themselves express c-*sis*, but also components of the vessel wall such as endothelium, macrophages, and smooth muscle cells (Barrett and Benditt, 1987), and placental cytotrophoblasts (Goustin et al., 1985). Transformation by v-*sis* involves, at least in part, secretion of PDGF-like molecules in an autocrine pathway (Huang et al., 1984). Other cellular genes that encode growth factors can become oncogenic upon transcriptional activation by a viral promoter (Lang et al., 1985; Stern et al., 1987), and the cellular onco genes *int*-1 and *int*-2 also may encode secreted proteins (Fung et al., 1985; Marx, 1987).

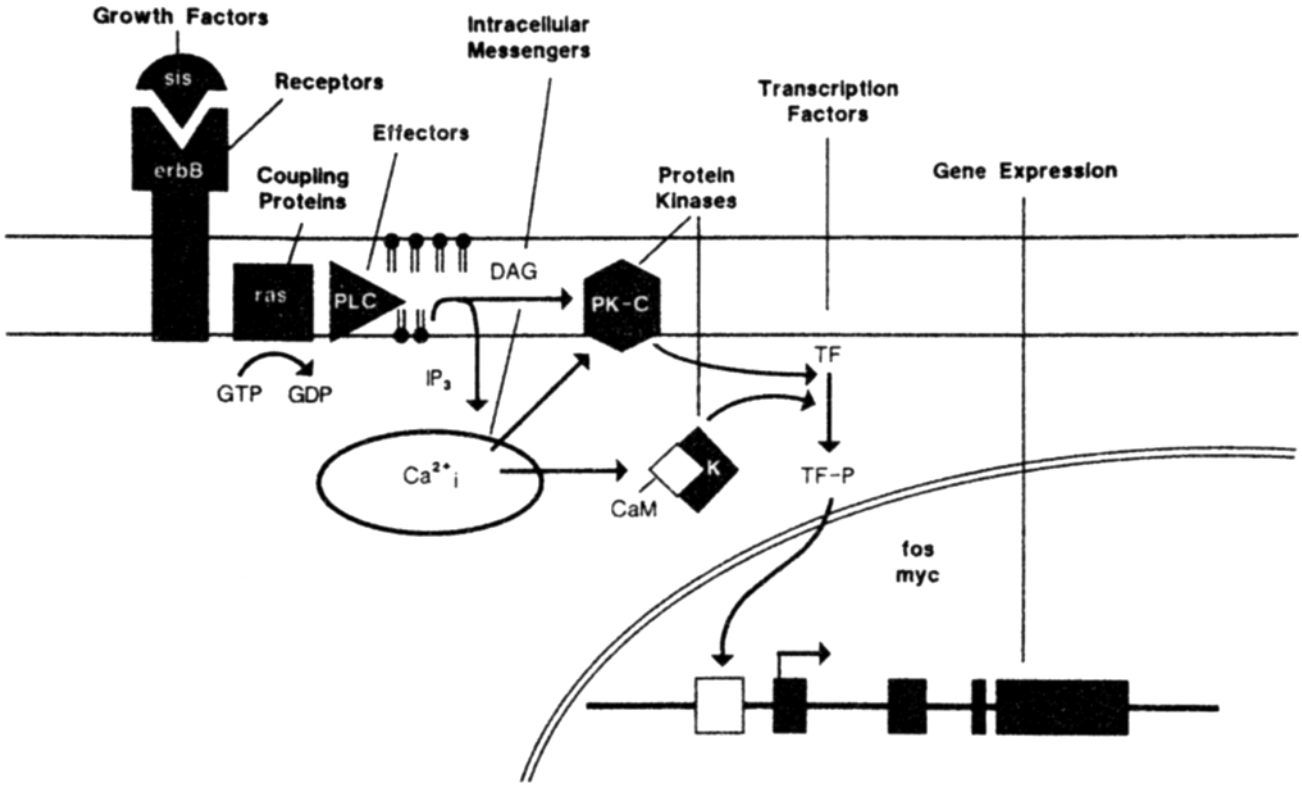

Fig. 2. Hypothetical regulation of muscle growth and differentiation by cellular oncogenes. Proteins encoded by cellular ("proto-") oncogenes include canonical growth factors (*sis*), growth factor receptors (*erb*B), and GTP-binding *ras* proteins that couple receptor occupancy to the generation of intracellular signals, including products of membrane phosphoinositol hydrolysis, that ultimately impinge on gene transcription. Membrane-associated tyrosine kinases such as *src* (not shown), similar to the insulin and EGF receptor cytoplasmic domains, also may exert their effects through *ras*, or *ras*-like proteins. Mitogens and "proximal" oncogenes also rapidly induce intranuclear proteins encoded by the oncogenes *fos* and *myc*, which themselves are implicated in DNA synthesis and control of gene expression. Abbreviations: PL-C, phospholipase C; DAG, diacylglycerol; IP_3, inositol-1,4,5-triphosphate; PKC, protein kinase C; Ca^{2+}, intracellular calcium; CaM, calmodulin, K, calmodulin-dependent protein kinase; TF, transcription factor; TF-P, phosphorylated transcription factor.

Oncogenes of a second class encode transmembrane proteins that bind and transduce mitogenic signals, including receptors for EGF (c-*erb*B: Ullrich et al., 1984; Downward et al., 1984) and the hematopoietic mitogen, colony-stimulating factor (c-*fms:* Scherr et al., 1985). Overproduction of c-*erb*B mRNA and, more rarely, amplification and rearrangement of the c-*erb*B gene are found in transformed cells that express high levels of EGF receptor. Transduction of c-*erb*B by the avian erythroblastosis virus or interpolation of a viral promoter can delete the amino-terminal domain necessary for ligand binding (Downward et al., 1984; Ullrich et al., 1984; Nielsen et al., 1985). The viral *erb*B protein also has undergone truncation of cytoplasmic sites for tyrosine phosphorylation (Ullrich, 1984) and is thought to act as a constitutive growth factor signal, since its tyrosine kinase activity cannot be regulated by ligand (Kris et al., 1985; Gilmore et al., 1985). Interestingly, insulin bound to an insulin receptor extracellular domain that has been fused to transmembrane and tyrosine kinase portions of the EGF receptor can induce the kinase, suggesting that the insulin receptor and c-*erb*B gene pro-

duct employ a common mechanism for transmembrane signal transduction (Riedle et al., 1986). Structural features of growth factor receptors also are found in a related cellular oncogene, *neu* (c-*erb*B-2), whose postulated ligand is unknown (Schechter et al., 1984).

Other oncogene-encoded proteins function distal to growth factor ligand binding (Hunter, 1984; Land et al., 1983b; Bishop, 1985). For example, the c-*src* protein is a membrane-associated protein similar to the tyrosine kinase domains of growth factor receptors; *ras* proteins may couple these receptors to intracellular enzymes; and the proteins encoded by c-*fos* and c-*myc* may be essential for growth factor effects within the nucleus. The subsequent discussion will be focused on *src*, *ras*, and *myc*, three complementary oncogenes whose functional properties during myogenesis have received the most thorough investigation to date.

Developmental Regulation of Cellular Oncogenes During Muscle Differentiation

Altered Expression of Cellular Oncogenes Accompanies Myogenesis In Vitro

Relatively little information is available concerning oncogene expression during embryonic and post-natal muscle development. In contrast, a broad survey of proto-oncogene expression has been reported using the muscle cell line, L6α1 (Leibovitch et al., 1986). Developmental changes observed upon growth arrest and myoblast fusion formed five categories of events (Table 1): (a) transcripts that were abundant in proliferative myoblasts and down-regulated markedly after fusion (*erb*B, *fes*, *fms*, Ki-*ras*, *fos*, c-myc, *fgr*); (b) lower-abundance messages that also down-regulated in myotubes (*sis*, *src*, *erb*A); (c) genes whose expression did not vary (*abl*, *myb*); (d) genes not detected at either stage (*mos*); and (e) one whose expression increased in myotubes (*N-ras*). Since proliferative myoblasts were contrasted with terminally differentiated cells, even if these changes have etiologic significance it is unclear whether they in fact accompany growth arrest, biochemical differentiation, fusion, or commitment to terminal differentiation. Dissection of these sequential events will clarify this ambiguity. For example, transient accumulation of c-*fos* on mitogen withdrawal precedes down-regulation of c-*fos*, exit from the cell cycle, and the induction of muscle-specific genes (Leibovitch et al., 1987). A number of these observations have been confirmed in other, contrasting myogenic systems including L6, C2, and BC_3H1 muscle cells, developing cardiac muscle, and primary cultures of rat skeletal myocytes (Sejersen et al., 1985; Endo and Nadal-Ginard, 1986; Schneider et al., 1986; Zimmerman et al., 1986; Schneider et al., 1987; Payne et al., 1987; Olson et al., 1987a; Spizz et al., 1987). For example, in concordance with findings in many other lineages, marked down-regulation of c-*myc* accompanies muscle cell differentiation. By contrast, little or no change occurs in the expression of c-Ha-*ras*.

The physiological significance of certain findings is especially perplexing. For example, c-*fms* encodes the receptor for a macrophage-specific growth factor, colony-stimulating factor-1 (CSF-1; reviewed in Sherr et al., 1985). The cognate viral oncogene (v-*fms*) can transform both fibroblasts and epithelial cells, neither of which is a target for CSF-1, suggesting that the effects of an activated oncogene can be promiscuous, rather than confined to cell types in which the related proto-oncogene product may function. Whereas tissue phagocytes may account for c-*fms* transcripts in liver, lymph nodes, brain, and placenta, clonal lines overcome this ambiguity. Yet it is open to question whether L6α1 cells actually express a c-*fms*-coded peptide, whether it resembles the c-*fms*

Table 1
Expression of Cellular Oncogenes During Myogenic Differentiation

Functional Classification	Oncogene	Transcript Abundance	System
Growth factor	*sis*	down-regulated	L6
Growth factor receptor	*erb*B	down-regulated	L6
	fms	down-regulated	L6
Tyrosine kinase	*src*	down-regulated	L6
	abl	constitutive	C2,L6
GTPase	H-*ras*	constitutive	BC_3H1,C2,L6
	K-*ras*	down-regulated	L6
	N-*ras*	up-regulated	L6
Nuclear protein	*fos*	down-regulated	L6
		constitutive/inducible	BC_3H1
	c-*myc*	down-regulated/inducible	BC_3H1,C2,L6
	myb	constitutive	L6
	*erb*A	down-regulated	L6

[a]The results shown are summarized from reports discussed in Section IV of the text. For clarity, "L6" denotes a number of independent clonal lines derived from parental L6 cells, which each express the myogenic phenotype following growth arrest (L6E9, L6α1, L6J1: Endo and Nadal-Ginard, 1986; Leibovitch et al., 1986, 1987; Sejersen et al., 1985).

gene product formed in macrophages, whether this polypeptide binds CSF-1 itself or perhaps a related growth factor, and whether signal transduction by the protein might control growth or differentiation in myogenic cells.

Abnormal Regulation of Cellular Oncogenes Is Found in Muscle Cell Lines that Cannot Differentiate

To interpret the possible functional significance of developmental changes in proto-oncogene expression, differentiating L6α1 cells were contrasted with two fusion-defective subclones (Leibovitch et al., 1987). However, neither the M4 nor RMS4 cells are contact-inhibited; both proliferate even in low serum concentrations, are anchorage-independent, and are tumorigenic in rats. Thus, it is conjectural whether they do not differentiate because of failure to exit the cell cycle, or because of transformation *per se*. Unlike proliferating L6α1 cells, during log-phase growth some defective cells do not express *src*, *erb*A, *erb*B, *fos*, or *sis*. Thus, proliferative myoblasts that can undergo myogenic differentiation express transcripts that distinguish them from defective myoblasts, though it cannot be inferred that these anomalies contribute to the cells' abnormal phenotype. Paradoxically, each of the defective lines also contained high levels of N-*ras*, whose abundance in L6α1 cells increases

during differentiation. This unanticipated finding is especially intriguing in view of the consequences of transfection with N-*ras* expression vectors.

Disparities exist among muscle cell lines that are defective for differentiation. In marked contrast to the deregulation of c-Ki-*ras* in a tumorigenic L6 clone (Leibovitch et al., 1986), c-Ki-*ras* down-regulated normally in growth-arrested cells of a subclone of L6 resistant to α-amanitin (Sejersen et al., 1985). This latter cell line bearing a mutation in RNA polymerase II can undergo contact inhibition and can also down-regulate c-*abl* and histone H2a mRNA (a marker of S phase), yet these cells cannot fuse or differentiate. It is noteworthy that c-*myc* persisted after mitogen withdrawal in both defective muscle cell lines. Although it was surmised that down-regulation of c-*myc* might be obligatory for activation of muscle-specific genes (Sejersen et al., 1985), this evidence is unable to distinguish between divergent possibilities: that failure to down-regulate c-*myc* might be a contributing cause of failure to differentiate, or might merely be a molecular marker of a less developed phenotype.

Induction of c-myc is Neither Sufficient nor Necessary to Prevent the Expression of Muscle-Specific Genes

Because c-*myc* levels decline precipitously prior to activation of muscle-specific genes, this reciprocal temporal relationship suggested that *myc* might function as a negative regulator of myogenesis (Sejersen et al., 1985; Spizz et al., 1986). However, it was demonstrated in L_6E_9 cells that terminal differentiation does *not* involve a block to induction of c-*myc*, since fetal calf serum could evoke c-*myc* even in myotubes that had exited the cell cycle irreversibly (Endo and Nadal-Ginard, 1986). More importantly, activation of c-*myc* was insufficient to suppress the differentiated muscle phenotype, and had no effect on myosin heavy chain, α-actin, troponin-T, or myosin light chain$_2$ mRNA in serum-challenged myotubes. Moreover, nuclear runoff transcription measurements revealed that c-*myc* and muscle-specific genes can be transcribed simultaneously in terminally differentiated myocytes (Endo and Nadal-Ginard, 1986). However, a potential role for c-*myc* during entry into the differentiated state cannot be disproven by these results alone. For example, terminally differentiated myotubes also are refractory to TGF-β, the most potent inhibitor of myogenic differentiation identified thus far (Olson et al., 1986; Massagué et al., 1986; Florini et al., 1986).

The ability of TGF-β to prevent differentiation without stimulating cell proliferation made it possible to examine the interrelationship between c-*myc* and the myogenic phenotype, without complications resulting from cell division itself (Spizz et al., 1987). Differentiation of BC_3H1 cells was accompanied by a precipitous decline in c-*myc* levels, whereas no modulation in the steady-state level of c-*fos* was seen. Myoblasts subjected to TGFβ during mitogen withdrawal failed to induce *mck* mRNA, but down-regulated c-*myc* mRNA normally. TGF-β induced both c-*fos* and c-*myc* in quiescent myoblasts, with transient kinetics for *fos* induction resembling serum effects in various cell types; c-*myc* mRNA was induced within 30 min and accumulated for 4 h. This contrasts with the delayed induction of c-*fos* and c-*myc* by TGF-β in certain nonmuscle cells through an autocrine mechanism mediated by c-*sis* (Leof et al., 1986). Together these results demonstrate that repression of muscle-specific genes by growth factors does not require persistent induction of c-*myc*. Whether transient expression of c-*fos* or c-*myc* might be necessary remains an open question.

Src and Other Tyrosine Kinases

The src Gene Product is a Tyrosine-Specific Protein Kinase Implicated in Cell Growth and Differentiation

More than seventy-five years ago, Peyton Rous reported that the cell-free filtrate of a chicken sarcoma could itself propagate tumors in injected animals (Rous, 1911). The transforming retrovirus bearing his name, isolated subsequently, has remained a prototype for discovry in the biology of oncogenes. For example, *src* was the first transforming gene of an RNA tumor virus to be dissected by mutation and mapped in the viral genome (Martin, 1970; Vogt 1971; Wang et al., 1976). Furthermore, the protein it encodes, $pp60^{v\text{-}src}$, was the first transforming protein to be identified biochemically (Purchio et al., 1978), assigned an enzymatic function (Collett and Erikson, 1978; Levinson et al., 1978; Hunter and Sefton, 1980), and localized in transformed cells (Willingham et al., 1979). Together with transmembrane and membrane-associated oncogene proteins of more recent vintage, $pp60^{v\text{-}src}$ shares with the cytoplasmic domain of certain growth factor receptors the ability to phosphorylate protein specifically on tyrosine residues (Hunter and Cooper, 1985). Finally, the ancestral gene c-*src* was the first cellular oncogene to be demonstrated within a vertebrate genome, as a target for retroviral transduction (Stehelin et al., 1976).

The kinase activity of $pp60^{c\text{-}src}$ can be increased by phosphorylation of amino-terminal tyrosine induced by the mitogen PDGF (Ralston and Bishop, 1985; cf. Bolen et al., 1984). Though over-expression of c-*src* cannot itself transform cells or propel mitotic growth (Iba et al., 1984; Shallway et al., 1984), missense mutations in its tyrosine kinase domain suffice to generate foci in monolayer cultures and solid tumors in inoculated chichs, a more stringent test (Levy et al., 1986; cf. Tanaka and Fujita, 1986). It is unknown whether these substitutions or those that endow $pp60^{v\text{-}src}$ with transforming potential might also alter the specificity of *src* gene products for their respective protein substrates. In addition, $pp60^{v\text{-}src}$ whose myristylation has been prevented by mutagenesis can phosphorylate all known polypeptide substrates of the *src* kinase, yet cannot transform cells (Kamps et al., 1986). Presumably, one or more essential substrates for *src*, perhaps located in or on the plasma membrane, have not been identified thus far. Despite the innate attractiveness of observations that protein tyrosine kinases such as *src* and *ros* might also phosphorylate membrane phosphoinositides (Sugimoto et al., 1984; Macara et al., 1984), no definitive proof of this notion has been reported.

Expression of Certain c-src Transcripts may Increase during Differentiation

That cellular oncogenes might have a role alternative to mitotic signal transduction alone was suggested first by the finding that $pp60^{c\text{-}src}$ is especially abundant in certain nondividing, differentiated cells, such as neurons of the retina, cerebellum, and spinal ganglia (Cotton and Brugge, 1983; Sorge et al., 1984), as well as platelets (Golden et al., 1986). Alterations of $pp60^{c\text{-}src}$ accompany neuronal differentiation and augment its tyrosine kinase activity (Brugge et al., 1985; Bolen et al., 1985; Cartwright et al., 1987), but whether the primary structure of the src protein itself is altered in differentiated neurons was unknown. An 18-nucleotide insertion within neuronal c-src mRNA contributing to the NH_2-terminal domain maps precisely to the junction of c-src exons 3 and 4: thus, the neuronal and nonneuronal c-src transcripts appear to be generated through alternative splicing pathways (Martinez et al., 1987). Although c-src mRNA in de-

veloping retina increases in parallel with pp60$^{c\text{-}src}$ (Vardimon et al., 1986), increased src tyrosine kinase activity during differentiation does not always involve increased expression of src mRNA (Gee et al., 1986; Barkenow and Gessler, 1986). The prediction from *in situ* hybridization studies of Drosophila that c-src might also accumulate in developing smooth muscle (Simon et al., 1985) has not yet been tested in mammalian systems.

Conditional Mutants of v-src Reversibly Block Differentiation in Many Cell Types

The availability of conditional mutants of Rous sarcoma virus facilitated studies of the impact of viral oncogenes on differentiation in muscle and other lineages. For example, pp60$^{v\text{-}src}$ can block the appearance of melanin in melanoblasts (Boettiger et al., 1977) and suppress a neuronal tetrasialoganglioside and high-affinity neurotransmitter uptake in the embryonic retina (Brackenbury et al., 1984; Casalbore et al., 1987). Conversely, v-src can induce anamalous expression of genes such as b-globin in RSV-infected fibroblasts (Groudine and Weintraub, 1980). Dramatic evidence that the ability of an oncogene to impinge on gene expression was contingent on cell type, and not the properties of the oncogene alone, was illustrated by the ability of RSV to suppress fibronectin and type I procollagen mRNA in fibroblasts (Howard et al., 1978; Fagan et al., 1981), yet enhance their expression in infected chondroblasts (Adams et al., 1982). As discussed below for ras sarcoma viruses and oncogenic c-ras proteins, infection of pheochromocytoma (PC12) cells with v-src, paradoxically, can provoke growth arrest and the formation of neurites, functioning as a surrogate for nerve growth factor (Alemà et al., 1985).

The Viral src Gene Prevents Myoblast Differentiation

Certain pioneer studies examining the impact of viral oncogenes on muscle development demonstrated that the wild-type Rous sarcoma virus (Prague, Schmidt-Ruppin, or Bryan strains) could successfully infect myogenic cells and are generally regarded as illustrations that v-*src* can block cell fusion and the formation of striated myofibrils (Kaighn et al., 1966; Lee et al., 1966, 1968; Easton and Reich, 1972; Fiszman and Fuchs, 1975; Holtzer et al., 1975; Hynes et al., 1976). In fact, these accounts suggest that myotubes might actually arise after RSV infection, then undergo vacuolation and cytolysis (Easton and Reich, 1972; Holtzer et al., 1975; Fiszman and Fuchs, 1975). Furthermore, fusion can be uncoupled from induction of muscle-specific genes, and is a potentially inaccurate measure of myogenic differentiation. To overcome the objection that virus may be found selectively in mononuclear cells that could not differentiate, mutant strains of virus were subsequently used, which were conditional for transformation. At temperatures "permissive" for its tyrosine kinase activity, pp60$^{v\text{-}src}$ can prevent the induction of representative tissue-specific gene products including myosin, ACh receptors, acetylcholinesterase, desmin, and muscle creatine kinase (Holtzer et al., 1975; Hynes et al., 1976; Fiszman, 1978; Moss et al., 1979). Moreover, cell-free translation of myosin heavy chain mRNA suggested that a *src* oncogene might in fact control muscle development at the level of gene transcription or mRNA processing (Moss et al., 1979).

This block to differentiation by *ts* RSV is stable yet can be released at a "nonpermissive" temperature even after propagation for 20–100 generations in an undifferentiated state (Monterras and Fiszman, 1983; Alemà and Tatò, 1987). Myoblasts formed at 41°C by clones of

quail myoblasts that contain *ts* RSV can synthesize both the fast and slow isoenzymes of myosin light chains, as well as subforms of α-tropomyosin, in proportions equivalent to those found in myotubes derived from uninfected quail myoblasts (Monterras andFiszman, 1983). However, other properties of RSV-infected myotubes are abnormal, such as ACh receptor aggregation into clusters and down-regulation of receptor number (Anthony et al., 1985; Alemà and Tatò, 1987). It is unknown whether these effects result from "leak" of tyrosine kinase activity at the nominally prohibitive temperture or from some residuum of the transformed state. Conversely, populations of "revertant" myotubes accumulate even at permissive temperatures and may involve as much as 20–30% of a culture, correlated with levels of $pp60^{v\text{-}src}$ activity and progeny virus (Tatò et al., 1983). The development of revertants even in clonal lines derived from successfully infected myoblasts argues against thepossibility that revertants merely are uninfected cells.

Effects of src on Muscle Differentiation May Be Independent of Cell Proliferation

These effects of *src* on myogenesis could not be accounted for by a block to fusion alone, since *ts*-RSV-infected myoblasts prevented from fusing in calcium-deficient medium could express skeletal myosin heavy chain at the nonpermissive temperature (Moss et al., 1979). Significantly, a permissive temperature could maintain infected myoblasts in a proliferative state, arrested by a shift to 41°C (Moss et al., 1979). Since withdrawal from the cell cycle (not cell fusion) is obligatory to activate muscle-specific genes, these results question whether suppressive effects of v-*src* on myogenic differentiation are direct, or merely contingent on a block to growth arrest. Since differentiation also failed to occur in RSV-infected myoblasts made quiescent with mitomycin C (Falcone et al., 1984), the effects of *src* on myogenesis are thought to be independent of cell proliferation (reviewed in Alemà and Tatò, 1987), in contrast to mechanisms discussed below for *myc*. Moreover, this interpretation agrees with evidence that v-*src* can prevent differentiation in chondroblasts, a contrasting lineage in which proliferative growth and tissue-specific gene expression coexist (Pacifici et al., 1977), and that activation of *ts* $pp60^{v\text{-}src}$ in myotubes at 35°C can suppress the synthesis of muscle differentiation products in post-mitotic cells (West and Boettiger, 1983). Conflicting reports have left unresolved the claim that v-*src* might reactivate DNA synthesis in myotubes (Lee et al., 1968; Holtzer et al., 1975; Kobayashi and Kaji, 1978; cf. Yaffe and Gershon, 1967). It may be pertinent that v-*src* disrupts the cAMP-dependent inhibition of protein kinase C in RSV-infected L6 myoblasts (Narindrasorasak et al., 1987).

Cooperation with v-erbA May Be Necessary for the v-erbB Gene to Prevent Differentiation

To determine whether functional effects on myogenic differentiation might be shared among oncogenes that code for tyrosine kinases, analogous investigations were performed with v-*erb*B. Myoblasts transformed with wild-type AEV or with *ts* v-*erb*B (and cultured at the permissive temperature) failed to form multinucleate myotubes (Falcone et al., 1985). As demonstrated with v-*src*, AEV-infected myoblasts were prevented from morphological differentiation, and expression of muscle-specific myosin, desmin, nACh receptors, and muscle creatine kinase was inhibited (Falcone et al., 1985). However, fewer "revertant" myotubes

formed, and at the nonpermissive temperature differentiation was incomplete. Apart from potential disparities in temperature sensitivity, these effects also may be accounted for by the coexistence in AEV of an additional cell-derived gene, v-*erb*A.

Deletion mutants of AEV that contain only v-*erb*B successfully transform fibroblasts and erythroid cells in culture, and produce sarcomas as well as erythroblastosis in inoculated chickens (Frykberg et al., 1983; Yamamoto et al., 1983). By certain criteria (anchorage-independent growth, plasminogen activator, hexose transport), *erb*A$^-$*erb*B$^+$ cells are indistinguishable from AEV-transformants, yet more modest effects on actin cables and fibronectin expression suggest an incompletely transformed phenotype, intermediate between wild-type and normal cells (Frykberg et al., 1983). Analogously, erythroid cells transformed by v-*erb*B alone acquire increased proliferative capacity but, unlike AEV-infected cells, become anchorage-independent with low probability, remain dependent on erythropoietin and high concentrations of chicken serum, and frequently can synthesize hemoglobin and erythrocyte surface antigens (Graf and Beug, 1983; Beug et al., 1984). Complementary studies revealed that the v-*erb*A gene by itself does not transform fibroblasts or erythroid precursors but can potentiate the transforming effect of v-*erb*B in fibroblastic cells (Frykberg et al., 1983). Furthermore, v-*erb*A acts synergistically with v-*erb*B (and even with v-*src*, v-*fps*, v-*sea*, or v-Ha-*ras*) to prevent erythroid differentiation, blocked weakly by the latter oncogenes alone (Kahn et al., 1986). Though v-*erb*A itself does not suppress the formation of normal myotubes (Alemà and Tatò, 1987), the cooperative action of v-*erb*A in AEV-transformed myoblasts may explain both the relative paucity of revertants and the failure of a temperature shift to correct the block to muscle cell differentiation.

Analogously, in BC_3H1 cells transfected only with v-*erb*B and the neomycin resistance gene, despite marked morphological transformation, the induction of muscle differentiation products such as *mck* mRNA, ACh receptor, and voltage-gated Na^+ and Ca^{2+} channels was partially inhibited, not prevented (Caffrey et al., 1987a; Schneider and Olson, unpublished observations) and channel density was equivalent to control cells after 14–20 d of mitogen withdrawal. These findings support the inference that v-*erb*B may require complementation by v-*erb*A to block the differentiation of muscle cells fully, but experimental evidence to confirm this interpretation is not presently available. The potential role of v-*erb*A in myogenic development has assumed particular significance with the recent discovery that the c-*erb*A gene product appears to be the receptor protein for thyroid hormone (Weinberger et al., 1986; Sap et al., 1986), whose modulatory effects on transcription of myosin heavy chain genes are well-known (Izumo et al., 1986). Preliminary results suggest the possibility that another nuclear oncogene, c-*myc*, also might act synergistically with v-*erb*B (Caffrey et al., 1987a).

v-fps Blocks Differentiation in Muscle Cells but Promotes Differentiation in Other Lineages

Myogenesis also was disrupted by a third tyrosine kinase, v-*fps*, the transforming gene of Fujimani sarcoma virus (Falcone et al., 1985). Quail myoblasts infected with *ts* v-*fps* could not form myotubes or express muscle-specific proteins at 35°C, but differentiated at the nonpermissive temperature. Reciprocal effects were provoked by v-*fps* in chicken myeloid cells, which differentiated into macrophages without exogenous colony-stimulating factors (Carmier and Samarut, 1986). Despite their activity interchangeable from v-*fps* in myogenic cells, neither v-*src* nor v-*erb*B promoted myeloid differentiation, even though both RSV and

AEV could infect and replicate in macrophages. Thus, the hypothetical consequences of a viral or cellular oncogene in a particular lineage cannot be simply predicted from effects on other systems.

To date, investigations of tyrosine kinases in myogenesis have emphasized the use of v-*src* and related viral oncogenes. Although analysis of the possibly contrasting effect of their cellular homologs will require future studies, a portion of this information is already available for the cellular *ras* genes.

The *Ras* Multi-Gene Family

Ras *Proteins Confer Growth Signals from the Cell Membrane to the Nucleus*

The mammalian *ras* family comprises three genes, Harvey (*H*)-*ras*, Kirsten (*K*)-*ras*, and *N*-*ras*, which encode 21 kdalton GTP binding proteins localized to the cytoplasmic surface of the plasma membrane (Parada et al., 1982; Der et al., 1982; Shimizu et al., 1983). Like G-proteins that modulate adenylate cyclase, other membrane-associated enzymes, and ion channels, ras proteins are converted to an activated state upon binding of GTP and in this form are believed to couple cell surface growth factor receptors to membrane enzymes involved in intracellular signaling (Hurley et al., 1984; Tanabe et al., 1985; McGrath et al., 1984; Sweet et al., 1984). Termination of transduced signals is achieved by hydrolysis of bound GTP *ras* Genes bearing point mutations, generally at codons 12 or 61, have been isolated from a wide range of tumors, and oncogenic *ras* proteins frequently (but not always) exhibit diminished endogenous GTPase activity (Lacal et al., 1986; Der et al., 1986). Thus, "activated" *ras* proteins might deliver persistent growth signals to cells and may not require extracellular growth factors.

ras Proteins appear to participate in transduction of mitogenic signals by interacting with cell surface receptors and other proximal elements of the cascade triggered by growth factors. For example, EGF or insulin can stimulate the GTP-independent phosphorylation and guanine nucleotide-binding activity of certain *ras* proteins (Kamata and Feramisco, 1984). Furthermore, in some cell types, missense mutations of *ras* can supplant exogenous mitogens that are normally indispensable for proliferative growth. Microinjection with p21 *ras* can induce membrane ruffling and pinocytosis (Bar-Sagi and Feramisco, 1987), stimulate the amiloride-sensitive Na^+/H^+ antiporter, increase intracellular pH (Hagag et al., 1987), and induce quiescent cells to express transiently high levels of the nuclear oncogene c-*fos* (Stacey et al., 1987), eliciting the early effects of exogenous mitogens, and, finally, can enable cells to transverse the cell cycle, replicate DNA, and divide (Feramisco et al., 1984; Stacey and Kung, 1985; cf. Mulcahy et al., 1985; Durkin and Whitfield, 1986).

Conversely, microinjection of "blocking" antibodies that bind p21 *ras* can block serum stimulation of DNA synthesis and restore normal growth responses to *ras*-transformed cells as well as to cells transformed by oncogenes such as *src* and *abl* that encode proteins "proximal" to *ras* in the hypothetical cascade for transduction of growth signals. *ras*-Transformed cells also exhibit elevated levels of diacylglycerol and other products of membrane inositol phosphate hydrolysis (Fleischman et al., 1986). Together, these studies suggest that *ras* can mediate the transduction of growth factor signals that lead to DNA synthesis and mitotic division. Growth factor synthesis and transcriptional activation of c-*sis* itself may be induced by activated *ras* alleles (Anzano et al., 1985; Stern et al., 1986; Owen and Ostrowski, 1987). However, *ras* like v-*src* is unable to elicit proliferative growth in senescent cells (Lumpkin et al., 1986), and evokes growth arrest and differ-

entiation in neuronal pheochromocytoma cells (Noda et. 1985; Bar-Sagi and Feramisco, 1985). Thus, the biological effects of *ras* are not intrinsic to the gene product but are dependent on both cell lineage and cell age.

Oncogenic ras Alleles Block Fusion and Muscle-Specific Gene Expression

In view of the importance of *ras* proteins for transmembrane signaling of growth factor effects, C2 and BC_3H1 myoblasts were modified by transfection with mutationally activated H- and N-*ras* oncogenes (Caffrey et al., 1987a,b; Olson et al., 1987; Payne et al., 1987). The valine-12 allele of human H-*ras* driven by its own promoter completely prevented the manifestations of differentiation in BC_3H1 cells, including *mck*, α-actin, desmin, ACh receptors, and Na^+ and Ca^{2+} channels (Caffrey et al., 1987; Payne et al., 1987; Olson and Capetanaki, unpublished results). The absence of functional Ca^{2+} channels involved a block to the expression of the transmembrane dihydropyridine receptor protein itself (Caffrey et al., 1987b). Potassium channels, which are expressed constitutively in both proliferating and biochemically differentiated BC_3H1 cells, were not affected by the *ras* gene, whose effects thus were specific for ion channels whose formation is contingent on mitogen withdrawal (Caffrey et al., 1987a). Similarly, in C2 myoblasts, fusion was abolished by the val12 H-*ras* gene, and biochemical differentiation did not occur (Olson et al., 1987). Like FGF and TGFβ, the oncogenic *ras* allele did not abrogate cells' requirement for growth factors to divide and inhibited differentiation through a mechanism independent of cell proliferation. At the level of expression caused by its own promoter, the normal glycine-12 H-*ras* gene had no effect on establishment or maintenance of a differentiated phenotype in either muscle cell line (Olson et al., 1987; Payne et al., 1987).

Oncogenic *ras* genes also blocked the downregulation of c-*myc* and other molecules whose expression normally declines in the pathway toward the differentiated state, such as β- and γ-cytoplasmic actin, vimetin, and ornithine decarboxylase, which continued to be expressed at elevated levels following growth arrest (Hu et al., 1987; Olson et al., 1987; Payne et al., 1987; Olson and Capetanaki, unpublished results). Thus, *ras* oncogene proteins may activate a subset of the responses that occur during transit out of G_0 toward S phase but are insufficient, by themselves, to evoke DNA synthesis in BC_3H1 and C2 myoblasts. As discussed below, autonomous expression of c-*myc* does not prevent induction of muscle-specific genes in *myc*-transfected myoblasts. Therefore, persistent expression of c-*myc* cannot account for the inability of *ras*-transfected myoblasts to differentiate.

An Inducible ras Gene Blocks Muscle Differentiation Reversibly but Does Not Suppress Muscle-Specific Gene Expression in Terminally Differentiated Cells

Myoblasts bearing chimeric N-*ras* oncogenes, activated by missense mutation ($glu^{61} \rightarrow lys^{61}$) and linked to the steroid-inducible mouse mammary tumor virus promoter, were used to demonstrate that inhibitory effects of *ras*, like FGF and TGFβ, are rapidly reversible and require continuous generation of intracellular signals (Gossett and Olson, 1988; cf. McKay et al., 1986). Conversely, to test whether muscle-specific genes in terminally differentiated myotubes might remain susceptible to suppression by *ras*, myotubes were

allowed to form then were exposed to dexamethasone. Induction of N-*ras* failed to down-regulate muscle-specific genes in myotubes: thus, *ras* may act proximal to the transitional events that confer irreversibility to "terminally" differentiated cells. The basis for the reported contrasting effect of *ts* v-*src* in this setting is not known (West and Boettiger, 1983). Because oncogenic *ras* proteins generate growth factor-like signals even in the absence of extracellular growth factors, the failure of growth factors to inhibit tissue-specific genes in myotubes might involve events apart from the fall in growth factor receptor number that accompanies fusion (cf. Clegg and Hauschka, 1987).

Little is known of the transmembrane signaling pathways by which FGF and TGFβ inhibit myogenesis. Similarities between the effects of these growth factors and of activated *ras* gene products on myocytes raise the intriguing possibility that these regulators of muscle differentiation may operate by a common intracellular pathway. Inhibitory effects of *ras* on myogenic differentiation might be caused by direct activation of an intracellular event that impinges on differentiation, or alternatively, a secondary mechanism involving release of autocrine factors such as TGFs. C2 myoblasts differentiated normally in *ras*-conditioned medium, indicating that oncogenic *ras* genes might not act on C2 cells via an autocrine mechanism (Olson et al., 1987). It will be interesting to test the hypothesis that the receptors for FGF or TGFβ interact with cellular *ras* proteins to initiate the intracellular cascade that culminates in suppression of myogenic differentiation. The possibility that TGFβ and FGF function through a *ras*-dependent mechanism is supported by prepiminary evidence that myoblasts expressing elevated levels of proto-oncogenic *ras* proteins might exhibit hypersensitivity to suppression by these growth factors. (Gosse and Olson, unpublished results).

Nuclear Oncogenes

The Intranuclear myc *Protein May Be Essential for Cells to Replicate DNA and Divide*

Activation of the cellular c-myc gene can be caused by insertion of retroviral DNA or by chromosomal translocation (Cole, 1985; Bishop, 1985; Erikson et al., 1986), events that place c-myc under novel transcriptional control, adjacent to a potent viral or cellular enhancer. Most often, these rearrangements increase steady-state levels of c-myc mRNA markedly, and altered growth is ascribed either to myc abundance per se or to its inappropriate expression following anti-proliferative signals. Amplification of the c-myc gene also has been reported, in se,veral types of tumors (Little et al., 1983; Schwab et al., 1985). Altered myc mRNA structure resulting from translocation or a shift in promoter usage (Battey et al., 1983) has been proposed to account for the physiological effects of certain activated myc genes, involving alternative translation products (Hann and Eisenmann, 1984), translational efficiency (Saito et al., 1983; Darveau et al., 1985), point mutations (Lee et al., 1985) or transcript stability (Piechaczyk et al., 1985). Phenotypic consequences of altered myc alleles have been more difficult to ascertain than for ras, but it appears, at least, that the potential to complement a mutant ras gene might require only augmented expression of a normal c-myc protein (Lee et al., 1985).

Initial evidence linking c-myc to control of cell replication was the demonstration that c-myc abundance could be provoked transiently in quiescent cells by a variety of growth-promoting signals including mitogenic lectins, serum, polypeptide growth factors such as PDGF, agonists that elicit membrane phospholipid hydrolysis, direct activators of protein kinase C, and calcium ionophores (Kelly et al.,

1983, Cochran et al., 1983; Müller et al., 1984; Greenberg and Ziff, 1984; Campisi et al., 1984; Zullo et al., 1985; Ran et al., 1986; Moolenaar et al., 1986). Conversely, down-regulation of c-myc was found to be an early response to treatments that lead to growth arrest, such as mitogen withdrawal, contact inhibition, and "differentiating" agents (Reitsma et al., 1983; Jonak and Knight, 1984; Campisi et al., 1984; Dony et al., 1985; Dean et al., 1986; for N-myc, see Thiele et al., 1985). Thus, commonly, c-myc gene expression is activated during the transition from G_0 to G_1, is maintained at a lower level throughout the cell cycle in proliferating cells (Thompson et al., 1985; Rabbits et al., 1985), and is deinduced upon quiescence. Though few studies to date have addressed the molecular pathways used by physiological growth signals in vivo, partial hepatectomy and protein starvation have been shown to evoke transient and sequential expression of cellular oncogenes including c-myc prior to DNA synthesis in the liver, resembling events found during transit through the cell cycle in vitro (Kruijer et al., 1986; Horikawa et al., 1986). Early results concerning the potential contribution of *myc* transcription rates to steady-state *myc* abundance were conflicting (e.g., Blanchard et al., 1985; Greenberg and Ziff, 1985), and were resolved at least in part by the discovery that decreased transcription of c-*myc* in differentiated cells largely involves a block to message elongation rather than control of initiation (Bentley and Groudine, 1986). Regulation at the level of mRNA stability also has been shown (Dani et al., 1984; Blanchard et al., 1985; Knight et al., 1985; Dony et al., 1985).

The putative functional role of c-*myc* to trigger cell growth has been corroborated by complementary mechanistic studies. For example, either autonomous *myc* genes or microinjected *myc* protein can substitute for "competence" factors (Armelin et al., 1984; Kaczmarek et al., 1985). Conversely, antibodies directed against *myc* protein or c-*myc* anti-sense oligodeoxynucleotides specifically block DNA replication (Studzinski et al., 1986; Heikkila et al., 1987).

Contrasting with the usual correlation (and implied functional relationship) between activation of c-*myc* and transit through the cell cycle, several intriguing exceptions have been noted. First, in cultured cardiac muscle cells, c-*myc* expression can be provoked by norepinephrine, which elicits muscle growth and protein synthesis in the absence of DNA replication (Starkesen et al., 1986). Analogously, a hemodynamic load in vivo can increase both cardiac mass and c-*myc* expression (Mulvagh et al., 1987). Moreover, c-*myc* levels also are uncoupled from DNA replication during early *Xenopus* development (Taylor et al., 1986; Godeau et al., 1986), and it has been suggested that c-*myc* expression might play a role, instead, in the synthesis of ribosomal RNA. Furthermore, c-*myc* transcripts in early human embryos are restricted to only a subset of proliferating cells (Pfeiffer-Ohlsson et al., 1985), and c-*myc* mRNA levels increase transiently in postmitotic Purkinje cells during arborization of dendritic processes (Ruppert et al., 1986). Taken together, these events argue against a role for c-*myc* exclusively in mitotic growth.

Furthermore, these observations are compatible with evidence that c-*myc* also can be upregulated in certain model systems, by agents that promote growth arrest. First, as discussed below for c-*fos*, acute induction of c-*myc* is stimulated in rat pheochromocytoma (PC12) cells by nerve growth factor, a peptide that provokes withdrawal from the cell cycle, neurite extension, neurotransmitter synthesis, and formation of voltage-gated ion channels (Curran and Morgan, 1985; Greenberg et al., 1985). Second, during differentiation of mouse erythroleukemia cells or keratinocytes, *myc* mRNA abundance undergoes complex sequential changes, not a simple monotonic decline (Lachman and Skoultchi, 1984; Dotto et al., 1986). That c-*myc* expression might be important for commitment of MEL cells to terminal erythroid dif-

ferentiation was tested subsequently by gene transfer (*see below*). Effects to dissect the process of commitment in erythroleukemia cells have had important implications for studies of the functional properties of c-*myc* in myogenesis.

Autonomous Expression of c-myc can Substitute for Peptide Mitogens and Enhance Cells' Proliferative Capacity

In contrast to *ras* oncogenes, which confer morphological alteration and anchorage independence, the phenotypic effects of *myc* alleles can be less obvious and may account for the failure to detect lesions in c-*myc* by formation of foci. Indeed in several established lines and early-passage rat embryo fibroblasts, an SV40-driven c-*myc* gene evoked no change in cell morphology or tumorigenicity (Land et al., 1983a), though striking exceptions have been noted (Palmieri et al., 1983; Keith et al., 1983; Rapp et al., 1985). Whereas transformation of embryonic fibroblasts by *ras* was incomplete, addition of a deregulated *myc* gene enabled *ras* to produce transfected cells that could form foci even in dense monolayers and form tumors in host animals. Since *ras* was sufficient by itself to impart morphological alteration and tumorigenicity only to cells adapted to long-term culture (nominal "establishment" or "immortality"), these complementary effects of *myc* served as a facsimile of establishment, and led to the operational designation of *myc*, together with the cellular oncogene *p53* and the viral oncogenes polyoma large-T and adenovirus E1a, as an "immortalization" gene (Land et al., 1983a,b; Ruley et al., 1983).

These results were cautiously described as showing important functional similarities between *myc* and establishment, not that *myc* itself necessarily can immortalize cells, nor that spontaneous immortalization might involve activation of c-*myc* (Land et al., 1983a). However, it has become clear that either transcriptionally activated c-*myc* genes or the viral *myc* gene can augment cells' response to exogenous growth factors and substitute at least partially for mitogens that induce c-*myc* in quiescent cells. For example, autonomous expression of c-*myc* can abrogate the requirement of fibroblasts for "competence" factors like PDGF (Armelin et al., 1984) or supplant the need of macrophages for interleukins -2 and -3 (Rapp et al., 1985). Furthermore, c-*myc* expression vectors can enable cells to proliferate even in low concentrations of serum that cannot otherwise support mitotic growth (Keath et al., 1985; Mougneau et al., 1984), and increase cells' sensitivity to growth factors needed to form colonies in agar (Vennström et al., 1984; Sorrentino et al., 1986). Though muscle cells' irreversible withdrawal from the cell cycle involves down-regulation of multiple growth factor receptors, in contrast, *myc* genes enhance responsiveness to mitogens through a mechanism that does not increase the affinity or number of receptors for PDGF (Vennström et al., 1985), EGF (Stern et al., 1986), or IL-3 (Rapp et al., 1985). Furthermore, in contrast to *ras* and *src*-transformed cells (Adkin et al., 1984; Anzano et al., 1985; Stern et al., 1986), enhanced growth resulting from deregulated *myc* expression does not appear to involve an autocrine mechanism (Rapp et al., 1985; Stern et al., 1986).

Conversely, autonomous expression of c-*myc* also can make cells resistant to certain inducers of differentiation that normally produce growth arrest. Indeed, although *myc* expression vectors could block the onset of hemoglobin synthesis in mouse erythroleukemia cells, these observations must be interpreted in the context of the cells' inability to respond to DMSO and exit the cell cycle (Coppola and Cole, 1986; Prochownik and Kukowska, 1986; cf. Lachman et al., 1986). As in myogenesis, growth arrest is obligatory for establishment of a differentiated state in erythroid cells. There-

fore, any regulatory influence that propels constitutive growth would prevent entry into a differentiated state. Evidence that the impact of *myc* on differentiation might be indirect, through altered cell proliferation, was supplied using contrasting cell types whose mitotic growth coexists with tissue-specific gene expression. For example, *myc*-transformed chondroblasts readily express cartilage-specific proteins (Alemà et al., 1985), and neurons from embryonic chick retina "immortalized" with v-*myc* continued to express neuronal surface antigens, neurotransmitter uptake systems, and neurofilament protein (Casalbore et al., 1987), in agreement with the persistence of these properties in proliferating retinal cells. Contrasting reports that retinal neurons and rat macrophages could not be established with v-*myc* alone and seemed to require v-*mil*/v-*raf* in concert may have been confounded by conditions that limited the virus to a small proportion of the cells (Bechade et al., 1985; Blasi et al., 1985). As discussed below, contiguity with normal population can restore both growth control and tissue-specific gene expression to *myc*-transformed cells (Alemà and Tatò, 1987).

v-myc Blocks Muscle Differentiation Indirectly, through a Mechanism that Depends on Continued Myoblast Proliferation

Potential effects of *myc* on myogenic differentiation have been analyzed both in primary cultures and in a variety of muscle cell lines. Quail embryo myoblasts infected with v-*myc* acquire anchorage independence and become unable to fuse or express most muscle-specific proteins (Falcone et al., 1985). To ensure that v-*myc* was not merely selecting for nonmuscle cells, clonal strains of myoblasts produced using *ts* v-*src* were superinfected with v-*myc*: the viral *myc* gene prevented differentiation of these clonal myoblasts even when v-*src* was inactivated (Falcone et al., 1985). However, *myc*-transformed myoblasts that failed to differentiate were distinct from *src*-transformants in at least three ways. First, like MEL cells that bear autonomous c-*myc* genes, myoblasts infected with MC29 continued to proliferate after mitogen withdrawal. Second, resembling "undifferentiated" muscle precursor cells, *myc*-transformed myoblasts expressed low amounts of desmin, the muscle-specific subunit of intermediate filaments (Alemà and Tatò, 1987; cf. Devlin and Emerson, 1978). Finally, cultivation together with normal mammalian fibroblasts (C3H10T1/2 cells) could arrest the growth of *myc*-transformed myoblasts, permitting fusion and formation of striated myotubes to occur (Alemà and Tatò, 1987).

Down-Regulation of c-myc Is Not Obligatory for the Induction of Muscle-Specific Genes

The inference that growth arrest, not down-regulation of c-myc, was required for myogenesis was supported by investigations of BC_3H1 cells modified by an SV40-driven c-myc gene (Schneider et al., 1987; Caffrey et al., 1987a). After mitogen step-down, *myc*-transfected BC_3H1 cells exited the cell cycle within 48 h, even at subcofluent densities (cf. Falcone et al., 1985; Alemà and Tatò, 1987). Thus, the c-*myc* vector was unable by itself to abrogate the dependence of BC_3H1 cells on exogenous mitogens to proliferate. In transfected cells that had become quiescent, levels of the truncated c-*myc* transcript were at least 5-fold higher than in serum-challenged BC_3H1 cells (20- to 40-fold higher than in quiescent controls). However, unlike differentiation-defective mutants in which the endogenous *myc* gene could not be down-regulated (Sejersen et al., 1985; Olson et al., 1987; Payne et al., 1987), *myc*-transfected BC_3H1 muscle cells were able to induce tissue-specific gene products at least 50-fold after mitogen withdrawal (Schneider et al., 1987; Caffrey et al., 1987). Thus, activation of muscle-specific genes is not coupled obligatorily to

down-regulation or c-*myc*. However, accumulation of *mck* mRNA and ACh receptor protein was partially inhibited, and induction of Na^+ and Ca^{2+} channels was delayed ~2 d, by the chimeric SV40:c-*myc* gene. Ca^{2+} channel density after 14 to 20 d of mitogen withdrawal was equivalent to that in control BC_3H1 muscle cells. Taken together, these results show that introduction of a deregulated c-*myc* oncogene into mammalian muscle cells is not sufficient to prevent myogenic differentiation, complementing evidence that reinduction of c-*myc* in L_6E_9 myotubes does not suppress muscle-specific genes (Endo and Nadal-Ginard, 1986) and that autonomous expression of c-*myc* in transgenic mice does not disrupt normal muscle development (Leder et al., 1986). Contrasting results with v-*myc* in primary quail myoblasts might therefore signify differences in the cells or procedures employed (most notably, the stringency of mitogen withdrawal), quantitative differences in *myc* protein expression, or potential disparities between the viral and cellular *myc* proteins. Nonetheless, interpretation of these three contrasting studies coincides: withdrawal from the cell cycle, not down-regulation of myc, is required for the normal induction of muscle-specific genes.

Transient Expression of c-fos Is Provoked by Diverse Transmembrane Signals

Activation of c-*myc* by serum growth factors is preceded by the induction of a second intranuclear protein, c-*fos*, the cellular homolog of the Fujinami osteosarcoma virus (FBJ) transforming protein. High levels of c-*fos* mRNA are detected in extraembryonic fetal membranes and certain macrophage-like cell lines, but in few if any normal adult cell types in vivo (Müller et al., 1983). However, the control of c-*fos* in culture has begun to explicate mechanisms that might couple membrane signals to long-term changes in cell growth, differentiation, or function. Transcription of c-*fos* accompanies growth factor binding within 5 min, precedes the activation of c-*myc* and is, in fact, the earliest known nuclear event triggered by growth factor receptor occupancy (Kruijer et al., 1984; Müller et al., 1984; Greenberg and Ziff, 1984). Transient accumulation of c-*fos* after serum stimulation requires both an upstream element which resembles known transcriptional enhancers, together with 3' sequences that may allow *fos* mRNA to be degraded rapidly (Treisman, 1985, 1986). Both positive and negative *trans*-acting cellular factors regulate c-*fos* gene expression (Sassone-Corsi and Verma, 1987). Since pre-existing proteins are sufficient for serum to induce c-*fos*, it has been suggested that post-translational modifications provoked by serum might cause decreased binding of a negative factor for the regulatory sequences, or increased affinity of the positive factor.

Rapid, transient expression of c-*fos* can be provoked by competence factors such as PDGF or FGF (e.g., Kruijer et al., 1984), mitogenic lectins (Moore et al., 1986), thyroid stimulating hormone (Colletta et al., 1986), and phorbol esters, but not by the "progression" factors in platelet-poor plasma (Bravo et al., 1985). EGF, a canonical progression factor, is only a weak agonist for c-*fos* induction in 3T3 cells (Müller et al., 1984; Kruijer et al., 1984), but is sufficient to drive DNA synthesis and stimulate c-*fos* in cultured rat hepatocytes (Kruijer et al., 1986). c-*fos* also is induced in L6 myoblasts by the progression factor IGF-1 (Ong et al., 1987) and in BC_3H1 myoblasts by FGF and TGFβ (Spizz et al., 1987). The c-*fos* protein is extensively modified after translation, and is degraded with a half-life of ~2 h (Kruijer et al., 1984). Physiological signals including partial hepatectomy and wounding a confluent monolayer also provoke transient expression of c-*fos* (Kruijer et al., 1986; Verrier et al., 1986). Analogously, c-*fos* can be evoked in the heart and salivary gland by isoproterenol, a β-adrenergic agonist which produces hyperplastic and hypertrophic growth

of these organs (Barka et al., 1986). Preliminary evidence suggests that induction of c-*fos* may require the amiloride sensitive Na^+/H^+ antiporter (Kruijer et al., 1986; Moore et al., 1986), whose stimulation by growth factors precedes the onset of c-*fos* transcription.

Conversely, expression of c-*fos* also is provoked by interventions that cause differentiation to a post-mitotic state, for example, during the differentiation of monomyelocytes to macrophages (Gonda and Metcalf, 1984; Müller et al., 1985; Mitchell et al., 1985). However, c-*fos* expression may be neither sufficient nor obligatory for development of a differentiated phenotype (Mitchell et al., 1986; Dotto et al., 1986). In concordance with the "paradoxical" effects of activated *src* and *ras* genes that block cell growth and initiate neural properties in PC12 cells, c-*fos* expression in PC12 cells was elicited by nerve growth factor, but not by treatments which induce a chromaffin-like state (Kruijer et al., 1985; Curran and Morgan, 1985; Greenberg et al., 1985). Moreover, c-*fos* induction can be triggered in post-mitotic, differentiated PC12 cells by voltage-gated calcium currents activated via depolarization or by stimulatory dihydropyridines (Morgan and Curran, 1986; Greenberg et al., 1986). Evidence that a nuclear oncogene could be expressed in response to both ligand- and voltage-dependent cues associated with neuronal signaling has suggested that *fos* might couple membrane excitation to transcriptional events during long-term adaptation of the neuron (Goelet et al., 1986).

A Nuclear fos Protein that Blocks Myogenesis may Function in Trans as a Transcription Factor

Although c-*fos*, like c-*myc*, is rapidly produced in response to growth factors, there is no evidence that exogenous c-*fos* sequences can substitute for specific "competence" factors nominal immortality. One amino acid substitution activates the immortalizing potential of the *fos* gene transduced in FBR sarcoma virus (Jenuwein and Müller, 1987). Even the normal c-*fos* protein can induce morphological transformation, upon linkage of a viral LTR and disruption of 3' sequences that destabilize *fos* mRNA (Miller et al., 1984). Transfer of the normal c-*fos* gene into F9 teratocarcinoma cells could supplant retinoic acid and dibutyryl cyclic AMP, inducing certain of the proteins that signify endodermal differentiation (Müller and Wagner, 1984; Rüther et al., 1985).

Early evidence that the intranuclear *fos* protein might itself function as a *trans*-acting regulator of gene transcription was obtained by cotransfection of NIH 3T3 fibroblasts with a v-*fos* vector together with plasmids containing test promoters linked to chloramphenicol acetyltransferase (Setoyama et al., 1986). Consistent with the finding of increased type III collagen in v-*fos*-transformed cells, v-*fos* plasmids could stimulate the mouse a_1(III) collagen promoter. Indeed, c-*fos* may participate directly in *trans*-acting nucleoprotein complexes that regulate gene expression (Distal et al., 1987).

Conditionally-inducible constructs that express anti-sense *fos* RNA have been reported recently to decrease the abundance of "sense" *fos* transcripts and *fos* protein, to decrease markedly the synthesis of DNA in quiescent cells challenged with serum or PDGF (Nishikura et al., 1987), and perhaps also to inhibit logarithmic growth (Holt et al., 1986). Since no inhibition of *myc* was seen, induction of *fos* does not appear to be obligatory for the mitogenic activation of *myc*. It remains to be determined whether anti-sense *fos* constructs might impinge on differentiation, in systems where *fos* mRNA abundance increases after differentiating signals.

Myoblasts of the L6a1 line were infected with FBJ osteosarcoma virus as a source of v-*fos* and acquired the ability to form foci. Clones of the morphologically altered cells were unable to differentiate (Leibovitch et al., 1987). It is un-

certain whether these effects are direct (analogous to *src* and *ras*) or might be accounted for by the maintenance of a proliferative state (analogous to *myc*). This ambiguity might also be addressed through the use of a nonbiasing selectable marker, such as antibiotic resistance, rather than selection for growth in soft agar.

Summary and Future Directions

As summarized in Fig. 1, the stages of myogenic differentiation in developing muscle cells include: (a) "determination" of primitive mesodermal cells to a myogenic pathway; (b) proliferation of undifferentiated, mononucleate myoblasts; (c) withdrawal from the cell cycle into an initially reversible quiescent state; (d) induction of muscle-specific genes in mononucleate myocytes following growth arrest; and (e) "commitment" to nominally irreversible exit from the cell cycle, terminal differentiation, and myoblast fusion to form multinucleate myotubes. Thus, proliferating myoblasts defer the expression of muscle-specific genes until growth factor deprivation (or other anti-mitotic signals) can arrest cell replication. The seemingly disparate consequences of *myc* in developing skeletal myoblasts can be accounted for by the absence or presence of proliferative growth in the respective preparations. Thus, *myc* can delay or suppress myogenic differentiation, but only indirectly, through a potential block to exit from the cell cycle, and has no evident effect on muscle-specific gene expression when reinduced in myotubes. The intracellular signals generated by activated H- and N-*ras* proteins produce a phenotype indistinguishable from that elicited by the binding of FGF or TGFβ. *Ras* oncogenes, FGF and TGFβ each block the induction of all muscle-specific genes and gene products examined to date, and, conversely, can inhibit the expression of muscle-specific genes in (reversibly) differentiated mononucleate myocytes. The molecular events surrounding the intricately linked processes of commitment, terminal differentiation, and fusion, which place muscle-specific gene transcription beyond the control of *ras*, remain to be established.

A number of additional, intriguing questions remain unanswered (Fig. 2). For example, to what extent can the consequences of a viral oncogene be extrapolated to imply the involvement of its cellular homolog in myogenic differentiation? Would increased expression of a normal cellular oncogene suffice to prevent myogenesis, or require truncation or a missense mutation that confers transforming activity? Do structural alterations of *src* and *ras* permit these membrane constituents to influence, anomalously, cellular proteins that are not ordinarily their substrate? Does the block to formation of muscle-specific proteins by *src* and *ras* oncogenes involve an intracellular pathway shared with inhibitory peptides such as TGFβ? If *ras*, *src*, and TGFβ each inhibit the accumulation of muscle-specific gene transcripts, how might their effects impinge on the *trans*-acting factors that normally confer positive and negative regulation on these genes? What experimental strategies might resolve the conflicting effects of inducible *src* and *ras* vectors on suppression of muscle-specific genes in myotubes committed to nominally irreversible differentiation? Furthermore, it remains enigmatic precisely which growth factor signal(s) each *ras* protein might in turn convey, as well as how the mechanisms discussed here might interact with those which direct pluripotent cells along a myogenic pathway (Konieczny and Emerson, 1985) and generate diversification within the myogenic lineage (Schafer et al., 1987).

It has become feasible to manipulate the developing myoblast with conditionally inducible oncogene expression vectors, purified oncogene proteins produced in bacteria, blocking antibodies and anti-sense nucleotides. Together with alternative approaches to elucidate transmembrane signaling mechanisms, these

methods should begin to explain the cascade of events triggered by mitogens and other peptide growth factors, which converge on the nucleus and alter muscle-specific gene expression. Whether mitogens and cellular oncogenes control the formation of sodium and calcium channels through the same mechanisms that regulate the appearance and diversity of more familiar muscle-specific gene products is an open question, but one that has recently become amenable to study. It also is reasonable to anticipate that interventions such as TGF-β and activated *ras* alleles that prevent the accumulation of muscle-specific transcripts work, at least in part, through transcriptional mechanisms. If so, a mutant *ras* protein may suffice to block induction of transfected genes driven by upstream elements that confer developmentally regulated and muscle-specific expression, or, by itself, alter the binding of nuclear *trans*-acting factors to endogenous regulatory sequences, substituting for extacellular molecules that gate the entry of myoblasts into a differentiated state.

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