Factors Regulating Skeletal Muscle Development: Cell Culture and Transgenic Mouse Studies

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Abstract

The final steps of vertebrate skeletal muscle development involve withdrawal of determined muscle precursors from the cell cycle, expression of muscle-specific genes encoding myofibril components, and cell fusion to form terminally differentiated multinucleated myotubes. A great deal of this process has been adapted to and studied in cell culture for decades. While much has been learned about how a cell's environment influences differentiation decisions and what those decisions involve, only in the last few years has progress been made in understanding how the observed drastic changes in gene expression which accompany differentiation are regulated in the nucleus. This progress was spurred by the cloning of MyoD, a nuclear protein which can convert a variety of nonmyogenic cell types to skeletal muscle. Much evidence suggests that MyoD and its three relatives, myogenin, Myf-5, and MRF4-herculin-Myf-6, play critical roles in the transcriptional activation of skeletal muscle differentiation genes.

The studies presented in this thesis have addressed several important aspects of murine skeletal muscle development. Herculin, a novel member of the MyoD family of myogenic regulators, was cloned and characterized. Of the four known MyoD family members, herculin is the most abundant in adult skeletal muscle and therefore may be crucial for maintaining and/or enacting the mature muscle phenotype. Also, the c-myc proto-oncogene was shown to inhibit the ability of both MyoD and myogenin to initiate myogenic differentiation, even under conditions which normally promote it. This result is relevant for rationalizing, in part, why proliferating myoblasts which express MyoD do not spontaneously differentiate: c-myc is normally expressed in proliferating myoblasts but is down-regulated upon differentiation, perhaps allowing MyoD to become fully transcriptionally active. Finally, transgenic mice which express MyoD ectopically in the heart were produced. Transgenic hearts have morphological abnormalities and express myogenin and sarcomeric genes usually specific to skeletal muscle. This was the first

demonstration that MyoD can function during murine embryogenesis and, more generally, that targeted misexpression of a tissue-specific regulator during mammalian development can activate genes normally transcribed in an unrelated tissue.

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Chapter 1

Introduction

> "Until about the year 1800 it was generally believed either that a fully formed animal exists in miniature in the egg, needing only the stimulus of the sperm to initiate growth and unfolding, or that similarly preformed organisms, male and female, constitute the sperms and these merely enlarge when they get inside the eggs." (Arey 1965)

As the above quotation demonstrates, the fact that a complex organism such as the mouse develops from a single microscopic ovum can easily be explained, however wrong the explanation might be. Constraints that make such explanations more difficult to conjure up are three tenets of modern biology: 1) higher organisms are multicellular; 2) the fertilized egg is a single cell; 3) all cells arise from preexisting cells by cell division. From these simple rules arises the true challenge: if all cells arise from the zygote, how do they become different from one another during embryogenesis?

Observations by embryologists during the last century have led to detailed phenomenological descriptions of cellular differentiation as it occurs during development, but only recently has progress been made in understanding the molecular basis of histogenic differentiation. Extended progress, however, has been restricted to certain lineages. Many studies have centered on the identification, isolation, and characterization of tissue-specific nuclear proteins which bind to the promoters and enhancers of tissue-specific genes, since differential expression of regulatory proteins is likely to play a role in making tissue types different from each other. For example, several *trans*-acting factors which are specific to or are highly enriched in the liver bind to the control regions of liver-specific genes and activate transcription (Friedman *et al.* 1989; Descombes *et al.* 1990; Mendelzon *et al.* 1990; Sladek *et al.* 1990). The CCAAT/enhancer binding protein (C/EBP) found to be transcriptionally active in liver (Friedman *et al.* 1989) also seems to be

involved in activating adipocyte-specific genes during adipogenesis (Christy *et al.* 1989), and other fat cell *trans*-acting factors have been described (Wilkison *et al.* 1990; Graves *et al.* 1991). That C/EBP seems able to direct tissue-specific expression in both liver and fat suggests that there may be truly tissue-specific factors with which it interacts or cooperates, and this is a concept which will be discussed in more detail later. There also exist skeletal muscle-specific nuclear factors which bind to the regulatory sequences of muscle-specific genes *in vitro* (Buskin and Hauschka 1989; Gossett *et al.* 1989). Considered together with the finding that expression of a single transfected cDNA can cause nonmuscle cells to convert to the myogenic lineage and express muscle-specific genes (Davis *et al.* 1987), this led to experiments (Lassar *et al.* 1989) which suggest that the protein encoded by this cDNA, MyoD, plays a part in determining the muscle cell lineage by its binding to regulatory regions of muscle-specific genes and activating transcription during differentiation. Analogous regulators with such a power of transdetermination have not yet been isolated from any other tissue. Whether skeletal muscle is truly unique in this respect remains to be seen. Nevertheless, since a great deal about the partitioning of the skeletal muscle lineage during embryogenesis is known, and since cell lines which recapitulate some steps of myogenic differentiation exist, there is a great potential for discovering exactly how and to what extent the MyoD family of myogenic regulatory genes (Olson 1990; Weintraub *et al.* 1991) are responsible for determining and differentiating the skeletal muscle lineage.

All types of muscle--smooth, cardiac, and skeletal--are derived from mesoderm. Smooth muscle is derived from mesenchymal cells which aggregate, enlarge, elongate, and produce myofibrils. New smooth muscle needed in late fetal development arises either by recruitment of nearby mesenchyme or by mitotic division of fibers already present. The primitive heart forms from the cardiogenic plate in the splanchnic mesoderm and over a period of a few days evolves from a simple "tube within a tube" to a four-chambered organ

(Arey 1965). The heart, along with the blood vessels, comprise the first organ system to reach a functional state. This is because a circulatory system is necessary to distribute nutrients and oxygen to all parts of the embryo and to remove wastes. Cardiocytes retain the capacity for nuclear as well as cell division until shortly after birth, though binucleate cells are common. Subsequent growth of the heart is accomplished by an increase in cell size rather than cell number (Jackson *et al.* 1990).

Skeletal muscle is derived mostly from the somites. Somites are segmental blocks of mesoderm which form sequentially from head to tail in pairs on either side of the neural tube (Tam 1981). The first somites appear on day eight of embryonic development, the last one on day fifteen (Rugh 1968). Initially, somites are formed at a rate of about one pair per hour (Tam 1981). The mature fetus boasts 65 pairs of somites (Gearhart and Mintz 1972). The somite is composed of three discernable cell types: the dermatome, located dorsally, will eventually give rise to the dermal layer of the skin (Rugh 1968). The sclerotome, an aggregate of mesenchymal cells located ventrally in the somite, is destined to form the vertebrae and ribs (Arey 1965). Between the dermatome and scleratome lies the myotome, which contains precursors of almost all skeletal muscle cells, the exceptions being muscles of the head and neck, which are derived from mesenchyme of the branchial arches (Arey 1965). By day 10 of embryonic development, the most mature (anterior) somites begin to exhibit differentiated myotomal muscle and, as assayed by *in situ* hybridization, express cardiac α-actin, a marker for differentiated embryonic skeletal muscle (Sassoon *et al.* 1988). As embryogenesis proceeds, precursor myoblasts from the myotome proliferate, migrate to the limb buds, and terminally differentiate to form primary multinucleated muscle fibers (Rugh 1968; Vivarelli *et al.* 1988; Emerson 1990). After birth, muscle growth and repair is mediated by the satellite myoblast. Satellite myoblasts are normally quiescent and undifferentiated cells, but given the proper stimulus, such as muscle damage or growth signals, they will proliferate extensively and fuse with existing muscle fibers, thus

increasing the bulk of the muscle and providing new, healthy tissue (Allen and Rankin 1990).

> "There is a certain antagonism between cell division and cell differentiation. Cells undergoing rapid division are in a state of turbulence which is unfavorable to cytoplasmic specialization. On the other hand, cells that are producing cytoplasmic elaborations of a physical nature tend to lose the plasticity that is requisite to mitosis." (Arey 1965)

Proliferation is perhaps as important during embryogenesis as differentiation. Deficient proliferation would generally result in an abnormally small fetus, and, if the defect is due to a specific early event, whole cell lineages or parts of lineages might never arise. One protein which appears to play an important role in the proliferation of mesodermal (and other) cells, including skeletal muscle, is that encoded by the c-myc proto-oncogene. c-myc is a nuclear phosphoprotein and was first identified as the transforming component of the avian retrovirus MC29 (Cole 1986). There is evidence that c-myc is involved in the pathway which links stimulation by growth factors to actual cellular proliferation. For example, mitogens such as platelet-derived growth factor, fibroblast growth factor, and epidermal growth factor induce c-myc expression 10 to 20 fold in some cells. On the other hand, expression of c-myc is low in quiescent cultured cells and in nonproliferating somatic tissues (Cole 1986) such as mature liver and skeletal muscle. Studies of skeletal muscle cell lines indicate that c-myc RNA levels are relatively high during proliferation, but down-regulation of c-myc expression occurs upon differentiation (Sejersen *et al.* 1985; Endo and Nadal-Ginard 1986). This reduction in myc expression appears necessary for differentiation to occur efficiently, as transfection of some

muscle cell lines with DNA that directs high level expression of c-myc, even when the endogenous c-myc gene is down-regulated, inhibits differentiation (Denis et al. 1987). This phenomenon is not restricted to muscle; mouse erythroleukemia cells transfected with a constitutively expressed c-myc gene blocks their differentiation into erythrocytes (Coppola and Cole 1986). Interestingly, high level expression of c-myc in the developing hearts of transgenic mice does not prevent differentiation, but it does cause a doubling of myocyte number (Jackson *et al.* 1990). F9 teratocarcinoma cells normally down-regulate c-myc expression when they differentiate in response to retinoic acid. If c-myc is artificially down-regulated by constitutive expression of antisense myc RNA, differentiation occurs spontaneously in the absence of the inducing agent (Griep and Westphal 1988). Thus, a body of evidence supports the idea that c-myc plays an important role in the control of proliferation and differentiation.

What is the mechanism by which c-myc functions at the molecular level? Until recently none was known; while it was clear that c-myc was localized to the nucleus and had a general affinity for DNA, suggesting that it might be a transcription factor, no sequence-specific DNA binding was observed, and strong evidence that any cellular genes were directly activated (or repressed) by c-myc was lacking (Cole 1986). Great progress has been made, though, in understanding how the domains of the c-myc protein function in DNA binding, how c-myc heterodimerizes with a related protein to form a sequence-specific DNA binding complex, and what sequences this complex prefers as binding sites.

The c-myc protein contains three domains important for dimerization and DNA binding: a basic region, which provides sequence-specific affinity for DNA; a helix-loop-helix (HLH) region, which is likely to mediate dimerization; and a leucine zipper region, also important for inter-protein interactions (Blackwood and Eisenman 1991). c-myc is one member of a family of over 60 proteins which contains the basic and HLH regions; these are referred to as bHLH proteins and will be discussed in more detail later. Using

vastly different experimental approaches, two groups arrived at DNA sequences to which c-myc specifically bound that had identical core sequences (Blackwell *et al.* 1990; Prendergast and Ziff 1991). This core fits the consensus sequence of the E box (CANNTG) to which many other bHLH proteins bind and which is present in the regulatory regions of many tissue-specific genes (Blackwell and Weintraub 1990). The gene for Max, a protein which can dimerize with c-myc and form a complex which binds to the E box element, was isolated and shown to also contain the bHLH and leucine zipper (bHLH-Zip) motifs (Blackwood and Eisenman 1991). With this information, it should now be possible to identify genes which are specifically induced by c-myc, and, perhaps, a more specific function in the cell for c-myc can be determined.

> "The cell types resulting from the processes of differentiation are discrete entities, without transitional forms; for example, an intermediate between a muscle cell and nerve cell is never seen. In other words, once a cell becomes committed to any type of differentiation it cannot at the same time engage in another kind; nor can a cell abandon its original line of specialization and change to a different course." (Arey 1965)

Perhaps the most important discovery in the molecular biology of development of the last five--if not 10--years was that expression of a single gene, MyoD, was sufficient to convert fibroblasts to cells manifesting a skeletal muscle phenotype (Davis *et al.* 1987). The existence of such a gene was predicted by experiments in which transfection of bulk genomic DNA from a muscle cell line into fibroblasts produced the same result, but at a very low frequency consistent with transfection of a single gene (Lassar *et al.* 1986). An interesting feature of MyoD was its similarity to c-myc in what would become known as the basic helix-loop-helix (bHLH) region discussed above. Subsequent experiments

showed that a span of 68 amino acids of MyoD containing its bHLH region was necessary and sufficient to convert stably transfected C3H 10T1/2 fibroblasts to muscle cells (Tapscott *et al.* 1988).

Studies from several laboratories soon made it clear that MyoD was not alone in its ability to do convert fibroblasts to myoblasts. The *Myd* locus was identified by transfection of a human cosmid library into 10T1/2 cells; *Myd* converted those cells to myoblasts and activated the endogenous MyoD gene (Pinney *et al.* 1988). The molecular nature of the *Myd* locus has not yet been reported. Three genes with near-perfect amino acid conservation with MyoD (and each other) in the bHLH region, but sharing only short spans of homology elsewhere, can also convert fibroblasts to skeletal muscle cells. These genes are called: 1) myogenin (Edmondson and Olson 1989; Wright *et al.* 1989); 2) Myf-5 (Braun *et al.* 1989); and 3) MRF4 (rat--[Rhodes and Konieczny 1989]), herculin (mouse--[Miner and Wold 1990]), and Myf-6 (human--[Braun *et al.* 1990]). Consistent with their specific activity in cell culture, all four of these bHLH genes, known as the MyoD family (Olson 1990; Weintraub *et al.* 1991), are expressed exclusively in skeletal muscle in the animal. Additionally, *in situ* hybridization experiments have shown that MyoD and myogenin transcripts are skeletal muscle-specific during embryogenesis, appearing first in the myotome and then in the limb bud (Sassoon *et al.* 1989). Expression of myogenin precedes that of myosin heavy chain, suggesting that myogenin may be a pre-differentiation marker for muscle precursors in the embryo.

While all these genes are able to convert fibroblasts to myoblasts upon transfection, more extensive studies have shown that many cell types are successfully convertible to muscle by forced expression of MyoD; fat, cartilage, smooth muscle, liver, nerve, and pigment cells are examples (Weintraub *et al.* 1989; Choi *et al.* 1990). In some cases, ectopic expression of MyoD resulted not only in accumulation of skeletal muscle-specific proteins, but also in repression of the transformants' endogenous differentiation programs.

For example, primary chondroblasts and retinal pigmented epithelial (RPE) cells infected with a MyoD-expressing retrovirus differentiated into muscle fibers that were indistinguishable from those derived from true myoblasts. However, virtually no infected cells that expressed muscle proteins also expressed cartilage or pigment markers, respectively (Choi *et al.* 1990). On the other hand, when immortalized melanoma and neuroblastoma cells were infected with a MyoD expressing retrovirus, the endogenous developmental programs appeared to coexist with a MyoD-initiated muscle program. Most melanoma cells that expressed myosin heavy chain (MHC) also contained pigment granules, and neuroblasts that stained positively for MHC also sent out axon-like processes characteristic of neurites (Weintraub *et al.* 1989).

What are the molecular mechanisms by which the MyoD family converts nonmuscle cells to the muscle lineage? In one experiment which begins to address this question, it was shown that bacterially produced MyoD could bind to the same two sites of the muscle creatine kinase (MCK) enhancer that are bound by a factor called MEF1. MEF1 is found in nuclear extracts from differentiated myocytes, and the MEF1 binding sites are required for efficient activity of the MCK enhancer. Furthermore, an antibody directed against MyoD also recognized MEF1 (Lassar *et al.* 1989). The implication is that MyoD converts cells to muscle by binding to the regulatory regions of muscle-specific genes and activating transcription upon differentiation. Since MyoD (and/or one of its relatives) is present in undifferentiated, proliferating myoblasts, it may be poised and ready to bind to its target sites when differentiation is signaled. The evidence that MyoD is not bound inactively at its target sites in myoblasts comes from a dimethyl sulfate *in vivo* footprinting analysis of the MCK enhancer (Mueller and Wold 1989). The MEF1 sites of the MCK enhancer were shown to be unoccupied in proliferating myoblasts when the gene is silent, but upon differentiation into myotubes the sites became resistant to methylation, indicating that proteins were bound there. While there is no direct evidence as to which protein or

proteins were occupying the sites, *in vitro* binding experiments suggest that the binding complexes contain a ubiquitous factor (see below) heterodimerized with either MyoD (Murre *et al.* 1989b) or myogenin (Brennan and Olson 1990), as these were the only two myogenic regulators expressed in the cells used for the *in vivo* analysis (Mueller and Wold 1989).

The notion that a ubiquitous, non-tissue-specific factor participates in binding to muscle-specific enhancers comes from experiments which show that *in vitro*, heterodimers which contain MyoD, myogenin, or Myf-5 and one of the ubiquitous E2A proteins (originally called E12 and E47--[Murre *et al.* 1989a]) bind to the MEF1 site better than any of these proteins bind alone (Murre *et al.* 1989b; Braun *et al.* 1990; Brennan and Olson 1990). The heterodimerization of these proteins appears to be mediated by their helix-loop-helix regions, and binding to DNA requires an intact basic region (Davis *et al.* 1990). These complexes bind to sites present in the enhancer of other muscle-specific genes, and in all cases the binding site matches the consensus for the E box, discussed previously as a binding site for the c-myc/Max heterodimer.

Yet there is still an important problem with this model regarding the control of muscle-specific gene expression. MyoD and E2A proteins are both present in proliferating myoblasts, yet there is no expression of differentiation genes and presumably no binding to DNA (Mueller and Wold 1989). What resolves this conundrum is the existence and apparent function of the protein Id (Benezra *et al.* 1990). Id is a HLH protein which lacks a basic region and, therefore, cannot participate in binding to DNA. Through its HLH domain, Id dimerizes with E2A and MyoD *in vitro*; these complexes cannot bind DNA and thus should not be able to activate transcription *in vivo*. However, when mitogens are removed, Id RNA levels fall, whereas MyoD and E2A RNA levels remain high. As Id protein levels plummet, MyoD and E2A should then be free to dimerize productively with

each other rather than nonproductively with Id, allowing expression of terminal differentiation genes (Benezra *et al.* 1990).

Id and E2A proteins are both widely expressed in many tissues, so the possibility exists that the mechanism outlined above for muscle applies to many different tissues. This implies that there should be at least one bHLH protein analogous to MyoD for each tissue which exploits this model of molecular interplay. Its activity would be inhibited by Id; Id levels would drop concurrently with the signal for differentiation, thus allowing a productive dimerization with E2A. It is likely that unique, perhaps tissue-specific DNA binding motifs are formed by juxtaposition of tissue-specific bHLH protein basic regions with those of E2A (Blackwell and Weintraub 1990); otherwise the MCK gene and perhaps other muscle-specific genes would be activated in all tissues that utilize this HLH scheme of transcriptional control. Also, alterations of the basic region of MyoD has revealed that the transcriptional activation function of MyoD can be destroyed without affecting its ability to bind DNA (Davis *et al.* 1990). Thus, MyoD may actually bind to fat-specific promoters, for example, in muscle, but the MyoD basic region is "coded" for activation of muscle genes only, so fat genes are not expressed in muscle. On the other hand, there may be other factors (besides bHLH proteins) that are necessary for tissue-specific gene expression or that repress genes which are specific to different tissues that should remain silent (Weintraub *et al.* 1991). Since the family of bHLH proteins has grown to more than 60 in vertebrates, yeast, plants, and insects (Emerson 1990), and some of these are tissue-specific, the method by which only the proper tissue-specific enhancers which contain E box sequences are activated by these proteins may be discovered.

The existence of four different genes encoding bHLH proteins which can each activate as yet indistinguishable myogenic programs when transfected into fibroblasts suggests that there may be some functional redundancy. Recent experiments have begun to address this problem. For example, it was shown that while all four MyoD family

members can heterodimerize with E2A and bind to both MEF1 sites of the MCK enhancer *in vitro*, only MyoD, myogenin, and Myf-5 were able to *trans*-activate a MCK enhancer-driven chloramphenicol acetyltransferase gene when transiently transfected into C3H 10T1/2 and NIH 3T3 cells; MRF4 gave very little activity (Chakraborty *et al.* 1991). MRF4 also showed reduced ability to *trans*-activate the troponin I enhancer compared with MyoD and myogenin (Yutzey *et al.* 1990). However, these and future similar analyses may be complicated by the fact that in cell culture, a transfected muscle regulator is sometimes able to activate expression of not only its endogenous counterpart, but also one or more of its myogenic relatives (Emerson 1990; Olson 1990; Weintraub *et al.* 1991). Thus, activities attributed to a transfected gene may actually stem partly from an endogenous one that was activated. Interestingly, the ability to selectively activate expression of other regulators may turn out to be the best assay for differentiating between the activities of the MyoD family members. For example, in NIH 3T3 cells, a transfected MyoD gene can only activate myogenin expression, whereas a transfected herculin (MRF4) gene can activate MyoD, myogenin, as well as endogenous herculin expression (Miner and Wold 1990).

The expression pattern of the MyoD family during development itself seems to imply that functional differences exist. For example, in terms of gross RNA abundance, Myf-5 RNA is expressed early in embryos but is barely detectable in adult skeletal muscle. On the other hand, MRF4 expression begins late in embryogenesis and is the most abundant muscle regulatory factor transcript in adult skeletal muscle (Rhodes and Konieczny 1989; Miner and Wold 1990). Since adult muscle fibers are structurally and functionally different from the primary fibers which form during embryogenesis, differential expression of MyoD family members may partially account for this, as well as for the differences between fibers in the adult (Miller 1990).

As the molecular nature of the interactions between muscle regulatory factors, ubiquitous helix-loop-helix proteins, and DNA becomes clear through *in vitro* experiments, the important problems in the molecular biology of myogenesis will center on determining what interactions actually occur *in vivo*, and how the results of those interactions might reflect functional differences among the four known myogenic regulators. The solutions to these problems may require rather exotic experimental designs, such as swapping protein domains between family members; mis-expressing family members in transgenic mice, perhaps by promoter exchange; and eliminating gene function, through antisense methods or homologous recombination. How the regulators themselves are initially activated during development is another question which has not really been addressed, but because it may involve events which occur at a stage of development earlier than determination of the myotome, its resolution may shed new light on similar problems in other mesodermal lineages.

References

Allen, R. E., and L. L. Rankin. 1990. Regulation of satellite cells during skeletal muscle growth and development. *Proc. Soc. Exp. Biol. Med.* **194:** 81-86.

Arey, L. B. 1965. *Developmental Anatomy.* W. B. Saunders Company, Philadelphia.

Benezra, R., R. L. Davis, D. Lockshon, D. L. Turner, and H. Weintraub. 1990. The protein Id: A negative regulator of helix-loop-helix DNA binding proteins. *Cell* **61:** 49-59.

Blackwell, T. K., L. Kretzner, E. M. Blackwood, R. N. Eisenman, and H. Weintraub. 1990. Sequence-specific DNA binding by the c-Myc protein. *Science* **250:** 1149-1151.

Blackwell, T. K., and H. Weintraub. 1990. Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. *Science* **250:** 1104-1110.

Blackwood, E. M., and R. N. Eisenman. 1991. Max: A helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with myc. *Science* **251:** 1211-1217.

Braun, T., E. Bober, B. Winter, N. Rosenthal, and H. H. Arnold. 1990. Myf-6, a new member of the human gene family of myogenic determination factors: evidence for a gene cluster on chromosome 12. *EMBO J.* **9:** 821-831.

Braun, T., G. Buschhausen-Denker, E. Bober, E. Tannich, and H. H. Arnold. 1989. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. *EMBO J.* **8:** 701-709.

Brennan, T. J., and E. N. Olson. 1990. Myogenin resides in the nucleus and acquires high affinity for a conserved enhancer element on heterodimerization. *Genes Dev.* **4:** 582-595.

Buskin, J. N., and S. D. Hauschka. 1989. Identification of a myocyte-specific nuclear factor which binds to the muscle-specific enhancer of the mouse muscle creatine kinase gene. *Mol. Cell. Biol.* **9:** 2627-2640.

Chakraborty, T., T. Brennan, and E. Olson. 1991. Differential trans-activation of a muscle-specific enhancer by myogenic helix-loop-helix proteins is separable from DNA binding. *J. Biol. Chem.* **266:** 2878-2882.

Choi, J., M. L. Costa, C. S. Mermelstein, C. Chagas, S. Holtzer, and H. Holtzer. 1990. MyoD converts primary dermal fibroblasts, chondroblasts, smooth muscle, and retinal pigmented epithelial cells into striated mononucleated myoblasts and multinucleated myotubes. *Proc. Natl. Acad. Sci. USA* **87:** 7988-7992.

Christy, R. J., V. W. Yang, J. M. Ntambi, D. E. Geiman, W. H. Landschulz, A. D. Friedman, Y. Nakabeppu, T. J. Kelly, and M. D. Lane. 1989. Differentiation-induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte-specific genes. *Genes Dev.* **3:** 1323-1335.

Cole, M. D. 1986. The *myc* oncogene: its role in transformation and differentiation. *Ann. Rev. Genet.* **20:** 361-384.

Coppola, J. A., and M. D. Cole. 1986. Constitutive c-*myc* oncogene expression blocks mouse erythroleukemia cell differentiation but not commitment. *Nature* **320:** 760-763.

Davis, R. L., P.-F. Cheng, A. B. Lassar, and H. Weintraub. 1990. The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. *Cell* **60:** 733-746.

Davis, R. L., H. Weintraub, and A. B. Lassar. 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **51:** 987-1000.

Denis, N., S. Blanc, M. P. Leibovitch, N. Nicolaiew, F. Dautry, M. Raymondjean, J. Kruh, and A. Kitzia. 1987. c-*Myc* oncogene expression inhibits the initiation of myogenic differentiation. *Exp. Cell Res.* **172:** 212-217.

Descombes, P., M. Chijkier, S. Lichtsteiner, E. Falvey, and U. Schibler. 1990. LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein. *Genes Dev.* **4:** 1541-1551.

Edmondson, D. G., and E. N. Olson. 1989. A gene with homology to the *myc* similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. *Genes Dev.* **3:** 628-640.

Emerson, C. P. 1990. Myogenesis and developmental control genes. *Curr. Opinion in Cell Biol.* **2:** 1065-1075.

Endo, T., and B. Nadal-Ginard. 1986. Transcriptional and posttranscriptional control of c-*myc* during myogenesis: its mRNA remains inducible in differentiated cells and does not suppress the differentiated phenotype. *Mol. Cell. Biol.* **6:** 1412-1421.

Friedman, A. D., W. H. Landschulz, and S. L. McKnight. 1989. CCAAT/enhancer binding protein activates the promoter of the serum albumin gene in cultured hepatoma cells. *Genes Dev.* **3:** 1314-1322.

Gearhart, J. D., and B. Mintz. 1972. Clonal origins of somites and their muscle derivatives: evidence from allophenic mice. *Dev. Biol.* **29:** 27-37.

Gossett, L., D. Kelvin, E. Sternberg, and E. N. Olson. 1989. A new myocyte-specific enhancer binding factor that recognizes a conserved element associated with multiple muscle-specific genes. *Mol. Cell. Biol.* **9:** 5022-5033.

Graves, R. A., P. Tontonoz, S. R. Ross, and B. M. Spiegelman. 1991. Identification of a potent adipocyte-specific enhancer: involvement of an NF-1-like factor. *Genes Dev.* **5:** 428-437.

Griep, A. E., and H. Westphal. 1988. Antisense *Myc* sequences induce differentiation of F9 cells. *Proc. Natl. Acad. Sci. USA* **85:** 6806-6810.

Jackson, T., M. F. Allard, C. M. Sreenan, L. K. Doss, S. P. Bishop, and J. L. Swain. 1990. The c-*myc* proto-oncogene regulates cardiac development in transgenic mice. *Mol. Cell. Biol.* **10:** 3709-3716.

Lassar, A. B., J. N. Buskin, D. Lockshon, R. L. Davis, S. Apone, S. D. Hauschka, and H. Weintraub. 1989. MyoD is a sequence-specific DNA binding protein requiring a region of *myc* homology to bind to the muscle creatine kinase enhancer. *Cell* **58:** 823-831.

Lassar, A. B., B. M. Paterson, and H. Weintraub. 1986. Transfection of a DNA locus that mediates the conversion of 10T1/2 fibroblasts to myoblasts. *Cell* **47:** 649-656.

Mendelzon, D., F. Boissier, and M. M. Zakin. 1990. The binding site for the liver-specific transcription factor Tf-LF1 and the TATA box of the human transferrin gene promoter are the only elements necessary to direct liver-specific transcription *in vitro*. *Nucleic Acids Res.* **18:** 5717-5721.

Miller, J. B. 1990. Myogenic programs of mouse muscle cell lines: Expression of myosin heavy chain isoforms, MyoD1, and myogenin. *J. Cell Biol.* **111:** 1149-1159.

Miner, J. H., and B. Wold. 1990. Herculin, a fourth member of the *MyoD* family of myogenic regulatory genes. *Proc. Natl. Acad. Sci. USA* **87:** 1089-1093.

Mueller, P. R., and B. Wold. 1989. *In vivo* footprinting of a muscle specific enhancer by ligation mediated PCR. *Science* **246:** 780-786.

Murre, C., P. Schonleber McCaw, and D. Baltimore. 1989a. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, *daughterless*, *MyoD*, and *myc* proteins. *Cell* **56:** 777-783.

Murre, C., P. Schonleber McCaw, H. Vaessin, M. Caudy, L. Y. Jan, Y. N. Jan, C. V. Cabrera, J. N. Buskin, S. D. Hauschka, A. B. Lassar, H. Weintraub, and D. Baltimore. 1989b. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell* **58:** 537-544.

Olson, E. N. 1990. MyoD family: a paradigm for development? *Genes Dev.* **4:** 2104-2111.

Pinney, D. F., S. H. Pearson-White, S. F. Konieczny, K. E. Latham, and C. P. Emerson Jr. 1988. Myogenic lineage determination and differentiation: evidence for a regulatory gene pathway. *Cell* **53:** 781-793.

Prendergast, G. C., and E. B. Ziff. 1991. Methylation-sensitive sequence-specific DNA binding by the c-Myc basic region. *Science* **251:** 186-189.

Rhodes, S. J., and S. F. Konieczny. 1989. Identification of MRF4: A new member of the muscle regulatory factor gene family. *Genes Dev.* **3:** 2050-2061.

Rugh, R. 1968. *The mouse: Its reproduction and development.* Burgess Publishing Company, Minneapolis.

Sassoon, D., G. Lyons, W. E. Wright, V. Lin, A. Lassar, H. Weintraub, and M. Buckingham. 1989. Expression of two myogenic regulatory factors myogenin and MyoD1 during mouse embryogenesis. *Nature (London)* **341:** 303-307.

Sassoon, D. A., I. Garner, and M. Buckingham. 1988. Transcripts of α-cardiac and α-skeletal actins are early markers for myogenesis in the mouse embryo. *Development* **104:** 155-164.

Sejersen, T., J. Sumegi, and N. R. Ringertz. 1985. Density-dependent arrest of DNA replication is accompanied by decreased levels of c-myc mRNA in myogenic but not in differentiation-defective myoblasts. *J. Cell. Physiol.* **125:** 465-470.

Sladek, F. M., W. Zhong, E. Lai, and J. E. Darnell Jr. 1990. Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. *Genes Dev.* **4:** 2353-2365.

Tam, P. P. L. 1981. The control of somitogenesis in mouse embryos. *J. Embryol. Exp. Morph.* **65** (Supplement): 103-128.

Tapscott, S. J., R. L. Davis, M. J. Thayer, P.-F. Cheng, H. Weintraub, and A. B. Lassar. 1988. MyoD1: a nuclear phosphoprotein requiring a myc homology region to convert fibroblasts to myoblasts. *Science* **242:** 405-411.

Vivarelli, E., W. E. Brown, R. G. Whalen, and G. Cossu. 1988. The expression of slow myosin during mammalian somitogenesis and limb bud differentiation. *J. Cell Biol.* **107:** 2191-2197.

Weintraub, H., R. Davis, S. Tapscott, M. Thayer, M. Krause, R. Benezra, T. K. Blackwell, D. Turner, R. Rupp, S. Hollenberg, Y. Zhuang, and A. Lassar. 1991.

The *myoD* gene family: nodal point during specification of the muscle cell lineage. *Science* **251:** 761-766.

Weintraub, H., S. J. Tapscott, R. L. Davis, M. J. Thayer, M. A. Adam, A. B. Lassar, and A. D. Miller. 1989. Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. *Proc. Natl. Acad. Sci. USA* **86:** 5434-5438.

Wilkison, W. O., H. Y. Min, K. P. Claffey, B. L. Satterberg, and B. M. Spiegelman. 1990. Control of the adipsin gene in adipocyte differentiation. *J. Biol. Chem.* **265:** 477-482.

Wright, W. E., D. A. Sassoon, and V. K. Lin. 1989. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD1. *Cell* **56:** 607-617.

Yutzey, K. E., S. J. Rhodes, and S. F. Konieczny. 1990. Differential *trans* activation associated with the muscle regulatory factors MyoD1, myogenin and MRF4. *Mol. Cell. Biol.* **10:** 3934-3944.

Chapter 2

Herculin, a Fourth Member of the *MyoD* Family of Myogenic Regulatory Genes

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Herculin, a fourth member of the *MyoD* family of myogenic regulatory genes

(Myf-5/myogenin/MYC/muscle development)

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ABSTRACT **We have identified and cloned herculin, a fourth mouse muscle regulatory gene. Comparison of its DNA and deduced amino acid sequences with those of the three known myogenic genes (*MyoD*, myogenin, and *Myf-5*) reveals scattered short spans with similarity to one or more of these genes and a long span with strong similarity to all three. This long span includes a sequence motif that is also present in proteins of the myc, achaete–scute, and immunoglobulin enhancer-binding families. The herculin gene is physically linked to the *Myf-5* gene on the chromosome; only 8.5 kilobases separate their translational start sites. A putative 27-kDa protein is encoded by three exons contained within a 1.7-kilobase fragment of the herculin gene. When expressed under the control of the simian virus 40 early promoter, transfected herculin renders murine NIH 3T3 and C3H/10T½ fibroblasts myogenic. In doing so, it also activates expression of myogenin, *MyoD*, and endogenous herculin in NIH 3T3 recipients. In adult mice, herculin is expressed in skeletal muscle but is absent from smooth muscle, cardiac muscle, and all nonmuscle tissues assayed. Direct comparison of the four known myogenic regulators in adult muscle showed that herculin is expressed at a significantly higher level than is any of the others. This quantitative dominance suggests an important role in the establishment or maintenance of adult skeletal muscle.**

Cell lineage studies have led to a description of myogenesis as a stepwise developmental progression beginning with a multipotential mesodermal stem cell and ending in a terminally differentiated, multinucleated myotube. The first step in this process produces from a multipotential precursor cell a determined myoblast that still possesses the ability to proliferate but is now committed to the myogenic pathway. A second distinct step involves withdrawal of the myoblast from the cell cycle and activation of a battery of muscle-specific genes, such as those encoding contractile proteins and metabolic enzymes that identify the cell as a myocyte. Subsequent maturation into functional skeletal muscle includes fusion to form multinucleated myotubes of several distinct types.

A crucial step in understanding the regulation of this developmental pathway was the cloning by Davis *et al.* (1) of MyoD cDNA, which upon transfection converts a variety of nonmyogenic cells into functional myoblasts (2). Yet *MyoD* has turned out to be but one member of a family of myogenic genes, which also includes myogenin (3, 4) and *Myf-5* (5). Each can initiate myogenesis in a transfection assay, and their products share one very similar protein sequence that is also characteristic of a larger, extended family of nuclear proteins that includes the myc family, *Drosophila* achaete–scute complex products and immunoglobulin enhancer-binding proteins (1, 6). Some of these have been shown to be sequence-specific DNA-binding proteins, and the shared protein motif is essential for binding (7). MyoD, for example, binds to the mouse muscle creatine kinase enhancer *in vitro* (8).

While all skeletal muscle is thought to develop by the progression outlined above, the types of differentiated cells produced are phenotypically diverse. For example, several subtypes of fast- and slow-contracting fibers exist in fetal and adult skeletal muscle, and this diversity is thought to be a product of developmental history and, in some instances, function (reviewed in ref. 9). These skeletal muscle types express partially overlapping but distinct sets of muscle-specific gene products, implying the existence of a correspondingly diverse and subtle system of muscle gene regulation. This may be provided, at least in part, by different myogenic regulators of the MyoD type acting alone or in specific combinations. In this work we report the cloning and characterization* of herculin, a fourth member of the myogenic regulatory family. Among the presently known myogenic regulatory factors, it is the quantitatively dominant species in adult muscle, suggesting a major role in differentiation and/or maturation of adult skeletal muscle. Its capacity to activate expression of some other members of the family in cell culture experiments is shown, and implications of these results for regulatory pathways *in vivo* are discussed.

MATERIALS AND METHODS

Identification of Mouse *Myf-5*-Positive Plaques. The BALB/c spleen Charon 4A mouse library was a gift from R. Perlmutter and L. Hood (California Institute of Technology). Plaques were transferred to Hybond-N nylon circles (Amersham) and hybridized to human Myf-5 cDNA probe (a gift from H. Arnold, University of Hamburg Medical School) at 42°C overnight in a solution containing 40% formamide, 6× SSPE (1× SSPE = 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA), 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 50 mM sodium phosphate (pH 6.5), 0.5% $NaDodSO_4$, and 50 μg of salmon sperm DNA per ml.

DNA Sequencing. After restriction mapping, fragments of phage insert DNA were subcloned into Bluescript II KS+ (Stratagene) by standard methods (10) and sequenced with a Sequenase DNA sequencing kit (United States Biochemical) with T3 (Stratagene) and T7 (Promega) promoter primers.

Cell Culture and Analysis. NIH 3T3 cells were grown in 90% (vol/vol) Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) calf serum (both GIBCO). C3H/10T½ cells were grown in 90% DMEM/10% (vol/vol) fetal bovine serum (Hazleton Research Products, Lenexa, KS). Cells on 10-cm plates were transfected by a standard calcium phosphate coprecipitation with 5 or 10 μg of the herculin expression vector pSVhrc, 0.5 μg of pY3, which provides resistance to hygromycin B (11), and enough non-

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*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M30499).

specific plasmid or L-cell DNA to make 15 μg. After 12 hr of exposure to DNA, cells were fed fresh medium. After another 24 hr, the medium was changed to DMEM containing 200 μg of hygromycin B (Calbiochem) per ml and one of the following: 10% calf serum, 2.5% fetal bovine serum (NIH 3T3 cells), or 15% fetal bovine serum (C3H/10T½ cells) to select stable transformants. Alternatively, to induce myogenesis in a transient assay for myogenic activity, the medium was changed to DMEM containing 2% horse serum (Flow Laboratories) and 2 μg of insulin (GIBCO) per ml for 3 days, after which the cells were fixed and immunocytochemically stained for myosin heavy chain (as in ref. 1) with Amersham reagents.

RNA Isolation and Analysis. RNAs were prepared by the method of Chomczynski and Sacchi (12). Riboprobes were synthesized from Bluescript I- or II-based templates. Hybridizations for RNase protections were done in 30 μl of 80% deionized formamide/40 mM Pipes, pH 6.4/1 mM EDTA/0.4 M NaCl under mineral oil at 50°C for 12 hr. Unhybridized probes were digested with 300 units of RNase T1 (BRL) at 30°C for 30 min.

RESULTS AND DISCUSSION

A Phage Containing *Myf-5* Also Contains the Related Herculin Gene. To isolate a fragment of the mouse *Myf-5* gene, we screened a BALB/c spleen genomic library at reduced stringency with the 750 base pair (bp) *Pst* I–*Pvu* II fragment of human Myf-5 cDNA (5). This fragment, which does not contain the *myc* homology region of *Myf-5*, was chosen to avoid cross-reaction with *MyoD* and myogenin. Six reproducibly positive plaques were identified, and restriction analyses of their inserts indicated there were two distinct clones among them that represented both possible orientations of the same 14.5-kilobase (kb) genomic insert. More detailed restriction mapping and Southern DNA transfer analyses led to the map of the phage insert shown in Fig. 1. The analysis also revealed two separate *myc* homologies approximately 8 kb apart, suggesting the presence of two different genes. The DNA sequence within and adjacent to both *myc* homologies was determined, and it confirmed that one was indeed part of the mouse *Myf-5* gene and that the other, 8.5 kb upstream, was part of a new but related gene, which we have named "herculin." Their restriction maps are also shown in Fig. 1. The fact that two independent clones with identical insert maps were isolated suggested a true linkage, not linkage by cloning artifact. To confirm linkage in the genome, Southern blots of *Kpn* I-digested mouse genomic DNA were probed with two gene-specific fragments (see the legend to Fig. 1) either separately or together. From the phage map, *Kpn* I is expected to produce a 9-kb chromosomal segment containing both fragments, and in all three cases the probes did identify a single 9-kb hybridizing band (data not shown).

Sequence of the Herculin Gene Reveals Regions Similar to *MyoD*, Myogenin, and *Myf-5*. The nucleotide sequence and predicted amino acid sequence of herculin are shown in Fig. 2*A*. The DNA sequence extends from a consensus TATA element located just upstream of the *Sal* I site to the *Xba* I site located 3′ of an in-frame TAA stop codon. Assignments of internal exon boundaries were confirmed by sequence analysis of products derived by polymerase chain reaction amplification of herculin cDNA from skeletal muscle (which expresses herculin—see below).

The initial basis for proposing the putative 27-kDa protein to be a member of the MyoD family is its strong identity with MyoD, myogenin, and Myf-5 over the 57 amino acid span boxed in Fig. 2*A* and reproduced for comparisons in Fig. 2*B*. Similar domains are found in proteins of the MYC, *Drosophila* achaete-scute, and immunoglobulin enhancer-binding families and include the proposed helix–loop–helix motif (6), which is known to be important for protein dimerization and DNA binding (7). Within this region the herculin protein is most identical to the myogenic regulators myogenin (82%), MyoD (81%), and Myf-5 (79%). MyoD and Myf-5 are the most closely related pair in the family, having 89% identity in this region. By contrast, the relatedness of herculin to nonmyogenic molecules such as c-myc and E12 is only 33% and 36%, respectively.

In addition to the similarity delineated by the box, there are other amino acids conserved among all four myogenic regulators, the majority of which are in a region just upstream of the box, and these are shown as shaded in Fig. 2*A*. There are no other extended regions of amino acid similarity shared by all four molecules, but there are many scattered spans of homology shared by herculin and only one or two of the other myogenic molecules, the most unusual being a stretch between amino acid residues 214 and 229. In this region, the herculin protein sequence is similar to MyoD and Myf-5 but not to myogenin. However, the corresponding nucleic acid sequence reveals a striking homology shared by all four. This can be explained by a difference in reading frame caused by the apparent deletion of a base from myogenin relative to the other three. The surprising apparent conservation of 48 nucleotides of myogenin DNA following a frameshifting event may suggest that this was a relatively recent occurrence or that there is some independent selective pressure for conservation of this sequence in DNA or in RNA.

Expression of Herculin Is Confined to Skeletal Muscle in an Adult Mouse. Fig. 3 shows the result of a RNase protection experiment designed to detect herculin transcripts in the indicated adult mouse tissues. By this sensitive assay, expression of herculin was confined to skeletal muscle; no transcripts were detected in smooth muscle or cardiac muscle or in other tissues. This pattern is not surprising, since MyoD and myogenin are also exclusively expressed in skeletal muscle. However, *Myf-5*, the nearby chromosomal neighbor of the herculin gene, is reported to be expressed in both skeletal and smooth muscle (5). This indicates that despite their proximity in the genome, the genes for herculin and Myf-5 must be subject to independent regulation, at least in the context of smooth muscle. However, coregulation in

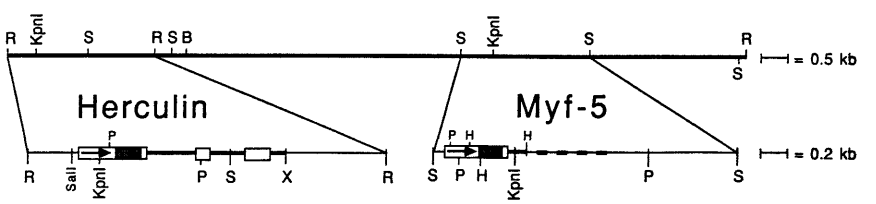

FIG. 1. Phage and plasmid maps showing the genomic organization of herculin and *Myf-5* genes. Boxes identify exons, shading indicates the shared *myc* sequence similarity, and arrows indicate direction of transcription. Only the first exon and part of the first intron of *Myf-5* have been mapped and sequenced; the dotted line represents a portion of the gene not yet analyzed. B, *Bam*HI; H, *Hin*dIII; P, *Pst* I; R, *Eco*RI; S, *Sac* I; X, *Xba* I. Southern blot probes were the *Pst* I–*Sac* I fragment of the herculin gene and the *Sac* I–*Hin*dIII fragment of *Myf-5*.

A

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            SalI                                                                                                       M  M  M  D  L  F  E  T  G     9
TATAAAGCTGGGTCGACTTATGTCACCGCACTAATTAAATGCCATCTGGGTGGCTCCTCTGGGTTTTTGAGTCCATCACCCAGATCAGATCAGTCAGAGGCCAAGGAGGAGAACATGATGATGGACCTTTTTGAAACTGG 140

 S  Y  F  F  Y  L  D  G  E  N  V  T  L  Q  P  L  E  V  A  E  G  S  P  L  Y  P  G  S  D  G  T  L  S  P  C  Q  D  Q  M  P  Q  E  A  G  S  D  S    56
CTCCTATTTCTTCTACTTAGATGGAGAAAATGTGACTCTTCAGCCATTAGAAGTGGCAGAGGGCTCTCCTTTGTATCCAGGGAGTGATGGTACCCTATCCCCTTGCCAGGACCAAATGCCCCAGGAAGCCGGGAGCGACA 280

 S  G  E  E  H  V  L  A  P  P  G  L  Q  P  P  H  C  P  G  Q  C  L  I  W  A  C  K  T  C  K  R  K  S  A  P  T [D  R  R  K  A  A  T  L  R  E]   102
GCAGTGGAGAGGAACACGTTCTGGCTCCCCCAGGCCTGCAGCCTCCCCACTGCCCTGGCCAGTGTCTGATCTGGGCTTGCAAGACTTGCAAGAGAAAATCTGCCCCCACAGATCGTCGGAAAGCAGCTACCCTGCGCGAA 420

[R  R  R  L  K  K  I  N  E  A  F  E  A  L  K  R  R  T  V  A  N  P  N  Q  R  L  P  K  V  E  I  L  R  S  A  I  S  Y  I  E  R  L  Q  D  L  L  H]  149
AGGAGGAGACTAAAGAAAATCAACGAAGCCTTTGAGGCCCTGAAGCGTCGGACTGTGGCCAACCCCAACCAGAGACTGCCCAAGGTGGAGATTCTGCGGAGTGCCATCAGCTACATTGAGCGTCTACAGGACCTGCTGCA 560

 R  L  D  Q  Q  E  K  M  Q  E  L  G  V  D  P  Y  S  Y  K  P  K  Q  E  I                                                                          173
CCGGCTGGATCAGCAAGAGAAGATGCAGGAGCTGGGCGTGGACCCCTACAGCTACAAACCCAAGCAAGAAATTgtaagcccatatgcgaccaggccagggaaatgtaaaaggcctattaaaagtcttcctgagactgtat 700

cctccacctgcttggtgcctacccccttcttgcccgcccctagcctctctaatgaacctccagtgaccctagaagatagagcccctgtcctaagcaggaaatgcagtctggcccaagaaagcaggggagacacccccaa 840

                                                                              L  E  G  A  D  F  L  R  T  C  S  P  Q  W  P  S  V  S  D  H  S    194
gcctccacctcccacncctcctctccccccctgcgtatttgtttcttttctaatctgctgctttggttttccttccagCTTGAGGGTGCGGATTTCCTGCGCACCTGCAGCCCCCAGTGGCCAAGTGTTTCGGATCATTCC 980

R  G  L  V  I  T  A  K  E  G                                                                                                                   204
AGGGGCCTCGTGATAACTGCTAAGGAAGgtaacttaaaagaaagggcactgggctgtactggaagcatctaggctcagcacagcacagcacagcacagcacagcacagcacagcacagcacagcacagcacagcacagca 1120

cagcacagcacagcacagcacagcacagcatagcacaggagtgctctggctgagcacaaagagctcaaagctagcaacagccaaatccggcgcttttttttttttttttctctcagggatgcaatctcctgcgtggcc 1260

                                                                          G  A  N  V  D  A  S  A  S  S  S  L  Q  R  L  S  S  I  V  D  S  I  S    227
ttggtagccttctgtggggcacacagaggaatgctacaggcgcggtttgtgtttgttctctgtgcgcagGAGGAGCAAACGTGGATGCCTCAGCCTCCAGCAGTCTTCAGCGCCTTTCTTCCATCGTGGACAGTATTTCT 1400

S  E  E  R  K  L  P  S  V  E  E  V  V  E  K  end                                                                                               242
TCAGAGGAACGCAAACTCCCCAGCGTGGAGGAGGTGGTGGAGAAGTAACTCAGCCAGCATTTGGAACATTCTTCACCCAGCAGGAAGACCCCCCTTTCCACCTAATCATTTAGATTAGGGCTCACAGATGCCAGCTTTTA 1540

TGAAAGGCGAGAGATTTAGTATTTAAAAAGAAACTTCTCACCACCTCAAGTGAAAGTCCTNCCTTGGGGCTTTTATTATGATACTTATTATATATCGGAACGGCTAGTAGCTTAGCTCTAGA                   1662
                                                                                                                      XbaI
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B

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MyoD       DRRKAATMRERRRLSKVNEAFETLKRCTSSNPNQRLPKVEILRNAIRYIEGLQALLR
Myf-5      DRRKAATMRERRRLKKVNQAFETLKRCTTTNPNQRLPKVEILRNAIRYIESLQELLR
Myogenin   DRRRAATLREKRRLKKVNEAFEALKRSTLLNPNQRLPKVEILRHAIQYIERLQALLS
Herculin   DRRKAATLRERRRLKKINEAFEALKRRTVANPNQRLPKVEILRSAISYIERLQDLLH
                   *              *    * **            *    *     *   *   *
```

FIG. 2. Nucleotide sequence of the herculin gene and the deduced amino acid sequence of herculin (*A*) and comparison of the myc similarity motifs from the indicated proteins (*B*). The boxed amino acids in *A* comprise this shared motif, and shading outside the box denotes other amino acid spans common to all four proteins. Stars in *B* denote amino acid positions with the most variability among the molecules in this region.

skeletal muscle by one or more shared cis-acting elements is possible, since both genes are expressed there.

Herculin Is Myogenic in NIH 3T3 and C3H/10T½ Cell Backgrounds. To determine whether the sequence similarities between herculin and the other MyoD-related molecules reflect functional similarities, the 1.7-kb *Sal* I–*Xba* I fragment of the herculin gene, which contains the entire putative coding region, was inserted into the expression vector pECE (13). pECE contains a polylinker flanked by the simian virus 40 early promoter and the tumor antigen polyadenylylation signal sequence. The resulting plasmid, pSVhrc, was transfected by calcium phosphate coprecipitation into NIH 3T3 and C3H/10T½ cells together with a selectable marker. These host cells are not myogenic prior to transfection, but herculin expression was found to induce myogenesis in both lines. This was demonstrated in a transient assay by the appearance of myosin heavy chain in individual cells detected by immunostaining (data not shown). The efficiency with which the herculin vector induced myogenic cells was approximately the same as that of an analogous MyoD vector in a parallel experiment. Transfection with the pECE vector alone did not produce any myogenic cells.

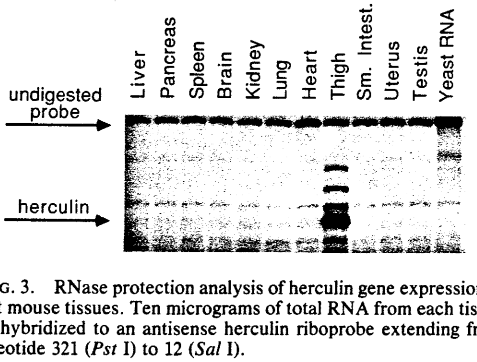

FIG. 3. RNase protection analysis of herculin gene expression in adult mouse tissues. Ten micrograms of total RNA from each tissue was hybridized to an antisense herculin riboprobe extending from nucleotide 321 (*Pst* I) to 12 (*Sal* I).

An interesting and potentially important property of MyoD is that it can, in some recipient cells, activate expression of endogenous *MyoD*, demonstrating a positive autoregulatory pathway (14). We asked whether cells stably transfected with herculin similarly activated the endogenous herculin gene or any of the other cloned myogenic regulatory genes. Stable herculin-transfected NIH 3T3 cells were pooled and cultured under conditions that promoted either proliferation (12.5% serum) or differentiation (2% serum for 3 days). Total cellular RNA was extracted and analyzed by RNase protection assays for expression of herculin, myogenin, and *MyoD* (Fig. 4). Myogenin was activated by herculin but only under differentiation conditions; this is a pattern consistent with myogenin's behavior in other myogenic cell lines (Fig. 4*B*), where it is always expressed upon differentiation. The MyoD and herculin probes are designed to distinguish between endogenous transcripts and those derived from the transfected constructs. The data in Fig. 4*C* demonstrate that exogenous herculin activates endogenous *MyoD* in NIH 3T3 cells, and this activation is most pronounced in cells that have differentiated. The results in Fig. 4*A* show that herculin transformation of NIH 3T3 cells can also activate expression of the endogenous herculin gene, and preliminary experiments suggest a low-level activation of *Myf-5* in these cells as well (J.H.M., unpublished observations). We conclude that herculin can act as a powerful member of the myogenic regulatory network. Its capacity to activate, either directly or indirectly, *MyoD*, myogenin, endogenous herculin, and *Myf-5* argues for a potentially complex network of interactions involving the MyoD family members. It is clear that host-cell characteristics also play a significant role in defining the

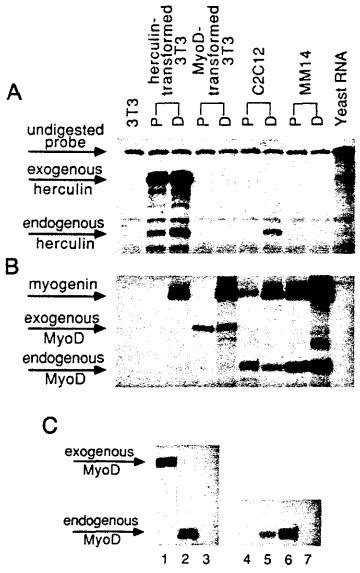

FIG. 4. RNase protection analyses of herculin, myogenin, and *MyoD* gene expression in cell lines under conditions of proliferation (lanes P) or differentiation (lanes D). (*A* and *B*) Separate experiments in which 10 μg of total RNA from each cell line was hybridized to the following riboprobes: herculin, which was the same as in Fig. 3; myogenin, which extended from nucleotide 218 (*Sma* I) to 35 (*Sty* I) (4); MyoD, which extended from an extragenic *Bam*HI site in the MyoD expression vehicle pEMc11s to nucleotide 1692 (*Sty* I) of MyoD cDNA (1). The low level of myogenin expression in proliferating C2C12 and MM14 populations is probably due to a small number of spontaneously differentiated cells. (*C*) Results of additional studies with the MyoD riboprobe alone. Lanes: 1, differentiated MyoD-transformed NIH 3T3 cells; 2, differentiated aza-myoblasts; 3, yeast RNA; 4, proliferating herculin-transformed NIH 3T3 cells; 5, differentiated herculin-transformed NIH 3T3 cells; 6, differentiated C2C12 cells; and 7, yeast RNA. C2C12 cells were grown in 80% DMEM/20% fetal bovine serum and differentiated in 98% DMEM/2% horse serum/2 μg of insulin per ml for 2 days. MM14 cells were grown and differentiated as in ref. 15.

network. In the NIH 3T3 cells studied here, MyoD transfection activates myogenin expression but fails to activate herculin (Fig. 4*A*), *Myf-5* (data not shown) or endogenous *MyoD* (Fig. 4*C*). By contrast, in C3H/10T½ cells, exogenous MyoD does activate its endogenous counterpart (14). Therefore, we expect that host-cell properties are influencing herculin regulatory activity in our cell culture experiments and, very likely, play a significant role in the developing animal as well. Although the importance of host-cell factors cautions against a facile generalization from cell culture model systems to myogenesis *in vivo*, the potent activity of the herculin gene in transfection studies does reveal a circuit for extensive cross-talk between herculin and other family members. This circuitry may prove to be important *in vivo* for initiating a developmental decision or for reinforcing one, once made.

A variety of other muscle cell lines was also examined, and C2C12 (16) was found to be the only one that, under our culture conditions, expressed herculin at detectable levels. MM14 cells (17) and aza-myoblasts (derived by 5-azacytidine treatment of C3H/10T½ cells) (1) did not express herculin (Fig. 4*A* and data not shown). Thus, some independently derived lines can differentiate without detectable expression of herculin, demonstrating that it is neither essential for maintenance of the myoblast phenotype nor for differentiation into muscle. Also, it is notable that in this range of cell types, expression of MyoD or myogenin has not activated expression of herculin. It is not known, however, if any of these lines expressed herculin at some earlier point in their developmental history. The tissue explant-derived line that does express herculin, C2C12, also expresses all of the other family members, and in this respect is similar to herculin-transfected NIH 3T3 cells. Other lines examined express, in the differentiated state, various pairwise combinations of myogenin with only one other member.

Expression of Herculin *in Vivo* Is High Compared with MyoD, Myogenin, and Myf-5. To begin to compare herculin expression in the animal with its activity in cell culture, we measured the relative levels of expression of muscle regulatory genes in adult skeletal muscle by RNase protection assays (Fig. 5) using riboprobes for myogenin and Myf-5 (lane 1), herculin (lane 2), and MyoD (lane 3). Probes were synthesized such that the nuclease-protected probe fragments for each of the four had identical specific activities on a molar basis. Thus, the intensity of each band is proportional to its relative abundance in total RNA from adult skeletal muscle. It is evident that herculin RNA is by far the most prevalent.

Because herculin is the most abundant of the four known myogenic molecules in this tissue, it seems likely that it plays a significant role in the maintenance of the skeletal muscle phenotype in adults. However, it remains to be determined whether herculin is globally expressed in all mature adult skeletal muscle or is restricted to a specific subset of muscle cell types or physiological states. The possibility that herculin is differentially expressed among different muscle cell populations is part of the more general question concerning the roles played by each MyoD family member in muscle tissue and its developmental precursors. Lineage studies suggest that there are different precursor myoblast populations in developing muscle that give rise to distinct muscle fiber types (9). It seems likely that the ultimate differentiated phenotype of these species of skeletal myocytes may be dictated, at least in part, by their patterns of expression of MyoD, myogenin, Myf-5, herculin, and perhaps Myd (18) and ski (19) during development and upon maturation. A comprehensive picture of the pattern of expression for each of these regulators during the life history of a given myogenic lineage is not presently available, but an initial study of MyoD and myogenin in the developing mouse embryo has found significant diversity among different cell populations. By hybridization to RNA *in situ*, Sassoon *et al.* (20) found at least two different

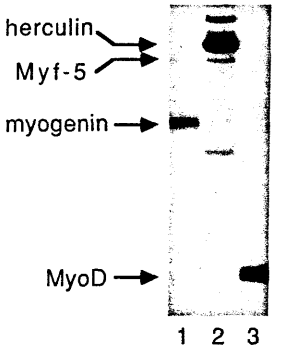

FIG. 5. RNase protection analyses of herculin, Myf-5, myogenin, and MyoD transcripts in adult skeletal (thigh) muscle. Total RNA (10 μg) was hybridized to the following riboprobes: Myf-5 and myogenin (lane 1), herculin (lane 2), and MyoD (lane 3). The Myf-5 probe was synthesized from a 382-bp *Hin*dIII fragment that includes the end of the first exon (see Fig. 1). The other probes were as described in the legends to Figs. 3 and 4.

patterns of expression coincident with two different types of muscle, myotomal and limb. They also reported the presence of determined myoblasts in the limb bud that expressed no detectable MyoD or myogenin, though some of these myoblasts did express detectable levels after 4 or 5 days in culture. Were these myoblasts initially determined by expression of a different myogenic molecule, such as herculin, Myd, or Myf-5, which later activated expression of MyoD and myogenin? Such a temporal sequence of expression of these molecules during development might arise from the circuitry and changing host-cell influences identified in our cell culture experiments. This regulatory history could then lead to a final set of regulators that governs the functional identity of a mature myocyte.

Note Added in Proof. With respect to expression of Myf-5 (5), Arnold and coworkers (21) have continued investigation and recently reported that it is absent from smooth muscle. Therefore, herculin and *Myf-5* (as well as *MyoD* and myogenin) are similar in their restriction of expression to skeletal muscle.

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1. Davis, R. L., Weintraub, H. & Lassar, A. B. (1987) *Cell* **51,** 987–1000.
2. Weintraub, H., Tapscott, S. J., Davis, R. L., Thayer, M. J., Adam, M. A., Lassar, A. B. & Miller, A. D. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 5434–5438.
3. Wright, W. E., Sassoon, D. A. & Lin, V. K. (1989) *Cell* **56,** 607–617.
4. Edmonson, D. G. & Olson, E. N. (1989) *Genes Dev.* **3,** 628–640.
5. Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E. & Arnold, H. H. (1989) *EMBO J.* **8,** 701–709.
6. Murre, C., Schonleber McCaw, P. & Baltimore, D. (1989) *Cell* **56,** 777–783.
7. Murre, C., Schonleber McCaw, P., Vaessin, H., Caudy, M., Jan, L. Y., Jan, Y. N., Cabrera, C. V., Buskin, J. N., Hauschka, S. D., Lassar, A. B., Weintraub, H. & Baltimore, D. (1989) *Cell* **58,** 537–544.
8. Lassar, A. B., Buskin, J. N., Lockshon, D., Davis, R. L., Apone, S., Hauschka, S. D. & Weintraub, H. (1989) *Cell* **58,** 823–831.
9. Miller, J. B. & Stockdale, F. E. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 3860–3864.
10. Maniatis, T., Fritsch, E. R. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
11. Blochlinger, K. & Diggelmann, H. (1984) *Mol. Cell. Biol.* **4,** 2929–2931.
12. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
13. Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A. & Rutter, W. J. (1986) *Cell* **45,** 721–732.
14. Thayer, M. J., Tapscott, S. J., Davis, R. L., Wright, W. E., Lassar, A. B. & Weintraub, H. (1989) *Cell* **58,** 241–248.
15. Mueller, P. R. & Wold, B. (1989) *Science* **246,** 780–786.
16. Blau, H. M., Pavlath, G. K., Hardeman, E. C., Chiu, C. P., Silberstein, L., Webster, S. G., Miller, S. C. & Webster, C. (1985) *Science* **230,** 758–766.
17. Linkhart, T. A., Clegg, C. H. & Hauschka, S. D. (1981) *Dev. Biol.* **86,** 19–30.
18. Pinney, D. F., Pearson-White, S. H., Konieczny, S. F., Latham, K. E. & Emerson, C. P., Jr. (1988) *Cell* **53,** 781–793.
19. Colmenares, C. & Stavnezer, E. (1989) *Cell* **59,** 293–303.
20. Sassoon, D., Lyons, G., Wright, W. E., Lin, V., Lassar, A., Weintraub, H. & Buckingham, M. (1989) *Nature (London)* **341,** 303–307.
21. Braun, T., Bober, E., Buschhausen-Denker, G., Kotz, S., Grzeschik, K. H. & Arnold, H. H. (1989) *EMBO J.* **8,** 3617–3625.

Chapter 3

c-myc Inhibition of MyoD and Myogenin-Initiated Myogenic Differentiaion

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c-*myc* Inhibition of MyoD and Myogenin-Initiated Myogenic Differentiation

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In vertebrate development, a prominent feature of several cell lineages is the coupling of cell cycle regulation with terminal differentiation. We have investigated the basis of this relationship in the skeletal muscle lineage by studying the effects of the proliferation-associated regulator, c-*myc*, on the differentiation of MyoD-initiated myoblasts. Transient cotransfection assays in NIH 3T3 cells using MyoD and c-*myc* expression vectors demonstrated c-*myc* suppression of MyoD-initiated differentiation. A stable cell system was also developed in which MyoD expression was constitutive, while *myc* levels could be elevated conditionally. Induction of this conditional c-*myc* suppressed myogenesis effectively, even in the presence of MyoD. c-*myc* suppression also prevented up-regulation of a relative of MyoD, myogenin, which is normally expressed at the onset of differentiation in all muscle cell lines examined and may be essential for differentiation. Additional experiments tested whether failure to differentiate in the presence of *myc* could be overcome by providing myogenin ectopically. Cotransfection of c-*myc* with myogenin, MyoD, or a mixture of myogenin and MyoD showed that neither myogenin alone nor myogenin plus MyoD together could bypass the c-*myc* block. The effects of c-*myc* were further dissected by showing that c-*myc* can inhibit differentiation independently of Id, a negative regulator of muscle differentiation. These results lead us to propose that c-*myc* and Id constitute independent negative regulators of muscle differentiation, while myogenin and any of the other three related myogenic factors (MyoD, Myf-5, and MRF4/herculin/Myf-6) act as positive regulators.

During skeletal muscle development, multipotential precursor cells progress through a determined myoblast stage before differentiating to become myocytes. While precursors and myoblasts proliferate extensively, withdrawal from the cell cycle is required for differentiation (13). The onset of differentiation is characterized by the expression of genes that code for muscle-specific enzymes, contractile proteins, and receptors that are not expressed in myoblasts (reviewed in reference 48). The determination step that establishes a cell as a myoblast can be forced in many fibroblast cell lines by expression of any one of the four members of the MyoD family of myogenic regulatory genes. This family (reviewed in reference 42) includes genes encoding MyoD (17, 54), myogenin (20, 59), and Myf-5 (7) and the rat gene encoding MRF4 (47) which is called herculin in mice (37) and Myf-6 in humans (6). All share a highly similar 57-amino-acid B-HLH domain characterized by a strongly basic region in the amino portion followed by a potential helix-loop-helix (HLH) structure (40). Similar domains are also found in an extended family of nuclear proteins (reviewed in reference 31). In vertebrates, these include the *myc* family of protooncogenes (reviewed in reference 14), twist (28), achaete-scute (29), and the E12 and E47 proteins, first identified as binding to the immunoglobulin κ-chain enhancer (40).

In vitro studies of DNA binding and protein-protein interactions, together with mutagenesis studies, show that the B-HLH domain is functionally important (9, 16, 34, 41). The HLH portion mediates protein-protein interactions between molecules to yield homo- or hetero-oligomers. Dimers or higher order oligomers are the molecular species capable of binding to DNA in a sequence-specific fashion, and such binding is dependent on the presence of an intact basic region in each monomer. Some mutants with mutations in the basic region of MyoD which do bind DNA in vitro do not activate muscle-specific gene expression in vivo, suggesting that the basic region plays a complex role in gene activation (16).

Further support for the roles of the basic and HLH regions in producing active DNA-binding proteins comes from the nature of the protein Id (2). Id contains an HLH domain but not an adjacent basic region. It can associate in vitro with MyoD, E12, and E47 and inhibit their binding to DNA. In vivo, overexpression of Id inhibits the *trans*-activation of the muscle creatine kinase (MCK) enhancer upon cotransfection with MyoD. Thus, Id, which is widely expressed, appears to negatively regulate the differentiation functions of tissue-specific B-HLH complexes by sequestering components of such complexes in inactive hetero-oligomers (2).

The structural similarity of the MyoD family of regulators with the *myc* family of proto-oncogenes is interesting because c-*myc* is strongly implicated in the control of cell proliferation in several different cell lineages, including muscle (30, 51). During myogenesis, c-*myc* is expressed in proliferating myoblasts, but *myc* mRNA and protein levels drop substantially when these cells withdraw from the cell cycle and differentiate (22, 51). *myc* is further implicated in the proliferation-differentiation switch by the fact that differentiation-defective derivatives of some myoblast cell lines fail to down-regulate c-*myc* expression (51). Other studies show that ectopic *myc* expression can interfere with myoblast differentiation (18, 24, 50). Conversely, MyoD has recently been shown to inhibit the serum-induced progression of quiescent cells into S phase (15a, 52). Considered together, the pattern of *myc* expression, its sequence similarity to MyoD, its nuclear localization, and the apparent effects of ectopic expression make attractive the possibility that *myc* and MyoD play pivotal and opposing roles in controlling the differentiation decision.

In this study we show that c-*myc* can inhibit the differen-

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tiation of cells made myogenic by transfection of a gene encoding MyoD in both transient and stable assays of NIH 3T3 cells. An important virtue of this system is the ability to study the activities of ectopic MyoD, myogenin, and c-*myc* in the absence of Myf-5, herculin, and endogenous MyoD, as these genes are not activated in these cells by MyoD (37, 55). Our results show that MyoD and myogenin are not sufficient, acting alone or together, to drive muscle differentiation in the presence of high levels of c-*myc*. Moreover, the *myc* suppression can function independently of Id activity, suggesting that there are at least two negative regulators normally expressed in undifferentiated myoblasts that can prevent differentiation.

MATERIALS AND METHODS

Expression vector constructions. The MT-*myc* gene in 3T3 MT-*myc* cells contains the metallothionein-I promoter (*Eco*RI to *Bgl*II, [38]) fused to a c-*myc* minigene containing the last 33 nucleotides (nt) of the first intron (from the *Xba*I site), exons 2 and 3 (without intron 2) up to the *Nsi*I site (3), and the metallothionein-I polyadenylation signal sequence. The MyoD expression vehicle pEMc11s was a gift from Andrew Lassar (17). Simian virus 40 (SV40)-promoted genes used in the transient cotransfections were all constructed in pECE (21), a 2.9-kb vector which contains the SV40 early promoter and T antigen polyadenylation signal sequences flanking a multiple cloning site. pSVc11s contains the 1.8-kb *Eco*RI fragment (MyoD cDNA) from pEMc11s cloned into the *Eco*RI site of pECE. pSVmyc contains the 1.4-kb *Xba*I-to-*Xho*I fragment of c-*myc* (from MT-*myc*) cloned into the *Xba*I site of pECE via a three-step ligate-blunt-ligate procedure. pSVrGEN contains rat myogenin cDNA (a gift from Woody Wright) as a 1.5-kb *Eco*RI fragment from BS-11, which contains the cDNA (59), cloned into the *Eco*RI site of pECE. Standard procedures were used for all manipulations (36).

Cell culture and DNA transfections. NIH 3T3 cells obtained from the American Type Culture Collection were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Flow)–10% calf serum (GIBCO or Irvine Scientific) supplemented with penicillin (10 U/ml) and streptomycin (10 μg/ml) (Irvine Scientific). 3T3 MT-*myc* cells were grown in DMEM–10% fetal bovine serum (FBS) (HyClone). Prior to stable transfection with the MyoD expression vehicle pEMc11s, NIH 3T3 cells and 3T3 MT-*myc* cells were plated on 10-cm-diameter gelatin-coated plates in DMEM–10% FBS. They were transfected by calcium phosphate coprecipitation (57) with 10 μg of pEMc11s–1 μg of pY3 (which confers resistance to hygromycin B [4])–5 μg of sheared L cell DNA (as carrier). After 12 h, cells were treated with 15% glycerol in DMEM for 2 min, rinsed with phosphate-buffered saline, and fed DMEM–15% FBS. Twenty-four hours later the medium was supplemented with 200 μg of hygromycin B per ml (Calbiochem). After 10 days, surviving colonies were fed differentiation medium (DMEM, 2% horse serum [Flow Labs], and 8 μg of insulin per ml [GIBCO]). Colonies which began to show morphological changes characteristic of myocytes were picked after 36 to 48 h and expanded in DMEM–15% FBS.

NIH 3T3 cells used in transient transfections were plated on uncoated dishes in DMEM–10% calf serum. Transfections were performed as described above, but the glycerol shock was omitted. Plasmid mixtures are described in the figure legends. Forty-eight hours after transfection, plates were fed DMEM–2% horse serum–2 μg of insulin per ml to induce differentiation. Three days later plates were fixed and immunostained for the myosin heavy chain (MHC) with horseradish peroxidase as described by Rhodes and Konieczny (47) by using the monoclonal antibody MF20 (1) and Amersham secondary reagents.

RNA isolation and analysis. RNAs were prepared by the method of Chomczynski and Sacchi (12) from cells on 15-cm-diameter dishes by using 4 ml of guanidinium solution. Relevant transcripts were analyzed by ribonuclease protection assays. Hybridizations were done in 30 μl of 80% deionized formamide–40 mM PIPES (pH 6.4) [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)]–1 mM EDTA–400 mM NaCl under mineral oil at 50°C for 12 h by using 2.5 or 5 μg of total RNA and 5×10^9 radiolabeled probe molecules per hybridization. Unhybridized probes were digested by adding 300 μl of 10 mM Tris (pH 7.5)–5 mM EDTA–300 mM NaCl–1 U of RNase T1 (Bethesda Research Laboratories) per μl. In addition, 0.36 ng of RNase A (Sigma) per ml was used for human γ-actin and Id probe digestions. Ribonucleases were digested with proteinase K (Boehringer Mannheim), samples were extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and protected probes were isopropanol precipitated with 30 μg of yeast type III RNA (Sigma) as a carrier. Pellets were dissolved in 80% formamide and electrophoresed through 8% denaturing polyacrylamide gels, which were exposed to Kodak XAR-5 film at −70°C with an intensifying screen.

Probes for ribonuclease protections. Radiolabeled riboprobes were synthesized by using SP6 (Promega), T3, or T7 (Pharmacia or US Biochemical Corp.) RNA polymerase and [α-^{32}P]CTP or UTP (Amersham or New England Nuclear). pSP6 g-actin contains a fragment of the human γ-actin gene (26) in pSP64. When cut with *Hin*fI and transcribed with SP6 RNA polymerase, the resulting 145-nt probe protects a 65-nt fragment of mouse γ-actin RNA (23). The MyoD probe BH8 contains the ~600-bp *Hin*dIII-to-*Bam*HI fragment of pEMc11s inserted into Bluescript KS$^+$ (Stratagene). BH8 is cut with *Sty*I (nt 1692 in reference 17) and transcribed with T7 RNA polymerase to make a probe that produces a 116-nt protected fragment from transcripts off pEMc11s and a 92-nt fragment from transcripts off the endogenous MyoD gene. The mouse *myc* probe used extends from the *Eco*RV site in the second exon to the *Xba*I site in the first intron and was transcribed with T3 RNA polymerase. This probe distinguishes between transcripts from the MT-*myc* gene and the endogenous gene. The mouse myogenin plasmid SRmyo8 contains the 224-bp *Eco*RI-to-*Sma*I fragment (5′ end in reference 20) inserted into *Eco*RI- and *Sma*I-cut Bluescript II KS$^+$. This plasmid is cut with *Sty*I (nt 35 of myogenin) and transcribed with T7 RNA polymerase. The Id plasmid pMH18ΔR (2) was cut with *Dde*I at nt 817 and transcribed with T7 RNA polymerase to make a 174-nt probe, 110 nt of which is protected by Id RNA.

RESULTS

***myc* inhibits differentiation initiated by MyoD in transient cotransfection assays.** To test the effects of c-*myc* on MyoD-initiated myogenesis, we performed a series of transient cotransfections in which the absolute amount of MyoD was held constant and the amount of *myc* was varied. To avoid difficulties stemming from differences in promoter strength, both genes were placed under the control of the constitutive SV40 early promoter in the plasmid pECE (21). Also, to guard against artifactual effects due to competition for factors driving the SV40 promoter, these transfection mixtures

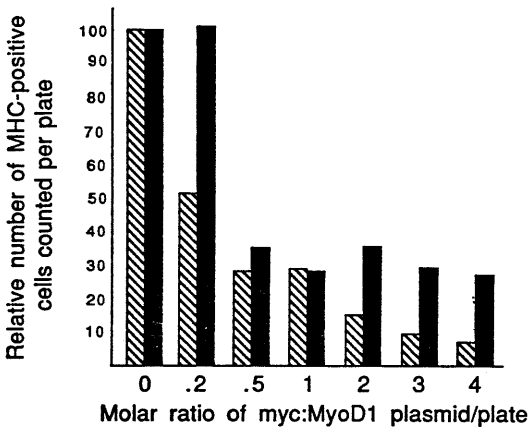


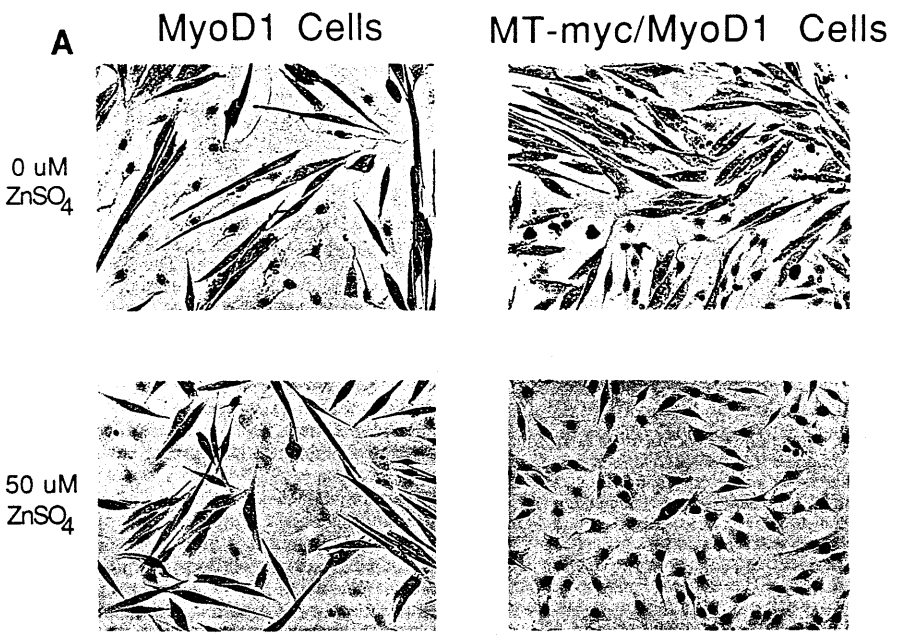
FIG. 1. Transient inhibition of MyoD-mediated myogenesis by c-*myc*. These experiments show that in transient cotransfections, differentiation is most efficient at low molar ratios of *myc* to MyoD plasmids and is least efficient at higher ratios. NIH 3T3 cells were transfected in two separate experiments (represented by hatched and black bars) using different *myc* plasmid preparations. DNA mixtures contained 5 μg of pSVc11s (SV40 early promoter-driven MyoD), enough of the *myc* plasmid pSVmyc (SV40 early promoter-driven *myc*) to make the indicated *myc*/MyoD ratios, enough pECE (SV40 promoter alone) to equalize the molar amount of SV40 promoter per plate, and either 1 μg of pY3, 1.5 μg of pSV2CAT, plus enough sheared L cell DNA to make 25.8 μg of DNA per 10-cm plate (for hatched bars) or 2 μg of pSV2CAT plus enough pT7T3-18 (Bethesda Research Laboratories) to make 25.3 μg of DNA per plate (for black bars). Chloramphenicol acetyltransferase activity was not significantly variable from point to point (data not shown). For the experiment represented by the hatched bars, 20 fields at 25× power on each of two plates were counted and averaged per point; 100 represents 1,960 MHC-positive cells. For the experiment represented by the black bars, 25 fields on one plate were counted per point; 100 represents 12,722 MHC-positive cells.

all contained the requisite amount of pECE vector such that the total molar amount of SV40 promoter in each transfection mixture was identical. NIH 3T3 cells were transfected with these mixtures along with the SV40-promoted bacterial chloramphenicol acetyltransferase gene (pSV2CAT) to control for significant transfection efficiency differences from plate to plate. After 3 to 4 days in differentiation medium, the cells were fixed and the number of MHC-positive cells were counted after immunostaining. The results of two such experiments (hatched and black bars) are shown in Fig. 1. As MyoD is held constant and c-*myc* is increased, the number of differentiated muscle cells diminishes by up to 90%. These results suggest that c-*myc* has a strong inhibitory effect on the ability of MyoD to promote differentiation in NIH 3T3 cells. By comparison with prior observations of retroviral inhibition of primary myoblast differentiation (24) or transfections of naturally occurring myoblast cell lines (e.g., reference 18) in which the complement of muscle regulators already being expressed is unknown, the results in this system specifically show inhibition of MyoD-initiated differentiation.

***myc* overexpression inhibits MyoD-initiated differentiation in stable transfectants.** There are likely to be important intermediate steps between expression of ectopic MyoD and activation of muscle-specific genes. Otherwise, any cell which expresses MyoD would become myogenic; but this is not the case (56). However, while the transient assays show that c-*myc* inhibits MyoD-initiated myogenesis, the small fraction of cells which actually take up and express DNA are not suitable for further characterization of the regulatory pathway. We therefore constructed a set of NIH 3T3-based cell lines that are myogenic because of constitutive expression of a transfected MyoD cDNA. Some of these lines also carry a c-*myc* gene under the control of the mouse metallothionein-I promoter (MT-*myc* gene) which can be induced up to 10-fold above endogenous *myc* protein levels by supplementing the culture medium with zinc sulfate (54a). However, these MT-*myc* cells do not appear to be growth transformed, as they do not form foci in culture or tumors in nude mice, even when treated with zinc. A useful feature of conditional *myc* expression is that it helps to ensure that the phenotypic effects observed are attributable to transient overexpression of c-*myc* and not to long-term secondary effects of chronic *myc* expression or to the emergence of differentiation-defective derivatives, a possible factor in prior studies of the effects of *myc* on myogenesis. Myogenic derivatives of these MT-*myc* cells and of the parental NIH 3T3 cells were made in parallel transfections by using the MyoD expression vector pEMc11s (17). The myogenic potential of MyoD-transfected cell clones was evaluated by immunostaining for expression of MHC after cultivation for several days in a mitogen-poor differentiation medium. Several independent myogenic transformants in both 3T3 and 3T3 MT-*myc* backgrounds were subcloned, and the most myogenic of these were chosen for further study.

MyoD myoblasts carrying the zinc-inducible MT-*myc* gene (MT-*myc*/MyoD1 cells) differentiate in the absence of added metal, but differentiation is suppressed in the presence of zinc. MyoD myoblasts lacking the metal-inducible *myc* gene (MyoD1 cells) are unaffected by zinc and differentiate to similar extents in the presence or absence of added metal. This result is visualized by the immunocytochemical stain for MHC shown in Fig. 2A, and a quantitation is presented in Fig. 2B. Differentiated cells were scored by counting cells that stain positive for MHC; similar results have been obtained by immunostaining for MCK and by quantitating MCK RNA levels (data not shown). Differentiation was not completely suppressed when *myc* was induced, a result which is expected if the quantity of c-*myc* expressed is crucial for suppression, since immunostaining for c-*myc* has shown that it does not accumulate to uniformly high steady-state levels in all cells of the MT-*myc* line (54a). We conclude that in these cells, differentiation is specifically suppressed by the induction of MT-*myc* expression.

***myc* suppresses differentiation even in the presence of MyoD.** How are the differentiation phenotypes in these experiments related to the levels of MyoD and *myc* expression? To begin to answer this question, the levels of *myc* and MyoD transcripts were measured by a ribonuclease protection assay. Results shown in Fig. 3 confirm that in the MT-*myc*/MyoD line, *myc* transcripts from the transgene accumulate to significantly elevated levels in the presence of added zinc (Fig. 3; in the 3.5-h exposure of the *myc* probe, compare lane 1 with 2 and lane 3 with 4 and in the longer exposure, compare lane 5 with 6). Lower *myc* levels are observed in the absence of added zinc when cells differentiated well, while the elevated *myc* levels are from cells that failed to differentiate efficiently (as in Fig. 2). It is relevant that by the criteria of appearance, adherence to the plate, yield of RNA, and level of expression of cytoplasmic γ-actin RNA (Fig. 3), all cells seemed equally healthy under all



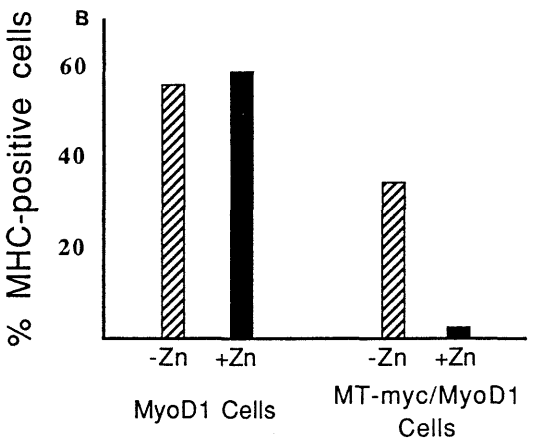


FIG. 2. Muscle differentiation suppressed by zinc-dependent induction of c-*myc* in MT-*myc*/MyoD1 cells but not in control MyoD1 cells. (A) Immunocytochemical staining for the presence of muscle MHC in MyoD1 cells and in MT-*myc*/MyoD1 cells. Differentiated cells stain brown, while cells that do not express MHC are counterstained light violet with hematoxylin. Cells were cultured for 6 days in a mitogen-poor differentiation medium (DMEM containing 1% horse serum and 5 μg of insulin per ml) with the concentration of zinc sulfate indicated. Zinc induces expression of *myc* from the MT-*myc* transgene in 3T3 MT-*myc* cells. Note that cell fusion does not occur in differentiated MyoD1 or MT-*myc*/MyoD1 cells. (B) Quantitation of an experiment similar to that described for panel A. Cells were cultured in differentiation medium with 50 μM (black bars) or no (hatched bars) zinc sulfate for ~6 days. Note that differentiation is suppressed in zinc-treated MT-*myc* cells, while zinc has no inhibitory effect on differentiation of MyoD1 cells lacking the MT-*myc* gene. Additionally, zinc had no significant effect on the average number of cells present in the random microscopic fields which were assayed.

conditions tested. Also, changes in *myc* RNA levels in 3T3 and MT-*myc* 3T3 cells have been found to reflect similar changes in *myc* protein levels (54a), although it remains possible that there is some additional control at the translational level for *myc* or for MyoD (54) in these cells.

Levels of MyoD expression were measured, and they revealed that under all conditions transgene MyoD was expressed. MyoD1 control cells expressed lower levels of MyoD than did MT-*myc*/MyoD1 cells, yet both cell lines differentiated well (unless *myc* was elevated). The probe used to measure MyoD discriminated between the transgene (shown in Fig. 3) and endogenous MyoD. In both cell lines the endogenous MyoD gene was silent (37) and therefore made no contribution to overall MyoD levels. In the MT-*myc*/MyoD1 line, levels of MyoD decreased modestly at later time points in the presence of metal (Fig. 3; for the MyoD probe, compare lane 3 with 4 and lane 5 with 6). However, this decrease is not likely to be the direct cause for inhibition of differentiation, because even the reduced MyoD levels substantially exceed amounts that are sufficient for differentiation in the control MyoD1 cell line (Fig. 3, lanes 7 through 12).

These data, taken together with the levels of *myc* expressed in MT-*myc*/MyoD1 cells and control MyoD1 cells,

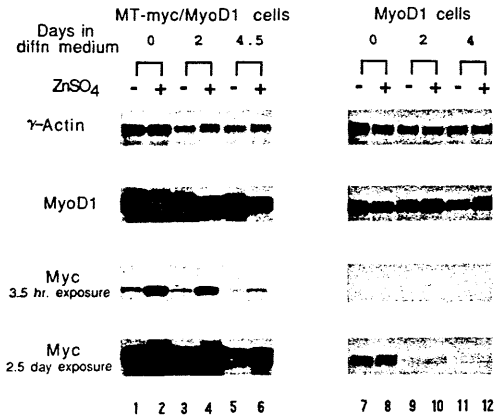


FIG. 3. Ribonuclease protection assays for *myc* and MyoD tNAs. Total RNA was extracted from MT-*myc*/MyoD1 and control lyoD1 cells were cultured as indicated. RNAs in lanes 1, 2, 7, and are from cells harvested 3 h after feeding with rich medium with or vithout zinc sulfate (as indicated by + or −). RNAs in lanes 4 and are from cells inhibited from differentiating, while lanes 3, 5, and through 12 contain RNAs from differentiated cells, as assayed by HC and MCK expression (data not shown). The relevant bands ave been excised from large gels. A probe for cytoplasmic γ-actin 23) was used to verify the quality and amount of RNA for each imepoint, which was 2.5 μg (determined spectrophotometrically). Note the two different exposures for *myc* as indicated.

lace some informative constraints on how c-*myc* and MyoD night be acting on the differentiation decision. Comparison f the absolute amount of total c-*myc* RNA in each of the wo cell lines in the absence of zinc (Fig. 3, lanes 1, 3, and 5 ompared with lanes 7, 9, and 11, respectively, of the 1.5-day exposure of the *myc* probe) shows that the two xpress significantly different levels. c-*myc* levels that are ermissive for differentiation in MT-*myc*/MyoD1 cells ex- eed those expressed in rapidly proliferating MyoD1 cells e.g., lane 7) and other myoblast cell lines (data not shown). Nevertheless, when the MT-*myc* gene is induced with zinc, *nyc* levels rise high enough—perhaps over a threshold—to uppress differentiation even in the presence of abundant mounts of MyoD (Fig. 3, lanes 4 and 6). In this context, the bserved decrease in MyoD RNA levels from lane 3 to 4 and ane 5 to 6 associated with added zinc may serve to lower ny threshold over which c-*myc* levels must rise to efficiently uppress MyoD-dependent differentiation. Therefore, we onclude that c-*myc* effects on myogenesis depend on the uantity of c-*myc*, not its mere presence or absence. The mount required for suppression of differentiation in these ells is high, exceeding levels that characterize myoblasts rior to differentiation. However, these cells also express igh levels of MyoD, suggesting that the ratio of c-*myc* to nyogenic HLH family gene products is the crucial parame- er.

Myogenin is expressed in differentiated cells but not in MT-*myc*/MyoD1 cells inhibited from differentiating. Mecha- isms that may rely on quantitative relationships among HLH molecules must also accommodate additional family nembers that may ultimately participate in MyoD-initiated nyogenesis. To further characterize the repertoire of HLH nuscle regulators in these cells, we performed RNase pro- ection experiments by using mouse Myf-5 and herculin

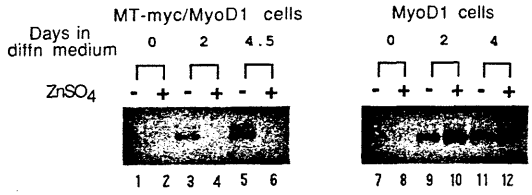


FIG. 4. Ribonuclease protection assays for myogenin RNA. The RNA samples used in Fig. 3 were assayed for expression of myogenin. The results show that differentiated cells express myogenin, but those inhibited by c-*myc* from differentiating do not (lanes 4 and 6). The amount of RNA used was 2.5 μg for each lane.

probes (37), and no expression was detected even after long autoradiographic exposures (data not shown). However, a similar experiment using a mouse myogenin probe (Fig. 4) shows that myogenin is expressed when cells differentiate (lanes 3, 5, and 9 through 12) but not when cells are proliferating (lanes 1, 2, 7, and 8) or are inhibited from differentiating (lanes 4 and 6). Myogenin is the only member of the MyoD family that has been detected in all differentiated skeletal muscle cell lines examined to date (5, 37, 39). Consistent with the hypothesis that myogenin has an important function in the differentiation decision, this experiment shows that when c-*myc* is induced, myogenin expression fails to be activated.

Can myogenin or MyoD-myogenin coexpression bypass the inhibitory effects of *myc*? The observation that up-regulation of myogenin and subsequent myogenic differentiation are inhibited by elevated c-*myc* levels led us to hypothesize that the inhibitory effect of c-*myc* might be bypassed if both myogenin and MyoD were provided. Furthermore, two facts suggested that both myogenic regulators should be required for such a bypass. First, observations of diverse skeletal muscle myocytes lead to a generalization that they always coexpress myogenin and at least one additional HLH myogenic regulator (5, 37, 39). Second, BC_3H1 myoblasts are efficiently inhibited from differentiating in the presence of myogenin-specific antisense oligonucleotides (10) even though they express Myf-5 (5, 39). Thus, in NIH 3T3 cells, perhaps both myogenin and MyoD are needed for activation of differentiation-specific genes, and only if both are provided can c-*myc* inhibition be relieved.

Since c-*myc* had already been shown to inhibit myogenesis in transient cotransfection assays with MyoD (Fig. 1), a similar experiment was done to test whether *myc* would suppress differentiation initiated by myogenin. The rat myogenin cDNA (59) was inserted into the pECE vector, and NIH 3T3 cells were transfected with the mixtures indicated in Fig. 5. The amount of *myc* plasmid used was one-half the total molar amount of myogenic plasmid. After 3 days in differentiation medium, cells were fixed and immunostained for MHC, and differentiated cells were counted. The results show that MyoD and myogenin act cooperatively when transfected together, in that they convert significantly more NIH 3T3 cells to myocytes than do the same molar doses of myogenin or MyoD alone (cf., reference 49). Nevertheless, c-*myc* inhibited differentiation whether MyoD, myogenin, or a mixture of the two was used to initiate myogenesis. Thus, although the activation of myogenin that normally accompanies differentiation of MyoD1 cells is suppressed by c-*myc*, myogenin provided ectopically cannot relieve the overall inhibition of differentiation. The straightforward interpreta-

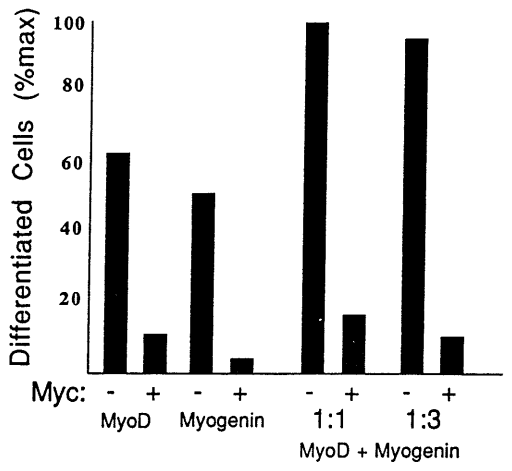


FIG. 5. Transient cotransfection of myogenin, alone or with MyoD, cannot bypass c-*myc* inhibition of myogenesis. NIH 3T3 cells were plated on 6-cm-diameter dishes. Each transfection mixture was divided among three dishes and contained the molar equivalent of 20 μg of pSVclls as its myogenic component. Note that the total molar amount of myogenic plasmid in each mixture of MyoD and myogenin is equal to their molar amounts when used individually. pSVmyc (9.1 μg) was sometimes included, as indicated, which was half that molar amount. Otherwise, 6.2 μg of pECE was included to balance the total SV40 promoter content. The transfection procedure was as described in Materials and Methods, except that 70 μM chloroquine was added to the medium during the first 6 h of transfection, after which the cells were rinsed with DMEM, fed fresh medium, and then switched to differentiation medium 24 h later for 3 days. After fixing and staining for MHC, positive cells in 15 fields were counted at a 25× magnification. In this experiment, the maximum was 670 differentiated cells counted. Two repetitions of this experiment yielded similar qualitative results.

tion is that in these cells, MyoD and myogenin together may be necessary for differentiation, but they are not sufficient to force differentiation in the presence of c-*myc*.

Expression of Id is not influenced by ectopic c-*myc* levels and is down-regulated even in cells inhibited from differentiating. The pattern of expression of Id, a negative regulator of MyoD-initiated muscle gene transcription (2), parallels the pattern of endogenous c-*myc* expression during myogenesis. Both are expressed at relatively high levels during proliferation compared with those expressed upon differentiation. This raised the possibility of a hierarchical relationship between the two in which elevated c-*myc* would exert its phenotypic effect by driving expression of Id. Thus, in our studies, *myc* might inhibit differentiation indirectly by positively regulating expression of Id in zinc-induced MT-*myc*/MyoD1 cells under mitogen-poor conditions, whereas Id levels would be down-regulated in the absence of zinc because of lower levels of c-*myc*. However, a direct experimental test of this possibility found Id to be down-regulated identically in the presence and absence of suppressing levels of c-*myc*. Figure 6 shows that even in cells that are inhibited from differentiating by c-*myc* (lanes 4 and 6), Id levels are no higher than those in cells that have differentiated (lanes 3, 5, and 9 through 12). Thus, while c-*myc* and Id are normally

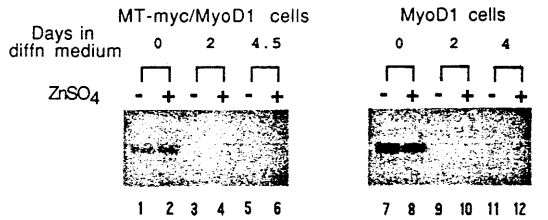


FIG. 6. Ribonuclease protection assays for Id RNA. The RNA samples from Fig. 3 were assayed for expression of Id, an inhibitor of differentiation. The results show that Id expression is properly down-regulated even in cells exhibiting c-*myc*-dependent suppression of differentiation (lanes 4 and 6). The amount of RNA used was 5 μg for each lane.

similar in their expression patterns under growth factor-rich and -poor conditions (Fig. 7A), we have detected no alteration in Id levels in response to ectopic c-*myc*.

DISCUSSION

c-*myc* is normally expressed in proliferating myoblasts and is down-regulated upon differentiation into myocytes (22, 51). We have begun to dissect the regulatory effects of c-*myc* on myogenesis initiated by MyoD, myogenin, or a combination of the two. In these experiments, expression of c-*myc* has been uncoupled from environmental and cellular factors which normally influence its regulation. The central result is that preventing the drop in c-*myc* expression that normally occurs at the onset of differentiation results in inhibition of muscle differentiation.

Comparison of *myc*-mediated inhibition with effects of other negative regulators. Our experiments suggest a significant role for c-*myc* in the differentiation decision which differs from the roles of other negative regulators tested in previous studies. Earlier steps in myogenesis are affected by perturbations in *ras* (32, 35, 43, 44, 53), proliferin (58), c-*fos* (35), and perhaps *src* (24), all leading to suppression of muscle differentiation, though there are conflicting results in some systems (27). Mutant-activated *ras* and excess *fos* have been shown to prevent or reduce transcription of the endogenous MyoD gene (32, 35), and in one study activated *ras*-dependent inhibition of myogenic differentiation was shown to be accompanied by an up-regulation of c-*myc* RNA, even in differentiation medium (43). Proliferin appears to act by reducing the level of MyoD expression or by altering the MyoD transcript, perhaps making it nonfunctional as MyoD mRNA (58). In contrast to these examples of myogenic inhibition, which act on regulation of MyoD, we have shown that c-*myc* is able to inhibit differentiation in cell lines at a more distal point in the regulatory pathway—even in the presence of constitutive MyoD. However, while our use of the NIH 3T3 cell line afforded experimental control of MyoD levels in the absence of endogenous MyoD expression, it prevented us from determining whether c-*myc*, like activated *ras* and *fos*, can down-regulate an active endogenous MyoD gene. A further observation in our study is that the c-*myc* inhibition of cellular differentiation cannot be overcome by expression of myogenin, alone or in conjunction with MyoD. By contrast, in the case of activated *ras* or *fos*, constitutive MyoD from a stably transfected cDNA bypassed their suppressing effects (32, 35).

While the present study shows that c-*myc* inhibits myogenic differentiation effectively in both transient and stable

A

		Multipotential Stem Cell	Determined Myoblast	Differentiated Myocyte
Culture Medium Growth Factor Level →		High	High	Low[a]
positive-acting myogenic regulators	MyoD[b]	−	+	+
	Myf-5[b]	−	+	+
	Myogenin[c]	−	−	+
	MRF4/ Herculin[d]	−	−	+
coregulators	E12/E47	+	+	+
negative-acting regulators	c-myc	+	+	−
	Id	+	+	−
mature muscle markers	MHC	−	−	+
	MCK	−	−	+
	α-actins	−	−	+

B

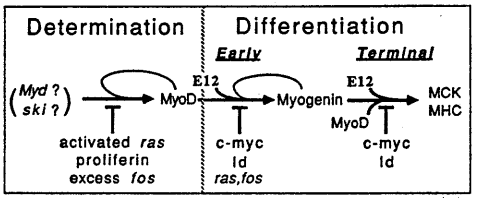


FIG. 7. (A) Expression patterns of genes involved in myogenic determination and differentiation in cultured cell lines. The data summarize expression patterns in naturally occurring skeletal muscle cell lines; ectopic expression of any one of the four MyoD family myogenic regulators by transfection can change these patterns. References: MyoD (17), myogenin (20, 59), Myf-5 (5, 39), MRF4/ herculin (37, 47), E12/E47 (40), c-*myc* (51), Id (2), MHC, MCK, and α-actins (48). [a] While many cell lines fuse into multinucleated myotubes concurrent with biochemical differentiation, BC_3H1 cells do not; however, transfection of a MyoD expression vector into these cells corrects the fusion defect (8). [b] While we find that differentiated C2C12 cells express all four myogenic regulators, many cell lines express only myogenin and either MyoD (e.g., MM14 cells) or Myf-5 (e.g., BC_3H1 cells) but not both. [c] Although we have detected myogenin RNA in nominally proliferating MM14 and C2C12 cells (37, 39), this is presumed to be contributed by spontaneously differentiated cells, which are frequently found in those cultures even in the presence of high levels of growth factors. [d] We detect herculin in differentiated C2C12 cells (37) and at much lower levels in both proliferating and differentiated BC_3H1 cells (36a); low level expression of MRF4 (the herculin homolog in rats) has been reported in differentiated L6 cells (47) and in L8 cells (6). Otherwise, while herculin is abundantly expressed in adult mouse skeletal muscle tissue, it is rarely expressed in cultured cell lines. However, *myf-6* (the herculin homolog in humans) is expressed in primary human myocytes in culture (6). (B) A speculative model for a myogenic regulatory network. Arrows represent activation, loops represent autoregulation, bars represent inhibition, and curves represent cooperative activation. E12 is meant to be representative of the several known daughterless homologs which can heterooligomerize with the MyoD family members.

transfection formats, the situation for *ras* and *fos* is more complicated. Their inhibitory effects can be bypassed by stable transfection with a MyoD expression vector, yet they cannot be bypassed when transiently cotransfected with MyoD and a muscle-specific chloramphenicol acetyltransferase reporter gene into C3H 10T1/2 cells (35). In that report (35), it was suggested that the negative activities of *ras* and *fos* dominate over the positive activities of MyoD in a transient assay but not in stable cell lines because the former may allow for a higher ratio of oncogene-to-MyoD activity. In addition, it is not certain what, if any, contribution is made to total MyoD levels by the endogenous gene in the transients (cf., reference 54), nor is it known whether myogenin is expressed or whether c-*myc* is up-regulated (as in reference 43). Whatever the explanation for the differences observed in the two assays, the results show that activated *ras* or excess *fos* may also act at a step later than MyoD expression, as c-*myc* clearly does.

Mechanisms of c-*myc* action. What are the possible mechanisms for c-*myc* inhibition of muscle differentiation? c-*myc* is localized in nuclei and contains motifs characteristic of sequence-specific DNA-binding proteins, including the B-HLH motif, essential for oligomerization and function of MyoD family regulators (16, 41), and a leucine zipper, a motif known to function in dimerization of other transcription factors such as *fos* and *jun* (33). These characteristics suggest two direct mechanisms for myogenic inhibition. In one, *myc* would bind to promoters or enhancers of downstream muscle-specific genes and act as a repressor. An alternative direct mechanism would have *myc* inactivate positive-acting muscle-specific transcription factors of the MyoD type by binding to them or to an essential limiting partner. Indeed, the apparent sensitivity of c-*myc* inhibition to the *myc*-to-MyoD ratio (shown in this study) is consistent with such a titration by c-*myc*. However, attempts to achieve dimerization of c-*myc* with these (or other) potential partners in standard in vitro assays have thus far failed (31). Moreover, strong evidence supporting sequence-specific DNA binding for *myc* is also lacking, though *myc* does have a general affinity for DNA (19, 45). Nevertheless, the initial negative results of such in vitro studies do not convincingly rule out the mechanisms outlined above.

Less direct mechanisms to explain c-*myc* effects on differentiation must also be considered. c-*myc* was first identified as an oncogene, thereby implicating it in the control of cell proliferation. More recent studies assign c-*myc* a significant role in regulating entry into G_1 and S phases of the cell cycle (11). This is relevant to skeletal myogenesis because it is well established that the permissive point in the cell cycle for the onset of differentiation is the G_0-G_1 boundary (13). Thus, since c-*myc* can drive a subset of quiescent NIH 3T3 cells out of G_0 and into G_1 and S phases, its ability to inhibit differentiation may be secondary to its ability to activate genes involved in progression through the cell cycle. Arguing against this view are two observations. (i) Up to 75% of MT-*myc*/MyoD1 cells differentiated in some of our experiments, and the extent of inhibition by c-*myc* induction exceeded 80%, thus involving a far larger fraction of MT-*myc* cells than can ever be forced into S phase by a *myc* induction, which is ~30% (36b). (ii) Although progression into S phase does not appear to fully explain the observed phenotype, a *myc*-mediated progression into a position in G_1 which is prohibitive for differentiation is possible. While definitive studies will require isolation and use of markers that subdivide progress into and through G_1, our data showing a lack of Id expression characteristic of cycling cells (Fig. 6) also argue against a mechanism dependent on cycling.

Positive and negative regulatory pathways of myogenesis in cultured cell lines. Figure 7A summarizes the expression patterns of positively and negatively acting myogenic regulatory genes in cell lines in culture and includes the expres-

sion pattern of some mature muscle markers. These data provide the biological context for a simple speculative model based upon the observations in this paper together with prior studies (Fig. 7B). It accommodates the effects of the positive- and negative-acting regulators presently known and integrates data from naturally occurring myogenic cell lines as well as transfection-initiated myoblast lines. The first step identified is determination, the transition from a multipotential stem cell to a myoblast. Here it is accomplished by expression of MyoD, although in other instances Myf-5 might substitute at this step. As indicated, MyoD is sometimes (but not always) able to establish an autoregulatory loop (55). The initial trigger of MyoD expression is presently unknown, but one plausible candidate is the genetically defined *myd* locus, which has been shown to activate MyoD when transfected into nonmyogenic cells (46). The *ski* oncogene, which can also convert fibroblasts to myoblasts in transfection assays (15), may also be involved. Negative regulators that act by disrupting MyoD expression include activated *ras*, excess c-*fos*, and proliferin, and they appear to be able to function even after the MyoD autoregulatory loop has been established (32, 35, 58). c-*myc* and Id have not yet been formally tested for a similar negative effect on MyoD expression in proliferating myoblasts. However, under growth factor-rich conditions that support proliferation of MyoD-positive myoblasts, significant levels of both c-*myc* and Id have been found in all cases, and we therefore think it is very likely that their expression is compatible with expression of MyoD.

At the next step in the pathway, MyoD-positive myoblasts are signalled to differentiate, which is accomplished in vitro by removing serum growth factors. A rapid response to reduced growth factors is a drop in Id and c-*myc* expression. One expected result will be the release of any B-HLH proteins that were previously nonfunctional because of sequestration by Id through protein-protein interactions. Indeed, E12 and MyoD both bind efficiently to Id in vitro, and this association inactivates their DNA-binding capacities. These observations led Benezra et al. (2) to propose that a MyoD/E12 hetero-oligomer is necessary for the onset of differentiation, and this activation step is indicated as the early phase of differentiation in Fig. 7B. Within the resolution of our kinetic measurements, myogenin expression begins concurrent with the drop in Id and c-*myc*; myogenin then appears to be able to positively autoregulate (5, 20). Experiments presented here and by Benezra et al. (2) indicate that this kinetically early step in differentiation (relative to MHC and MCK expression) is inhibited by c-*myc* and Id. Additionally, Lassar et al. (35) provide evidence that high levels of mutant *ras* or *fos* relative to MyoD also inhibit the myogenin activation step, and the results of Olson et al. (43) suggest that such inhibition by activated *ras* may actually be mediated by up-regulation of c-*myc* expression. Since myogenin has been found to be expressed in all differentiating myogenic cell lines examined (5, 37, 39), this step may be essential for differentiation. Direct support for this comes from studies which show that BC_3H1 and L6-A1 myoblasts are efficiently inhibited from differentiating in the presence of myogenin-specific antisense oligonucleotides (10, 25).

As differentiation proceeds, muscle-specific genes such as those encoding MHC and MCK are transcribed, and in some cases, cell fusion occurs to produce terminally differentiated myotubes. As E12 can dimerize with myogenin (9) as well as with MyoD (2, 41) and can bind to essential *cis*-acting elements in muscle-specific enhancers, both myogenin-E12 and MyoD-E12 complexes are good candidates for playing a central role in transcriptional activation of terminal differentiation genes. This final step in Fig. 7B is responsive to inhibition by ectopic c-*myc* expression, as demonstrated in our cotransfections using c-*myc*, myogenin, and MyoD. Id has not yet been formally tested for its ability to inhibit similarly in the presence of MyoD and myogenin. However, the molecular data showing that Id can inactivate E12 in vitro (2) suggest that ectopic Id expression will also block the final step of differentiation.

The group of positive and negative myogenic regulatory factors presented in Fig. 7 have been identified as such primarily by testing their activities in cultured cell lines and in in vitro biochemical studies. The repertoire of regulators described thus far is probably incomplete, but among those currently known some redundancy in both positive and negative regulators is already apparent. For example, all four MyoD family regulators can convert some nonmyogenic cells to myoblasts, yet some cell lines differentiate efficiently without expression of Myf-5 or herculin, while others can proceed without MyoD. Additionally, our results suggest that c-*myc* and Id can act independently as negative regulators. This leads to the prediction that even if Id expression is eliminated in myoblasts cultured in growth factor-rich medium, such myoblasts will not spontaneously differentiate because c-*myc* would provide an independent negative control (unless Id positively regulates c-*myc* activity—a testable possibility). Conversely, elimination of c-*myc* expression should not allow for differentiation if Id expression is maintained. Therefore, the minimal requirement for premature activation of muscle-specific genes in myoblasts would be the inactivation of both c-*myc* and Id.

The existence of at least two independent negative regulators of muscle differentiation may be useful to the organism, because such redundancy would ensure that differentiation does not occur before myoblasts have migrated to their proper locations in the developing embryo and have proliferated sufficiently to properly fill their compartments. Because of its positive effects on progression through the cell cycle and its negative effects on differentiation, c-*myc* appears well suited as a physiological regulator that simultaneously prevents premature differentiation and promotes expansion of precursor cell numbers in lineages where linkage of these activities is important.

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REFERENCES

1. **Bader, D., T. Masaki, and D. A. Fischman.** 1982. Immunochemical analysis of myosin heavy chain during avian myogenesis *in vivo* and *in vitro*. J. Cell Biol. **95**:763–770.
2. **Benezra, R., R. L. Davis, D. Lockshon, D. L. Turner, and H.**

Weintraub. 1990. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell **61**:49–59.

3. **Bernard, O., S. Cory, S. Gerondakis, E. Webb, and J. M. Adams.** 1983. Sequence of the murine and human cellular *myc* oncogenes and two models of *myc* transcription resulting from chromosomal translocation in B lymphoid tumors. EMBO J. **2**:2375–2383.
4. **Blochlinger, K., and H. Diggelmann.** 1984. Hygromycin B phosphotransferase as a selectable marker for DNA transfer experiments with higher eucaryotic cells. Mol. Cell. Biol. **4**:2929–2931.
5. **Braun, T., E. Bober, G. Buschhausen-Denker, S. Kotz, K. H. Grzeschik, and H. H. Arnold.** 1989. Differential expression of myogenic determination genes in muscle cells: possible autoactivation by the *Myf* gene products. EMBO J. **8**:3617–3625.
6. **Braun, T., E. Bober, B. Winter, N. Rosenthat, and H. H. Arnold.** 1990. *Myf-6*, a new member of the human gene family of myogenic determination factors: evidence for a gene cluster on chromosome 12. EMBO J. **9**:821–831.
7. **Braun, T., G. Buschhausen-Denker, E. Bober, E. Tannich, and H. H. Arnold.** 1989. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. EMBO J. **8**:701–709.
8. **Brennan, T. J., D. G. Edmondson, and E. N. Olson.** 1990. Aberrant regulation of MyoD1 contributes to the partially defective myogenic phenotype of BC_3H1 cells. J. Cell Biol. **110**:929–937.
9. **Brennan, T. J., and E. N. Olson.** 1990. Myogenin resides in the nucleus and acquires high affinity for a conserved enhancer element on heterodimerization. Genes Dev. **4**:582–595.
10. **Brunetti, A., and I. D. Goldfine.** 1990. Role of myogenin in myoblast differentiation and its regulation by fibroblast growth factor. J. Biol. Chem. **265**:5960–5963.
11. **Cavalieri, F., and M. Goldfarb.** 1987. Growth factor-deprived BALB/c 3T3 murine fibroblasts can enter the S phase after induction of c-*myc* gene expression. Mol. Cell. Biol. **7**:3554–3560.
12. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**:156–159.
13. **Clegg, C. H., T. A. Linkhart, B. B. Olwin, and S. D. Hauschka.** 1987. Growth factor control of skeletal muscle differentiation occurs in G_1-phase and is repressed by fibroblast growth factor. J. Cell Biol. **105**:949–956.
14. **Cole, M. D.** 1986. The *myc* oncogene: its role in transformation and differentiation. Annu. Rev. Genet. **13**:361–384.
15. **Colmenares, C., and E. Stavnezer.** 1989. The *ski* oncogene induces muscle differentiation in quail embryo cells. Cell **59**: 293–303.

15a. **Crescenzi, M., T. P. Fleming, A. B. Lassar, H. Weintraub, and S. A. Aaronson.** 1990. MyoD induces growth arrest independent of differentiation in normal and transformed cells. Proc. Natl. Acad. Sci. USA **87**:8442–8446.

16. **Davis, R. L., P.-F. Cheng, A. B. Lassar, and H. Weintraub.** 1990. The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. Cell **60**:733–746.
17. **Davis, R. L., H. Weintraub, and A. B. Lassar.** 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell **51**:987–1000.
18. **Denis, N., S. Blanc, M. P. Leibovitch, N. Nicolaiew, F. Dautry, M. Raymondjean, J. Kruh, and A. Kitzis.** 1987. c-*myc* oncogene expression inhibits the initiation of myogenic differentiation. Exp. Cell Res. **172**:212–217.
19. **Donner, P., I. Greiser-Wilke, and K. Moelling.** 1982. Nuclear localization and DNA binding of the transforming gene product of avian myelocytomatosis virus. Nature (London) **296**:262–266.
20. **Edmondson, D. G., and E. N. Olson.** 1989. A gene with homology to the *myc* similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. Genes Dev. **3**:628–640.
21. **Ellis, L., E. Clauser, D. O. Morgan, M. Edery, R. A. Roth, and W. J. Rutter.** 1986. Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. Cell **45**:721–732.
22. **Endo, T., and B. Nadal-Ginard.** 1986. Transcriptional and posttranscriptional control of c-*myc* during myogenesis: its mRNA remains inducible in differentiated cells and does not suppress the differentiated phenotype. Mol. Cell. Biol. **6**:1412–1421.
23. **Enoch, T., K. Zinn, and T. Maniatis.** 1986. Activation of the human β-interferon gene requires an interferon-inducible factor. Mol. Cell. Biol. **6**:801–810.
24. **Falcone, G., F. Tato, and S. Alema.** 1985. Distinctive effects of the viral oncogenes *myc*, *erb*, *fps*, and *src* on the differentiation program of quail myogenic cells. Proc. Natl. Acad. Sci. USA **82**:426–430.
25. **Florini, J. R., and D. Z. Ewton.** 1990. Highly specific inhibition of IGF-I-stimulated differentiation by an antisense oligodeoxyribonucleotide to myogenin mRNA. J. Biol. Chem. **265**:13435–13437.
26. **Gunning, P., P. Ponte, H. Okayama, J. Engel, H. Blau, and L. H. Kedes.** 1983. Isolation and characterization of full-length cDNA clones for human α-, β-, and γ-actin mRNAs: skeletal but not cytoplasmic actins have an amino-terminal cysteine that is subsequently removed. Mol. Cell. Biol. **3**:787–795.
27. **Harrington, M. A., F. Gonzales, and P. A. Jones.** 1988. Effect of cellular determination on oncogenic transformation by chemicals and oncogenes. Mol. Cell. Biol. **8**:4322–4327.
28. **Hopwood, N. D., A. Pluck, and J. B. Gurdon.** 1989. A Xenopus mRNA related to Drosophila *twist* is expressed in response to induction in the mesoderm and the neural crest. Cell **59**:893–903.
29. **Johnson, J. E., S. J. Birren, and D. J. Anderson.** 1990. Two rat homologues of *Drosophila* achaete-scute are specifically expressed in neuronal precursors. Nature (London) **346**:858–861.
30. **Kelly, K., B. H. Cochran, C. D. Stiles, and P. Leder.** 1983. Cell-specific regulation of the c-*myc* gene by lymphocyte mitogens and platelet-derived growth factor. Cell **35**:603–610.
31. **Kingston, R. E.** 1989. Transcription control and differentiation: the HLH family, c-*myc* and C/EBP. Curr. Opin. Cell Biol. **1**:1081–1087.
32. **Konieczny, S. F., B. L. Drobes, S. L. Menke, and E. J. Taparowsky.** 1989. Inhibition of myogenic differentiation by the H-*ras* oncogene is associated with the down regulation of the MyoD1 gene. Oncogene **4**:473–481.
33. **Landschulz, W. H., P. F. Johnson, and S. L. McKnight.** 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science **240**:1759–1764.
34. **Lassar, A. B., J. N. Buskin, D. Lockshon, R. L. Davis, S. Apone, S. D. Hauschka, and H. Weintraub.** 1989. MyoD is a sequence-specific DNA binding protein requiring a region of *myc* homology to bind to the muscle creatine kinase enhancer. Cell **58**:823–831.
35. **Lassar, A. B., M. J. Thayer, R. W. Overell, and H. Weintraub.** 1989. Transformation by activated *ras* or *fos* prevents myogenesis by inhibiting expression of MyoD1. Cell **58**:659–667.
36. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

36a. **Miner, J. H.** Unpublished data.

36b. **Miner, J. H., and S. V. Tavtigian.** Unpublished data.

37. **Miner, J. H., and B. Wold.** 1990. Herculin, a fourth member of the *MyoD* family of myogenic regulatory genes. Proc. Natl. Acad. Sci. USA **87**:1089–1093.
38. **Mueller, P. R., S. J. Salser, and B. Wold.** 1988. Constitutive and metal-inducible protein:DNA interactions at the mouse metallothionein I promoter examined by in vivo and in vitro footprinting. Genes Dev. **2**:412–427.
39. **Mueller, P. R., and B. Wold.** 1989. In vivo footprinting of a muscle specific enhancer by ligation mediated PCR. Science **246**:780–786.
40. **Murre, C., P. Schonleber McCaw, and D. Baltimore.** 1989. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, *daughterless*, *MyoD* and *myc* proteins. Cell **56**:777–783.
41. **Murre, C., P. Schonleber McCaw, H. Vaessin, M. Caudy, L. Y.**

Jan, Y. N. Jan, C. V. Cabrera, J. N. Buskin, S. D. Hauschka, A. B. Lassar, H. Weintraub, and D. Baltimore. 1989. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell **58**:537–544.

42. **Olson, E. N.** 1990. MyoD family: a paradigm for development? Genes Dev. **4**:1454–1461.
43. **Olson, E. N., G. Spizz, and M. A. Tainsky.** 1987. The oncogenic forms of N-*ras* or H-*ras* prevent skeletal myoblast differentiation. Mol. Cell. Biol. **7**:2104–2111.
44. **Payne, P. A., E. N. Olson, P. Hsiau, R. Roberts, M. B. Perryman, and M. D. Schneider.** 1987. An activated c-Ha-ras allele blocks the induction of muscle-specific genes whose expression is contingent on mitogen withdrawal. Proc. Natl. Acad. Sci. USA **84**:8956–8960.
45. **Persson, H., and P. Leder.** 1984. Nuclear localization and DNA binding properties of a protein expressed by human c-*myc* oncogene. Science **225**:718–721.
46. **Pinney, D. F., S. H. Pearson-White, S. F. Konieczny, K. E. Latham, and C. P. Emerson, Jr.** 1988. Myogenic lineage determination and differentiation: evidence for a regulatory gene pathway. Cell **53**:781–793.
47. **Rhodes, S. J., and S. F. Konieczny.** 1989. Identification of MRF4: a new member of the muscle regulatory factor gene family. Genes Dev. **3**:2050–2061.
48. **Rosenthal, N.** 1989. Muscle cell differentiation. Curr. Opin. Cell Biol. **1**:1094–1101.
49. **Schafer, B. W., B. T. Blakely, G. J. Darlington, and H. Blau.** 1990. Effect of cell history on response to helix-loop-helix family of myogenic regulators. Nature (London) **344**:454–458.
50. **Schneider, M. D., M. B. Perryman, P. A. Payne, G. Spizz, R. Roberts, and E. N. Olson.** 1987. Autonomous expression of c-*myc* in BC_3H1 cells partially inhibits but does not prevent myogenic differentiation. Mol. Cell. Biol. **7**:1973–1977.
51. **Sejersen, T., J. Sumegi, and N. R. Ringertz.** 1985. Density-dependent arrest of DNA replication is accompanied by decreased levels of c-myc mRNA in myogenic but not in differentiation-defective myoblasts. J. Cell. Physiol. **125**:465–470.
52. **Sorrentino, V., R. Pepperkok, R. L. Davis, W. Ansorge, and L. Philipson.** 1990. Cell proliferation inhibited by *MyoD1* independently of myogenic differentiation. Nature (London) **345**:813–815.
53. **Sternberg, E. A., G. Spizz, M. E. Perry, and E. N. Olson.** 1989. A *ras*-dependent pathway abolishes activity of a muscle-specific enhancer upstream from the muscle creatine kinase gene. Mol. Cell. Biol. **9**:594–601.
54. **Tapscott, S. J., R. L. Davis, M. J. Thayer, P. F. Cheng, H. Weintraub, and A. B. Lassar.** 1988. MyoD1: a nuclear phosphoprotein requiring a myc homology region to convert fibroblasts to myoblasts. Science **242**:405–411.

54a. **Tavtigian, S. V.** Unpublished data.

55. **Thayer, M. J., S. J. Tapscott, R. L. Davis, W. E. Wright, A. B. Lassar, and H. Weintraub.** 1989. Positive autoregulation of the myogenic determination gene MyoD1. Cell **58**:241–248.
56. **Weintraub, H., S. J. Tapscott, R. L. Davis, M. J. Thayer, M. A. Adam, A. B. Lassar, and A. D. Miller.** 1989. Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. Proc. Natl. Acad. Sci. USA **86**:5434–5438.
57. **Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin.** 1979. DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. Proc. Natl. Acad. Sci. USA **76**:1373–1376.
58. **Wilder, E. L., and D. I. H. Linzer.** 1989. Participation of multiple factors, including proliferin, in the inhibition of myogenic differentiation. Mol. Cell. Biol. **9**:430–441.
59. **Wright, W. E., D. A. Sassoon, and V. K. Lin.** 1989. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. Cell **56**:607–617.

Chapter 4

A Partial Skeletal Muscle Phenotype Initiated by Ectopic MyoD in the Hearts of Transgenic Mice

Submitted for Publication

A Partial Skeletal Muscle Phenotype Initiated by Ectopic MyoD in the Hearts of Transgenic Mice

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MyoD is a member of the basic/helix-loop-helix family of myogenic regulatory, sequence-specific DNA binding proteins, which are expressed solely in skeletal muscle cells (reviewed in ref. 1 and 2). Extensive studies in cultured cells have shown that MyoD can, upon transfection, cause diverse cell types to express a skeletal muscle phenotype[3,4]. It is not known, however, if MyoD expression can activate all or part of the skeletal muscle programme during embryogenesis. To determine whether and in what manner ectopic MyoD can alter the fundamental characteristics of a non-skeletal muscle tissue in the animal, we have produced transgenic mice which carry the MyoD protein coding sequence under the control of the mouse muscle creatine kinase enhancer/promoter[5]. This promoter directed overexpression of MyoD in skeletal muscle and ectopic expression in cardiac muscle; cardiac muscle is structurally, functionally, and developmentally distinct from skeletal muscle and normally expresses none of the MyoD family regulators. Transgenic hearts exhibit morphological abnormalities, and the foetuses die at 16-18.5 days of gestation. Of the four skeletal muscle regulators[1,2], transgenic hearts also express myogenin but not Myf-5, MRF4, or the endogenous MyoD gene. These hearts express sarcomere genes normally specific to skeletal muscle, though complete conversion to fused skeletal myotubes was not detected. We conclude that a partial induction of the skeletal muscle phenotype by MyoD is initiated in the presence of the foetal cardiac programme. Thus, earlier steps of the skeletal muscle lineage seem not to be required for MyoD activity, and heart cells contain whatever molecular machinery is needed for MyoD to activate genes normally destined to be silent in the heart.

Seven transgenic founder mice are listed in Table 1. Six of these have not produced live transgenic pups, though all were fertile. However, when offspring of four of these six founders were examined *in utero*, transgenics were discovered, suggesting that the transgene was associated with embryonic lethality in these cases. Transgenic foetuses in

several litters sired by founders MuD 14, MuD 42, and MuD 50 died at 16-18.5 days of gestation (E16-E18.5) and as early as E15.5 had visibly misshapen hearts (Fig. 1). Transgenic hearts did beat, but when removed from the foetus they were unable to fully pump blood out of both ventricles as hearts of wild-type (WT) littermates could. This pumping defect is one likely explanation for the lethality observed during the three days before birth. That viable transgenic founders were obtained at a frequency normal for microinjection[6] suggests that either many founders did not express the newly introduced transgene, or that they are genotypically mosaic in critical tissues. The MuD 30 founder, for example, was found to contain less than one copy of the transgene per cell in heart, liver, and tail (data not shown), and MuD 14, 42, and 50 all appear to be mosaic in the germ-line, transmitting the transgene at a frequency of only 10-30%.

These transgenics allowed us to test the ability of MyoD to act in the animal at the molecular level. Expression of the transgene and various cardiac and skeletal muscle genes was measured in hearts and whole limbs of transgenic foetuses and WT littermate controls (Fig. 2). The endogenous muscle creatine kinase (MCK) gene was expressed in both WT and transgenic heart and limb. As anticipated from prior transgenic studies with MCK regulatory sequences[7], the transgene, which contained the MCK promoter, was expressed in transgenic heart and limb. Limb skeletal muscle expressed endogenous MyoD in all cases, but transcripts from the endogenous MyoD gene were not detectable in transgenic hearts. Thus, in cardiac tissue, ectopic MyoD does not positively regulate its endogenous counterpart, a result also obtained with MyoD-transfected NIH 3T3 cells[8,9] but in contrast to the autoactivation observed in transfected C3H 10T1/2 cells[8]. Ectopic MyoD also failed to activate detectable expression of Myf-5 or MRF4/herculin/Myf-6 in transgenic hearts (data not shown), but myogenin expression was clearly turned on (Fig. 2). This may be biologically significant because myogenin is the only myogenic regulator to have been detected in all differentiated skeletal muscle cells in culture[1] and thus may be essential for

expression of the differentiated skeletal muscle phenotype. Hearts of the MuD 20 line, which showed no visible phenotype, expressed an aberrant MyoD transcript and did not express myogenin (data not shown).

Expression of MyoD and myogenin led to alterations in the expression of sarcomere components. All limbs and hearts expressed levels of cardiac a-actin RNA which appear unaffected by the transgene, but only limbs and transgenic hearts expressed detectable amounts of the skeletal isoform (Fig. 2). The most straightforward interpretation is that ectopic MyoD in the heart up-regulated the skeletal a-actin gene, as occurs in MyoD-transfected NIH 3T3 cells (J. H. M., unpublished). Alternatively, since skeletal a-actin is expressed in the absence of MyoD very early in the developing heart[10] and in hypertrophic adult hearts[11], the observed elevation of skeletal a-actin RNA may be secondary to physiological effects caused by the morphological abnormalities. However, other skeletal markers that are not subject to this qualification were also activated. Myosin heavy chain (MHC) isoform expresssion was analyzed by indirect immunofluorescence on frozen sections and by Western blotting using MHC isoform-specific mAbs (Fig. 3). Immunostaining analysis (Fig. 3*a-d*) shows that transgenic but not WT heart produced the embryonic MHC isoform, which is normally specific to skeletal muscle. Staining is restricted to portions of the ventricle, but we do not yet know whether this reflects a corresponding pattern of MyoD expression. (A similar pattern was observed using an antibody against nebulin, another skeletal muscle-specific sarcomere component [data not shown]). The staining results were confirmed by the western blot analyses (Fig. 3*e*), which show that both MuD 14 and MuD 42 transgenic hearts produced the embryonic MHC isoform. The MuD 42 transgenic heart expressed an additional skeletal muscle-specific MHC, the perinatal MHC isoform. We conclude that MyoD activated an exclusively skeletal regulator, myogenin, as well as several skeletal muscle-specific terminal differentiation genes. This is the first demonstration that targeted misexpression of

a tissue-specific regulatory gene can activate normally silent genes in a developing mammalian tissue.

Our results provide interesting comparisons with those obtained by Gurdon and colleagues. They ectopically expressed *Xenopus* MyoD and Myf-5 homologues in *Xenopus* animal caps[12,13], which normally give rise to ectodermal derivatives. In those experiments, endogenous MyoD was activated, and both cardiac and skeletal a-actin genes were expressed. However, it was concluded that skeletal myogenesis did not occur, since muscle-specific gene expression waned and the isolated caps differentiated normally into epidermis. Similarly, the MCK/MyoD transgenic hearts clearly exhibit a partial skeletal muscle phenotype, but by examination either of sections or of cardiocytes cultured from transgenic hearts, we have found no multinucleated myotubes characteristic of skeletal muscle fibers. These studies in developing embryos show that the ability of MyoD to function as a positive acting transcriptional regulator of skeletal muscle differentiation genes is not restricted to the artificial environment of cultured cells. While MyoD is also viewed as a positive regulator of determination because it is expressed in undifferentiated, proliferating myogenic precursors[14], it is not yet known whether MyoD has any essential, active regulatory role in such cells, or if its mere presence specifies the determined state. This issue is relevant because, in the present study, transgene MyoD was likely not expressed until E13, when MCK RNA is first detected in the heart (G. E. Lyons *et al.*, in preparation). The heart is a well-formed, beating organ by this time, and cardiac myocytes are already substantially differentiated along the cardiac pathway[15]. MyoD-expressing cardiocytes may fail to differentiate completely along the skeletal pathway because they have not executed early skeletal myoblast determination functions, should they exist. Alternatively, cardiac-specific differentiation may simply interfere with full expression of the skeletal programme. This is consistent with cell culture experiments in which MyoD-transfected melanoma and neuroblastoma cells do not become exclusively myogenic, but

coexpress some skeletal as well as their original differentiation functions[3]. On the other hand, death of the foetus may interrupt the terminal steps of skeletal muscle differentiation. Finally, Myf-5 and MRF4 may be necessary in the mouse for complete skeletal myogenesis, and neither is expressed in transgenic hearts. Additional transgenic experiments in which MyoD family regulators are specifically turned on at earlier points in cardiac and in other cell lineages should yield a more complete picture of MyoD regulatory potential.

References

1. Olson, E. N. *Genes Dev.* **4,** 1454-1461 (1990).
2. Weintraub, H. *et al. Science* **251,** 761-766 (1991).
3. Weintraub, H. *et al. Proc. natn. Acad. Sci. U.S.A.* **86,** 5434-5438 (1989).
4. Choi, J. *et al. Proc. natn. Acad. Sci. U.S.A.* **87,** 7988-7992 (1990).
5. Jaynes, J. B., Johnson, J. E., Buskin, J. N., Gartside, C. L. & Hauschka, S. D. *Mol. Cell. Biol.* **8,** 62-70 (1988).
6. Hogan, B., Costantini, F. & Lacy, E. *Manipulating the Mouse Embryo* (Cold Spring Harbor Laboratory, New York, 1986).
7. Johnson, J. E., Wold, B. J. & Hauschka, S. D. *Mol. Cell. Biol.* **9,** 3393-3399 (1989).
8. Thayer, M. J. *et al. Cell* **58,** 241-248 (1989).
9. Miner, J. H. & Wold, B. *Proc. natn. Acad. Sci. U.S.A.* **87,** 1089-1093 (1990).
10. Sassoon, D. A., Garner, I. & Buckingham, M. *Development* **104,** 155-164 (1988).
11. Schwartz, K. *et al. Circ. Res.* **59,** 551-555 (1986).
12. Hopwood, N. D. & Gurdon, J. B. *Nature* **347,** 197-200 (1990).
13. Hopwood, N. D., Pluck, A. & Gurdon, J. B. *Development* **111,** 551-560 (1991).

14. Davis, R. L., Weintraub, H. & Lassar, A. B. *Cell* **51,** 987-1000 (1987).

15. Rugh, R. *The Mouse, Its Reproduction and Development* (Burgess Publishing Co., Minneapolis, 1968).

16. Kramer Jr., A. W. & Marks, L. S. *J. Morph.* **117,** 135-150 (1965).

17. Chomczynski, P. & Sacchi, N. *Anal. Biochem.* **162,** 156-159 (1987).

18. Miner, J. H. & Wold, B. J. *Mol. Cell. Biol.*, **11**, 2842-2851 (1991).

19. Johnson, J. E. Ph. D. thesis, University of Washington, Seattle, 1988.

20. Laird, D. W. & Revel, J.-P. *J. Cell Sci.* **97,** 109-117 (1990).

21. Evans, D., Miller, J. B. & Stockdale, F. E. *Dev. Biol.* **127,** 376-383 (1988).

22. Schiaffino, S. *et al. J. Musc. Res. Cell Motil.* **10,** 197-205 (1989).

23. Miller, J. B. *J. Cell Biol.* **111,** 1149-1159 (1990).

24. Schiaffino, S. *et al. Dev. Biol.* **127,** 1-11 (1988).

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TABLE 1 Transgenic founder mice and summary of breeding data

Transgenic Founder:	MuD 14	MuD 18	MuD 20	MuD 28	MuD 30	MuD 42	MuD 50
Sex:	M	F	F	F	F	M	M
Transgenic Pups:	-	-	+	-	-	-	-
Transgenic Fetuses:	+	?	+	+	?	+	+

The MCK MyoD (MuD) transgene contains ~2.8 kb of the mouse MCK 5' region[5] (*Sal*I to *BstE*II) linked to a fragment of pEMclls[14] (*Rsa*I to *Xba*I) containing the MyoD coding region and SV40 T-antigen 3' processing signals. The transgene was purified away from vector sequences by agarose gel electrophoresis[7] and microinjected into pronuclei of single cell embryos[6] derived from a (C57BL/6 X DBA/2) F1 X (C57BL/6 X DBA/2) F1 cross. Potential founders were screened by Southern blot using tail DNA.

FIG. 1 Transgenic foetuses have malformed hearts. All hearts shown were dissected from ~E17 foetuses, fixed in 3.5% formaldehyde/PBS, and photographed frontally. The MuD 50 heart morphology is representative of that also exhibited by MuD 14 hearts. Transgenic ventricles have over-defined apices which point towards the right (i. e., towards the left atrium), whereas WT ventricle is more smoothly shaped and points down, away from the atria.

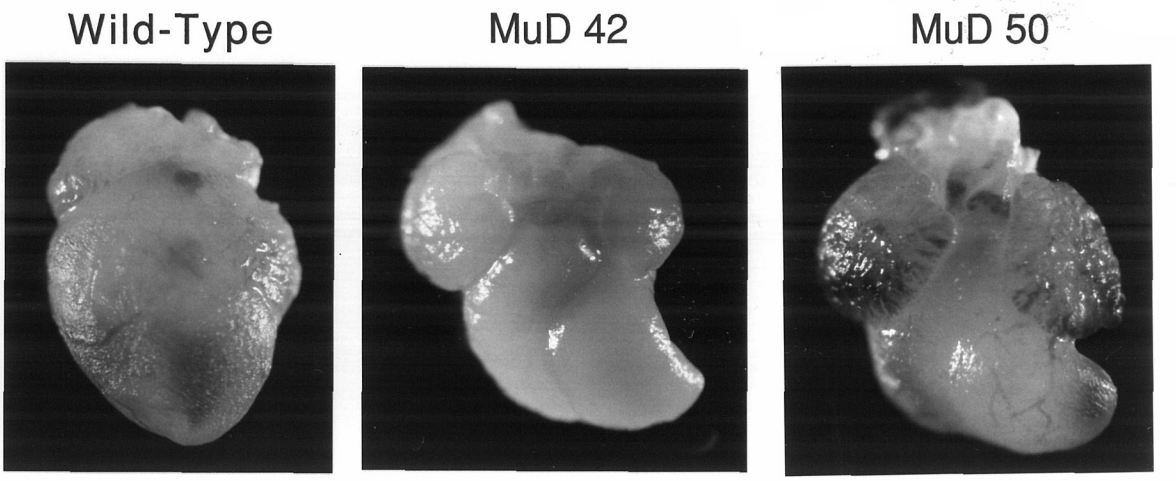

Wild-Type
MuD 42
MuD 50

FIG. 2 Ribonuclease protection analyses of expression patterns of the indicated genes. H, heart; L, limb (both whole). Transgenic hearts differ from WT in their expression of MyoD, myogenin, and skeletal α-actin. RNAs used in WT companion lanes are from littermates of transgenics. MuD 14 limb appears to overexpress myogenin relative to the companion WT limb, though corresponding MCK and α-actin levels are comparable. MuD 14 and WT companion RNA preparations were made from E18.5 tissues, except the MyoD assay used RNA from E16.5 tissues. All MuD 42 and WT companion RNA preparations were made from E16.0 tissues. In a similar experiment, long exposures revealed very low levels of transgene expression in liver and brain, and appreciable levels were found in lung (data not shown); however, the latter may be due to contaminating cardiomyocytes which are known to migrate into the lung through pulmonary blood vessels[16].

METHODS. RNA was prepared[17] and RNase protections performed[18] as described. Amounts of total RNA used were: for MCK and α-actins, 1 μg heart RNA and 3 μg limb RNA; for MyoD and myogenin, 8 μg heart RNA and 15 μg limb RNA. cRNA probes were as follows: MCK, a probe from exon 7[19]; MyoD, as described[18], except the restriction cut was at *Dde*I; myogenin, as described[18]. The template for the sarcomeric actin riboprobe was synthesized by PCR amplification of heart cDNA using one primer complementary to the unique 3' untranslated region of the cardiac isoform mRNA and a second primer from its coding region, which is very conserved between cardiac and skeletal isoforms. Cardiac α-actin mRNA protects the entire probe, whereas skeletal α-actin mRNA protects only coding region sequences, thus producing discrete bands.

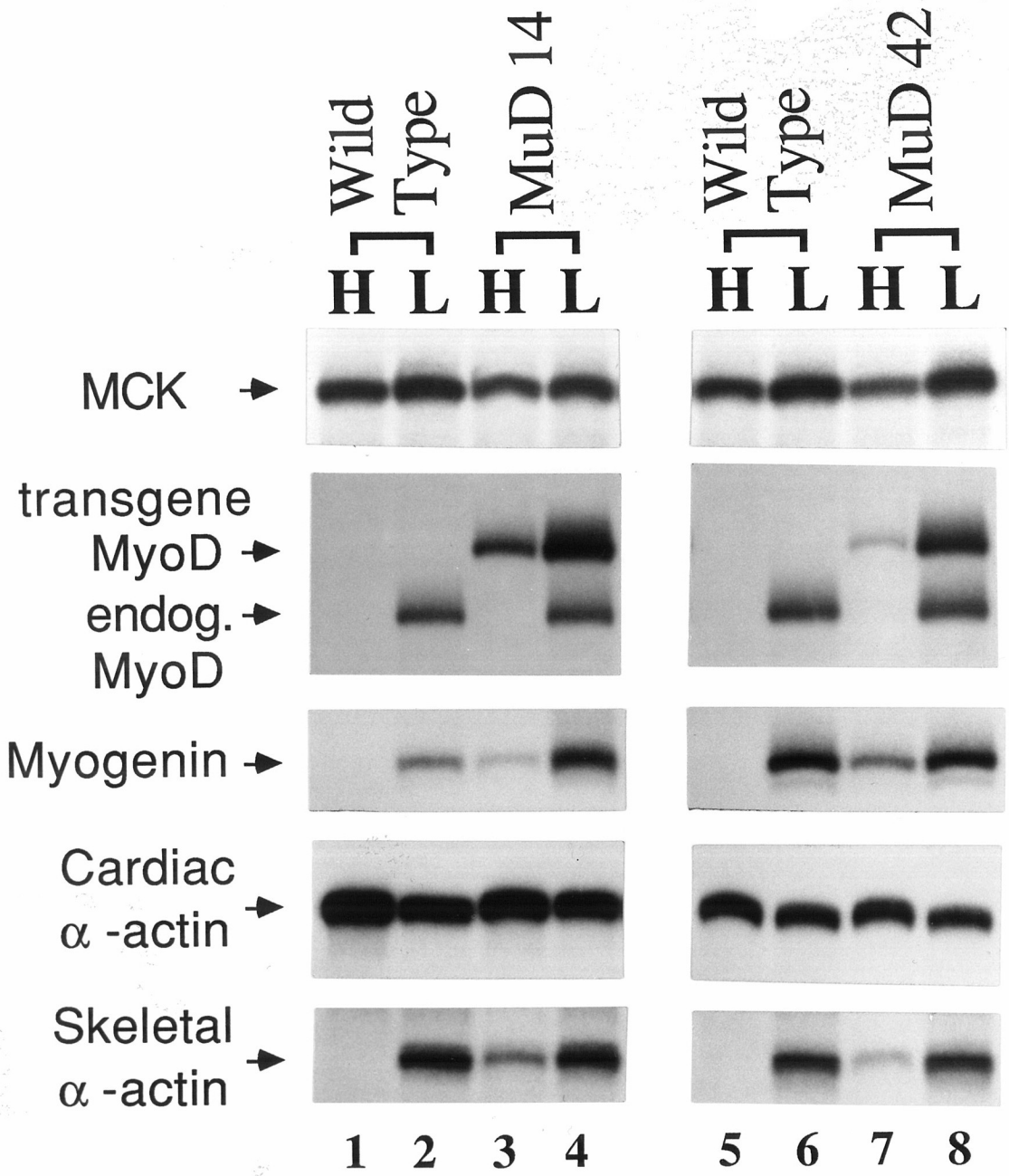
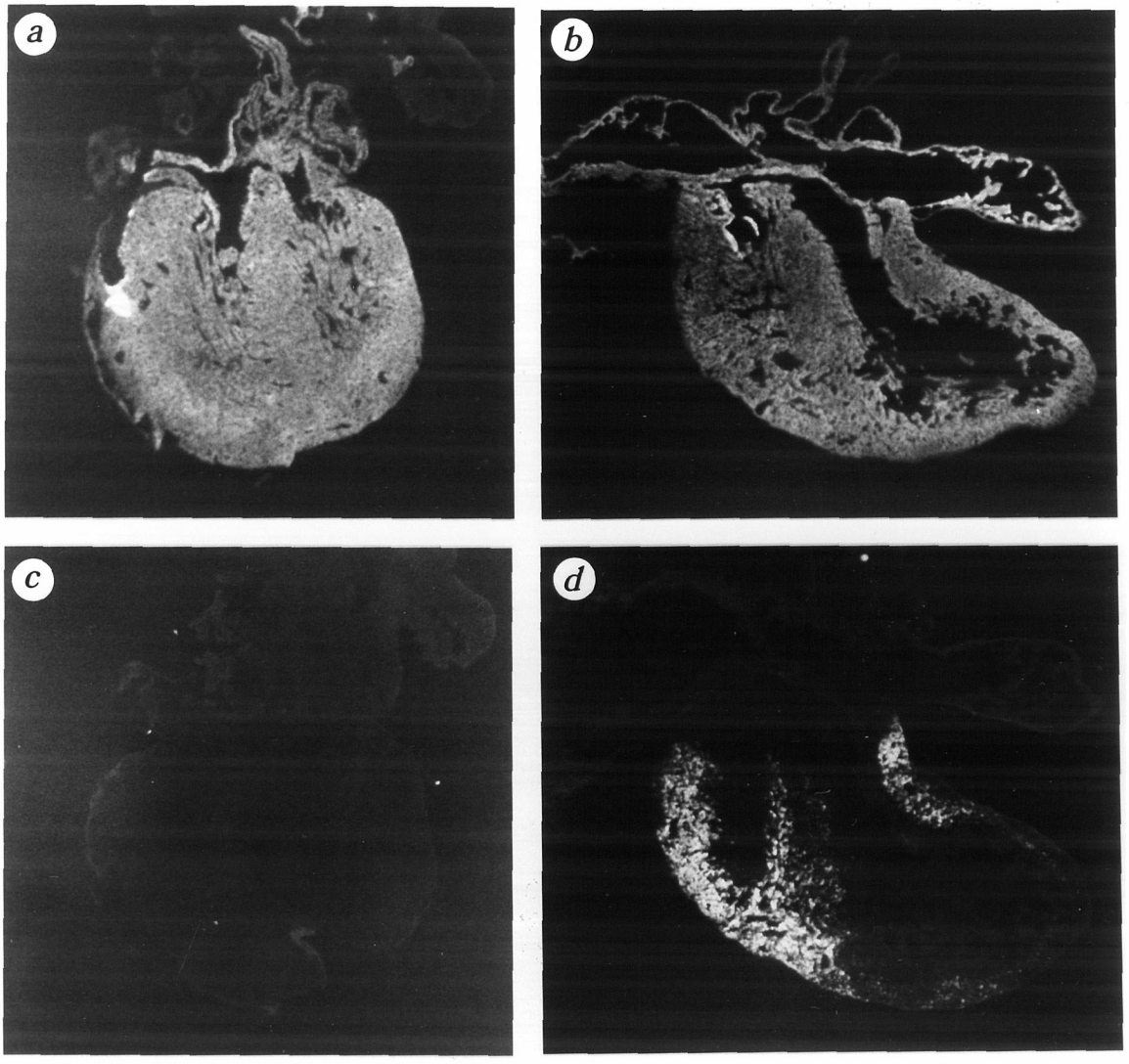
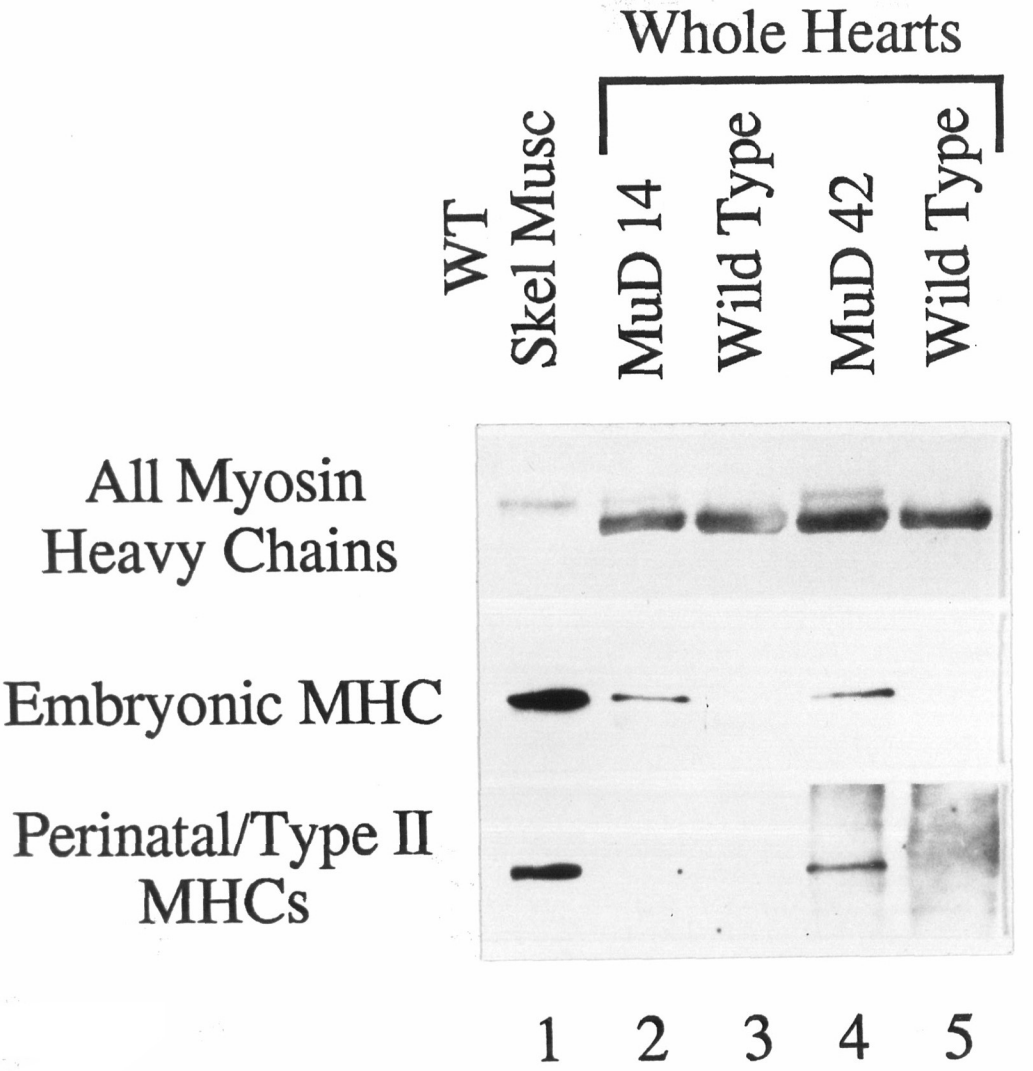

Wild Type
MuD 14
Wild Type
MuD 42
H L H L H L H L
MCK
transgene MyoD
endog. MyoD
Myogenin
Cardiac α -actin
Skeletal α -actin
1 2 3 4 5 6 7 8

FIG. 3 Skeletal muscle myosin heavy chain isoforms are expressed in transgenic hearts. *a-d*, Indirect immunofluorescence using either a general MHC monoclonal antibody[20] (*a, b*) or a skeletal muscle-specific embryonic MHC mAb (*c, d*) on 10 μM frozen sections of MuD 14 E16 heart (*b, d*) and WT control (*a, c*). While the nonspecific MHC mAb labels all atrial and ventricular musculature, only transgenic ventricle labels with the embryonic MHC isoform mAb. *e*, Western blot analysis of whole heart homogenates. The embryonic MHC expressed by MuD 14 and MuD 42 hearts was identified on the basis of electrophoretic mobility and reaction with an embryonic MHC-specific mAb. In addition to the embryonic MHC isoform, MuD 42 heart expressed the perinatal MHC isoform, as is indicated by reaction with a perinatal/Type II-specific mAb (shown) and with a perinatal-specific mAb (data not shown). Both the transgenic and non-transgenic hearts expressed the normal complement of cardiac MHC isoforms (top panel, lower bands).

METHODS For sectioning, dissected hearts were immediately placed into O. C. T. Compound and frozen into blocks on dry ice. 10 μM sections were cut on a cryostat and collected on subbed slides. Two 12-hour incubations with hybridoma supernatants were performed at room temperature in a humidified chamber, followed by a six-hour incubation with FITC-labeled goat anti-mouse secondary antibody. For western blot analysis, hearts were individually homogenized in 50 μL of high salt solubilization buffer[21], and the supernatants were mixed with an equal volume of glycerol. MHC isoforms were analyzed by SDS-PAGE in 6% polyacrylamide, 37.5% glycerol gels[22] followed by immunoblotting with mAbs F59[21,23] (top panel), BF-45[24] (middle panel), and F47[23] (bottom panel). Binding of mAbs was detected with a horseradish peroxidase detection system (Vectastain ABC kit) using a diaminobenzidine (F59) or chemiluminescent (BF-45 and F47) substrate.


a
b
c
d


Whole Hearts
WT Skel Musc
MuD 14
Wild Type
MuD 42
Wild Type
All Myosin Heavy Chains
Embryonic MHC
Perinatal/Type II MHCs
1
2
3
4
5

Conclusions

The studies presented in this thesis have involved several important aspects of mammalian skeletal muscle development, both in cell culture and in the animal. When the work was begun, MyoD was the only known myogenic regulatory gene. By the time herculin was isolated, the existence of myogenin and Myf-5 had already been reported. It is ironic that herculin (MRF4-Myf-6), which is the most abundant muscle regulatory factor transcript in adult skeletal muscle, was the last to be cloned and characterized. This was probably due to the rarity of its expression in myogenic cell lines in culture, which provided substrate RNA for most group's analyses. Herculin and Myf-6 were cloned not by the fact that they are expressed in muscle cells, but serendipitously by their tight linkage on the mouse and human chromosome to the Myf-5 gene. MyoD and myogenin, on the other hand, are neither linked to each other nor to the herculin-Myf-5 locus. While no extensive molecular evolutionary analysis has been reported, it appears that, in the helix-loop-helix region, herculin is most related to myogenin, while MyoD is most related to Myf-5. This suggests that the herculin-Myf-5 locus gave rise to myogenin and MyoD by duplication, respectively, and that the herculin-Myf-5 locus represents a duplication of an original myogenic regulatory gene at that locus.

Previous experiments which demonstrated inhibition of differentiation by c-myc over-expression in skeletal muscle cell lines were done before the cloning of the MyoD family. The similarity between c-myc and MyoD in the basic helix-loop-helix region suggested to us that these proteins might interact with similar DNA sequences or with similar partner proteins in order to carry out their functions, which were clearly antagonistic. Our experiments were designed to coexpress these antagonistic activities in a muscle cell background which was defined by the genes transfected and not by the endogenous myogenic program--thus, the use of NIH 3T3 fibroblasts, which are responsive to MyoD, as recipient cells. Only later did we learn of the auto- and cross-regulatory abilities of MyoD and its relatives. But since only myogenin was activated by

the transfected MyoD, the number of myogenic players remained low, and conclusions regarding the ability of c-myc to inhibit both MyoD- and myogenin-initiated myogenesis could be made. Additionally, it was shown that inhibition by c-myc occurred in the absence of Id, suggesting that c-myc prevents differentiation by an independent negative regulatory pathway or that c-myc is downstream from Id in a single pathway. This is relevant for explaining why proliferating myoblasts which express MyoD and/or Myf-5 do not differentiate. c-myc and Id are both expressed at relatively high levels in proliferating myoblasts, and they may be crucial for preventing premature differentiation and, at the same time, ensure expansion of the muscle precursor population during embrygenesis.

Finally, the production of transgenic mice which express MyoD ectopically in the heart has shown that MyoD's ability to initiate skeletal myogenesis is not restricted to the artificial environment of cell culture. Transgenic hearts express MyoD, myogenin, and sarcomere genes normally specific to skeletal muscle. These experiments are the first known to us to show that targeted mis-expression of a tissue-specific regulator during murine embryogenesis can activate genes normally destined to be silent. Transgenic mice should prove to be excellent for evaluating the regulatory potential of the remainder of the MyoD family as well as for other tissue-specific regulators.