Murine Twist is a bHLH Regulator that Inhibits Myogenesis by Multiple Molecular Mechanisms

Thesis by

Kyuson Yun

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1997

(November 22, 1996)

I would like to dedicate this thesis to the loving memory of my father who has always been the source of my strength and inspiration.

Acknowledgments

It has been a great blessing for me to have had the opportunity to work with so many bright, motivated colleagues in the last several years. I know that my progress through this Ph.D. program was touched by everyone who shared their thoughts and lives with me. I would particularly like to thank Ray Deshaies and his lab members for sharing valuable yeast-ology tips with me and sharing their incubator space. Also the same appreciation extends to Scott Fraser and his lab members for sharing their equipment and helpful tips. My transgenic experiments would not have been possible without the help of the Caltech vivarium staff and Dr. Jeff Mann at City of Hope, who allowed me to use his facility.

I am indebted to all members of the Wold lab, past and present, for sharing their ideas with me, helping me through experiments, and of course, for being available to go eat at any time of the day and night. It's been great fun to hang out with Ardem, Jeong, Albert, Susan, Chia-yu, and others. So I thank you all for your friendship as well as for your eagerness to share ideas and inspirations with me. I particularly want to thank two exceptional undergraduate students that I had the good fortune of working with: Albert Erives and Lawrence Schauffler. They challenged me and inspired me with their insatiable curiosity and energy for new knowledge and experience.

I would also like to thank all of my committee members, David Anderson, Scott Fraser, Howard Lipshitz, Paul Sternberg, and Jeff Mann for encouraging and criticizing my work. I cannot honestly say that every committee meeting was fun, but I can say that I learned something from each of those meetings. I thank them for believing in my ability and caring enough to push me. Needless to say, the single person who challenged and influenced me the most is my advisor Barbara Wold. So I thank her for having me in her lab and working with me all these years.

Last but not least, I would like to thank everyone in my family. Min and Sang have been the most supportive brothers, silently watching me and always available when I needed help. I could not have asked for a better set of parents-in-law, Don and Janet Gallup, who believed in my education more than myself at times. I am forever indebted to my parents for all the sacrifices they have made over the years to support my education. This is as much their thesis as it is mine. Finally, I would like to thank my incredibly patient, understanding, and supportive husband David without whose encouragement and love, I may not be writing this today. And I thank the newest addition to our family, Andrew, for making me smile. I am forever grateful that you are my family.

Abstract

Twist is a member of the basic helix-loop-helix (bHLH) class of transcription factors. From its expression patterns during fly and mouse embryogenesis, it was chosen to be studied as a potential upstream- or cross-regulator of myogenic regulatory factors (MRFs). Additional motivation for studying twist in the context of myogenesis comes from the shared structural motif among MRFs, E-proteins ("universal" partners for tissue-specific factors), and twist that would allow direct physical association. Helix-loop-helix domain allows combinatorial dimerization that results in distinct DNA-binding complexes. Depending on the partner choice and availability, twist and other bHLHs can form complexes with different activities.

In E8.5d mouse embryo, twist and myf5 overlap in their expression domains in the developing somites, indicating the physiological relevance of the twist and MRF interaction. This observation suggests that twist and myf5 (MRF) may interact with each other, directly or indirectly through E-proteins. Supporting data for this idea are presented in chapter 2. In addition, twist is demonstrated to form active DNA binding complexes with different E-proteins. Since twist titrates available E-proteins from MyoD *in vitro* and *in vivo*, twist acts as a dominant negative regulator of myogenesis. However, the fact that twist:E complex is an active DNA binding factor suggests that twist may regulate the expression of downstream target genes.

Studies using a tethered dimer between MyoD and E47 (MyoD~E47) support the likelihood of transcriptional regulaton by twist, presented in chapter 3. Results from chapters 2 and 3 suggest that although bHLH competition and MEF2 titration are viable mechanisms of myogenic inhibition by twist, the most potent inhibitory activity of twist is likely to involve another mechanism.

Since twist inhibits myogenesis initiated by all combinations of MRFs and MEF2 tested in transfected cells, the cell cycle status of these cells were tested since forced proliferation would account for failure to differentiate. MyoD expressing cells are found to arrest normally in the presence of twist, suggesting twist inhibition is specific to differentiation and not an overall inactivation of MyoD function. Furthermore, twist inhibits the onset of myogenin suggesting this early myogenic event is blocked. However, this is not the only restriction point targeted by twist since late twist expression (driven by the myogenin promoter) can still inhibit muscle differentiation. These observations together show that twist is a potent inhibitor of myogenesis and suggest that twist may be involved in regulating proper muscle differentiation in developing embryos.

Table of Contents

Chapter 1

Introduction

Skeletal Muscle Determination and Differentiation

Substantial portions of this chapter appear in a review in

Current Opinions in Cell Biology

by K.Y. and B.W.

Abbreviations

bHLH	basic helix-loop-helix
CDK	cyclin dependent kinase
CDI	cyclin dependent kinase inhibitor
FGF	fibroblast growth factor
HGF/SF	hepatocyte growth factor/ scatter factor
HLH	helix-loop-helix
IGF	insulin like growth factor
MADS	MCM1, agamous, deficiens, serum response factor
MCK	muscle creatine kinase
MEF2	myocyte enhancer factor-2
MRF	muscle regulatory factor
Rb	retinoblastoma protein

Abstract

Regulation of skeletal muscle determination and differentiation in the vertebrates centers on a core regulatory network that is composed of two families of transcription factors, the MyoD group basic helix-loop-helix (bHLH) muscle regulatory factors (MRFs) and the myocyte enhancer factor 2 (MEF2) group of MADS box regulators. Members of this network interact with each other genetically and physically, and together they cooperate to positively regulate transcription of downstream muscle specific differentiation genes. During development the myogenic network can be activated or repressed in response to patterning signals, some of which have recently been identified. Once activated, the powerful myogenic activity of the core network can be modulated and held in check by a remarkably large group of negative regulators that operate on network components by diverse mechanisms. Among these negative regulators, twist is a particularly potent inhibitor of myogenesis that can act through multiple molecular mechanisms. This chapter introduces the current knowledge and questions in the field of myogenesis and how twist fits into the overall picture.

Introduction.

Limb, head and trunk skeletal muscles in the vertebrates develop from separate lineages, but all enlist members of the same molecular network to regulate determination and differentiation (figure 1). Under the influence of multiple extracellular signals, both inductive and inhibitory, multipotent mesodermal precursor cells of the paraxial mesoderm become committed to a skeletal muscle fate. The resulting muscle precursor cells (myoblasts) often proliferate and sometimes migrate, but they do not yet express terminal differentiation genes that define differentiated skeletal muscle. These cells express determination class MRF family transcription factors (MyoD or myf5), then exit the cell cycle into a specialized and permanent G0/G1 arrest and commence cytodifferentiation to produce a differentiated muscle cell (myocyte). Initiation of the differentiation transition is preceded by expression of myogenin which is the MRF required for terminal cytodifferentiation and MEF2 family factors which enhance expression of differentiation genes. The differentiation transition is also marked by downregulation or inactivation of a large and diverse group of negative regulators of muscle differentiation. Differentiation specific genes such as MCK, AchR subunits, muscle specific myosins and actins are downstream targets, in both genetic and physical senses, for active MRF transcription factors acting together with MEF2 family of transcription factors. Most myocytes subsequently fuse with each other to form multinucleate myotubes, and these myotubes then mature into various classes of myofibers, a process that includes further adjustment and specialization in gene expression.

The discovery of MyoD in 1987 was a landmark event in establishing the molecular basis of vertebrate cell fate specification and determination [1], and the MyoD family of bHLH class transcription factors (MRFs) continues to loom large in the current picture

of skeletal myogenesis. A most striking property of the vertebrate MRFs (MyoD, myf5, myogenin or MRF4) is that when any one of the four is ectopically expressed in otherwise nonmyogenic cell types, many (though, tellingly, not all) will "differentiate" as skeletal muscle, expressing a wide array of muscle terminal differentiation genes and in some instances fusing. Enforced MRF expression can also cause proliferating cells to arrest independently of execution of muscle differentiation [2, 3], suggesting an additional dominant regulatory capacity. Recent work has clarified how this powerful gain of function works through a multi-factor network, why it is so potent, how it can be held in check, and how the system works during development.

The power is in the network and the network grows

Part of the explanation for MRF initiated skeletal myogenesis was appreciated early and appears mechanistically straightforward; MRF factors can bind to specific DNA consensus sites called E-boxes that are functionally important elements in transcriptional enhancers of muscle differentiation genes (reviewed [4]). However, a compelling case can now be made that it is not a single MRF factor acting in this way that specifies muscle fate or commands muscle differentiation; rather, it is the activation and combined function of a core regulatory network that does the job. In vertebrates this network is composed of the four MRFs plus MEF2 family regulators at least. Moreover, the developmental tasks performed by the myogenic network are not restricted to activation of muscle differentiation genes but also include regulation of myoblast functions that affect progenitor cell number and migration [5, 6].

Two networks in one - interacting proteins and interacting genes

The structure of the core myogenic network, as proposed in figure 2A, is interesting because at least two different kinds of molecular interactions link its members and influence the final output. The network itself is defined by a series of genetic

interactions that connect MRF (reviewed in [7-9]) and MEF2 [10] genes with each other and with myoblast and myocyte target genes. At the molecular level, these genetic relationships may (and likely often do) reflect direct binding of MEF2 and/or MRF factors with cis-acting DNA sequence elements in the target genes. However, the genetic relationships can also be indirect and may involve as yet unidentified intermediate regulators and cofactors. This means that the network may not exhibit all the relationships shown in figure 2A in every cell context. For example, MyoD autoregulation is observed in 10T1/2 fibroblasts but not in NIH3T3 fibroblasts. Exciting recent work has shown that a second class of interactions connects members of the core network in a different way. These are direct protein:protein associations between MRF and MEF2 factors that jointly elevate transcription of muscle differentiation genes [10**].

The cross-regulatory and autoregulatory genetic interactions diagrammed in figure 2A were mainly deduced from cell culture transfection studies which left uncertainty about their relevance to myogenesis *in vivo*. Subsequent gene disruption studies of the four mouse MRFs make it clear that some of the cross-regulatory relationships probably exist *in vivo* and are important, since different MRF genes serve at least two distinct developmental functions (summarized in Table 1 and reviewed in detail in [8, 9]). Myf5 and MyoD jointly act as determination genes while myogenin functions as a differentiation gene. Thus myf5 and MyoD double knockouts fail to produce or sustain a significant population of myoblasts. Myogenin null mice are highly (though not entirely) deficient in muscle differentiation, although they have a large pool of myoblast precursor cells where muscle fibers would normally be. Finally, the gain-of-function phenotype for MEF2 in cultured cells is a substantial enhancement of MRF-initiated myogenesis [10, 11], although the relevance of MEF2 function for skeletal muscle development *in vivo* is uncertain and awaits analysis of MEF2-knockout mice.

Activating the core network by different pathways in vivo.

During myogenesis *in vivo* the network is assembled by the sequential activation of at least one member from each of the three functional subgroups; determination MRF, differentiation MRF, and MEF2 factor. In the embryo, this is an ongoing process that has been best characterized for the axial and limb muscles, all of which arise from the somites. The myotome is a post-mitotic compartment that grows by continuous addition of new MRF expressing myoblasts drawn from a mitotic precursor pool that is thought to be located in the dermomyotome. There is now evidence that activation of the determination class MRFs in the somite occurs by at least two separate inductive signaling pathways that turn on different determination MRFs in distinct domains [12*]. The result is a myotome composed of apparently separate muscle sublineages, while limb muscle originates in an adjacent dermamyotomal domain under the influence of yet another set of signals (reviewed in detail in [13*, 14, 15*]) . A hypothetical model for building the axial and appendicular musculature is shown in figure 1. Myf5 expression is initiated in a dorsomedial domain in response to signals from the neural tube; Wnts 1,3 and 4 from the dorsal neural tube and sonic hedgehog from the notochord and ventral neural tube are candidate signal molecules [16**, 17**, 18], though it is not certain if they act directly or indirectly. These myf5 initiated cells are induced the earliest, define the dorsomedial-most domain of the myotome, and later contribute to the epaxial lineage that produces muscle of the deep back. A second group of cells is induced in response to signals from the dorsal ectoderm. It begins to appear later, is marked by the initial activation of MyoD [12*, 13*], and we suggest that these may be responsible for later producing hypaxial muscles of the body wall.

In all known skeletal lineages myf5/MyoD expression is followed, more or less promptly, by upregulation of myogenin and MEF2 at the beginning of terminal

cytodifferentiation. What governs the precise timing of their expression *in vivo* is an important but largely unanswered question. However, as with the MRFs, different members of the MEF2 family are upregulated differentially, with MEF2C RNA prominent at the medial lip of the early myotome, possibly overlapping with myf5 expression, and MEF2A, B and D appearing later [19, 20]. The last MRF to be activated in most muscle types is MRF4, though its functions in the medial domain of the myotome, where it is transiently expressed, or later in myofiber remains uncertain (reviewed[21*])

Head and limb skeletal muscle lineages also begin with precursor cells from the paraxial mesoderm and sequentially activate determination MRFs, MEF2 factors and myogenin. The limb lineage originates from a pool of cells in the ventrolateral region of the dermomyotome, and although their myogenic fate appears to be specified, they do not express MRFs until they have migrated out into the limb buds. A signal originating in the lateral plate mesoderm is needed for specification of these cells and bone morphogenetic protein (BMP) 4 is one candidate for this function [22**] . BMP4 can also suppress expression of MyoD and myf5, and this may help to explain the MRF-negative phenotype of limb-muscle precursors while they are in the somite. The signal from the lateral mesoderm is antagonized by signals from the neural tube[22], and recent studies showing that noggin and chordin molecules inhibit BMP4 in by direct binding in other systems [23, 24] would seem to suggest that molecules of this type may mediate the antagonizing medial function.

Another recent and very interesting discovery is that the c-met tyrosine kinase receptor is essential for migration of the limb muscle precursor cells away from the somite and into the limb buds. c-met is expressed in cells of the ventrolateral region of the dermomyotome while its ligand HGF/SF is expressed in the limb mesenchyme into

which the precursors migrate [25]. In animals null for the c-met receptor or its upstream regulator, Pax3, these cells fail to emigrate to the limb and also fail to differentiate as muscle, though neither hypaxial nor epaxial trunk muscles are affected [26*, 27]. The failure of limb progenitors to differentiate in the absence of Pax 3 and c-met is context dependent, since null precursors can differentiate if they are explanted to a limb site [28]. And while the absence of BMP4 signals may be one requirement for turning on myf5 in the limbs, it is not known what additional signals, if any, are needed to drive its expression in the limb domain.

MEF2 factors amplify MRF action by multiple mechanisms

In mammals four MEF2 genes, MEF2A-D, encode sequence specific DNA binding transcription factors of the MADS box family. The expression of any one of these substantially increases the efficiency of MRF initiated myogenesis in cotransfection assays [10, 11]. In contrast to the MRFs, however, the emerging consensus is that ectopic MEF2 expression cannot initiate myogenesis in nonmuscle cells [10, 15] and our unpublished data) despite one earlier report to the contrary [11]. How does MEF2 work in skeletal myogenesis? There is now evidence that the functional synergy of MEF2 with the MRFs can come from at least three different modes of action. First, a number of muscle specific transcriptional enhancers contain DNA binding sites for MEF2 factors, and cis-element mutagenesis has shown that these sites can make a substantial contribution to overall enhancer activity (reviewed[29]). Second, functionally significant MEF2 binding sites have been identified in regulatory sequences of myogenin and this could easily provide positive reinforcement of myogenin expression (and presumably its differentiation functions) [30, 31], even though MEF2 alone is not sufficient to turn on myogenin.

The third mechanism of MEF2 augmentation of MRFs is especially intriguing. Olson and colleagues recently reported that MEF2 protein can interact physically with MRF:E heterodimers[10**]. The surprise was that heterotypic MEF2/MRF:E complexes can bind DNA in a manner that requires the DNA binding capacity of only one member, either MEF2 or MRF:E. The physical interaction between MEF2 and MRF:E dimers requires the MRF bHLH domain. In particular, the conserved Ala and Thr amino acid residues that specify myogenicity among bHLH proteins are explicitly required. These residues are sufficient to convert a nonmyogenic bHLH to a myogenic one, and combined with other evidence, this had led Weintraub and collaborators to propose early on that an important cofactor may interact with MRFs in a manner that depends on these residues. And while the X-ray structure of the DNA/MyoD co-crystal now argues that the interaction is not with these residues per se [32], MEF2 has emerged as a credible candidate for this interaction. Similarly, the part of MEF2 required for binding to MRF:E factors maps to the MADS/MEF2 DNA binding and dimerization domains [10, 33]. This physical MEF2/MRF collaboration is especially attractive because it may help to explain how skeletal muscle specific transcriptional enhancers lacking MRF E-box binding sites, or enhancers from which E-boxes have been removed, can still be highly muscle specific [29, 34*] (Fig. 2b)

A threshold shifting effect for MEF2

An interesting feature of MEF2/MRF cotransfection results is that a larger proportion of transfected cells differentiate as myocytes when MEF2 is cotransfected with an MRF compared with MRF alone [10]. This is in contrast to a scenario in which the same number of recipient cells differentiate, but muscle reporter genes are expressed at a higher level in each cell. Also, the magnitude of MEF2 augmentation in our hands seems especially powerful at low levels of initiating MRF (our unpublished data). MEF2 seems to potentiate differentiation of cells that express MRF levels too low to

activate the pathway on their own, effectively downshifting the threshold MRF level needed. Open questions are whether there is a similar role for MEF2 during normal development and, if there is, whether myogenin activation is the immediate target. Consistent with this notion, mutation of a putative MEF2 binding site in myogenin regulatory sequences reduces the domain of axial myogenin expression [31]. Finally, the general conclusion that MEF2 factors act as amplifiers while the MRFs provide specificity fits agreeably with the character of MEF2 expression patterns, because unlike the MRFs, MEF2 factors are quite widely expressed in nonmuscle as well as muscle tissues [29].

Chipping away at necessary and sufficient

Several new observations temper the view that myf5 /MyoD and myogenin genes are both necessary and sufficient for determination and differentiation, respectively. We do not think they invalidate the overall picture for the determination and differentiation functions of specific MRFs, but they do emphasize the significant role played by cell context in defining MRF function. First, although most myoblasts remain undifferentiated in myogenin null animals, there is the intriguing observation that when these cells are explanted from myogenin null fetuses they differentiate robustly in primary cell culture [35, 36]. Moreover, a small but significant population of "escaped" differentiated myofibers was observed *in vivo* in myogenin null mice which lends weight to the question raised by the myogenin null cell culture phenomenon [35, 37]. More recent experiments show that animals that are doubly null for myogenin and MRF4 also produce some differentiated fibers *in vivo* and display similar differentiation behavior in cell culture; therefore it is not compensation by MRF4 , the other putative differentiation MRF, that accounts for this phenomenon (E. Olson, personal communication). These observations seem to be telling us two related things. Firstly, they suggest that there is no absolute requirement for a differentiation class MRF to

execute muscle differentiation. Secondly, the need for myogenin is conditional, and the conditions of cell culture or local microenvironment *in vivo* can liberate myoblasts from their usual myogenin requirement.

The basis for such conditionality is presently unknown, but studies of negative regulators of muscle differentiation suggest some attractive possibilities. The first of these is the Notch/delta signaling pathway and the second is growth factor stimulation. The Notch/delta pathway has been most extensively characterized in *Drosophila* and in *C. elegans* where it is responsible for mediating mutually exclusive choices of cell fate within a group of initially equivalent precursor cells (reviewed [38]). In mammals there are multiple genes related to Notch and Delta that are expressed in potentially relevant domains, and a dominant form of murine Notch can strongly suppress muscle differentiation [39]. More impressively, cells expressing the Notch ligand called jagged inhibit differentiation of Notch expressing C2C12 myogenic cells [40*]. The Notch pathway therefore seems a viable candidate for mediating local inhibition of the myogenic bHLH muscle differentiation function *in vivo*. A second group of environmental signals that likely differ when cells are removed to culture are the growth factors that include FGF, IGF, and TGFbeta family members. One or more of these may be produced at high levels to hold myoblasts in the undifferentiated state by autocrine or paracrine signaling. A postulated role for myogenin would be to downregulate either receptor or factor. This function would be served by cell dilution and media composition in culture.

The *in vivo* requirement for a determination class MRF gene can also be evaded under some conditions. A recent set of experiments asked what myogenic potential, if any, is possessed by embryonic stem cells that are null for both myf5 and MyoD (R. Jaenisch, personal communication). Based on the phenotype of the corresponding

doubly null mice, one might predict that such cells would never produce skeletal myocytes, but the result proved quite different. Under appropriate culture conditions, these double null cells activate myogenin expression and then differentiate into myocytes . Like the differentiation of myogenin null myoblasts in culture, this highlights a substantial role for the cell in defining "permissivity" for myocyte differentiation.

Cell cycle and myogenesis

Under normal conditions, terminal differentiation and proliferation are mutually exclusive events. Skeletal muscle cells do not re-enter cell cycle after they differentiate and accordingly, MyoD has been shown to be involved in coordinating cell cycle withdrawal and muscle differentiation. Specifically, in addition to activating transcription of muscle specific genes, MyoD has been shown to directly bind Rb [41, 42] and also to induce transcription of the p21 cyclin dependent kinase inhibitor [43, 44]. Recent studies suggest that although cell cycle arrest and muscle differentiation are often thought of as a single linked event, they can be separated into parallel pathways that intersect at points.

The close link between myogenic differentiation and cell cycle control is most directly demonstrated by genetic and physical interactions between MyoD and components of the cell cycle machinery. For example, direct Rb and MyoD binding suggests a simple mechanistic explanation for MyoD's ability to coordinate cell cycle arrest and terminal differentiation. Rb regulates G0/G1 to S-phase progression during cell cycle by regulating the expression of G0/G1 to S progression genes (reviewed [45, 46]). Upon terminal differentiation of many cell types, including neuronal, hematopoetic, and skeletal muscle cells, high levels of unphosphorylated or hypophosphorylated pRb are expressed. In this form, pRb binds to E2F to prevent G1-S transition. In cycling

cells, pRb is phosphorylated in late G1 to allow progression into S-phase. In vivo evidence of pRb function in terminal differentiation comes from the observation that in Rb -/- mice, cell death and large number of ectopic mitoses are observed in the nervous system [47-49]. Furthermore, although muscle differentiation does take place in Rb -/- mice, myotubes from these animals continue to synthesize DNA when challenged with growth factors in culture [42]. This is an interesting observation since Rb has been shown to directly interact with MyoD in vitro, and this association is thought to be important for myogenic differentiation. It is possible that p107, another Rb family members whose expression level is elevated in Rb -/- [42], complexes with MyoD and functionally substitute for Rb:MyoD interaction. Or, the Rb:MyoD interaction observed in vitro may not happen in vivo, as a recent study suggested. In either case, it is clear that muscle differentiation can take place in vivo in the absence of Rb although preventing those cells from further DNA synthesis requires Rb function.

MyoD has been shown to be intimately involved in both cell cycle regulation and terminal differentiation of myogenic cells. Studies using mutant forms of MyoD have demonstrated that cell cycle arrest and myogenic differentiation are separable events (2,3). Using CV1 cells, which fail to differentiate but do arrest upon MyoD expression, Sorrentino and his colleagues demonstrated that muscle differentiation and cell cycle withdrawal are regulated in parallel pathways [2]. They showed that a MyoD mutant, containing the basic region of E12, cannot induce myogenic differentiation but can inhibit cell proliferation. Crescenzi et al. observed similar effects of MyoD and their work showed that DNA binding activity is not necessary for induction of growth arrest by MyoD although it is required for differentiation [3]. Work described in chapter 4 further supports these observations by showing that twist expression affects MyoD's differentiation function independently of cell cycle arrest.

More directly linking MRF activity with cell cycle arrest is the observation that ectopic MyoD or myogenin induces p21 expression. p21 is a cyclin dependent kinase inhibitor (CDI) and its high level expression results in cell cycle arrest. Co-expression of MyoD with CDI's (p16, p21, and p27) enhances the myogenic reporter MCK-CAT activity by five to ten fold ([44], [Palmer, submitted #190], Chapter 4). Consistently, co-transfection of MyoD with cyclin D1, which forces cells through cycle, results in suppression of muscle reporter expression [50, 51]. This inhibition of MyoD by cyclin D1 has been postulated to be targeted at MyoD:E heterodimers since MyoD expression levels remain unaltered in co-transfected cells. Cyclin D1 also inhibits the action of the MyoD~E47 tethered dimer (Palmer et al., unpublished data). Perhaps in support of this post translational modulation of MyoD activity, Peverali et al. have shown that elevated level of Id expression is required for G1 progression[52]. Since many proliferating myoblasts express at least one MRF, often MyoD, one or more regulatory mechanisms apparently prevent MyoD from withdrawing cells from the cycle until proper time. Id is one likely candidate for suppressing the growth inhibitory MyoD function. These observations together demonstrate that muscle differentiation and cell cycle arrest are parallel pathways that intersect through several key regulatory molecules.

Multiple brakes on the myogenic accelerator

The strong positively reinforcing character of the core myogenic regulatory network has an attractive implication for development which was first suggested for MyoD autoregulation; once the "decision" to be a myogenic cell has been initiated by expression of an MRF, the decision is amplified and locked in place by positive autoregulation of the MRFs [53]. It is simple to extend this interpretation to embrace the cross-regulatory interactions of the expanded network. But this interpretation also raises a problem; how is myogenesis held in check during normal development, lest the initial expression of small amounts of MRF generate ectopic muscle or trigger

differentiation prematurely? Recent studies show that the possibility of ectopic or inappropriate myogenesis is not merely hypothetical, even though MRF expression is largely restricted to muscle and its immediate progenitors. For example, in the chick embryo epiblast [54], chick lateral plate mesoderm [55], and mouse brain [56], some MRF RNAs are expressed, even though these tissues do not give rise to skeletal muscle in vivo.

The striking overall observation from many studies is that a large number of diverse regulators can block muscle differentiation, and their downregulation or inactivation typically coincides with the onset of terminal differentiation. It is almost certain that additional negative regulators are yet to be identified, but those we know about fall into four classes; 1) regulators that promote G0/G1/S cell cycle progression and their associated upstream signal transduction apparatus; 2) regulators that dominantly specify other nonmuscle fates such as fat; 3) members of the Notch/Delta cell-cell signaling family; 4) inhibitory HLH and bHLH regulators such as Id and twist. This large and still incompletely described cast of negative regulators plays a major role in defining the "cellular context" for the action of the core myogenic network. When these negative regulators are inactive, a cell is "permissive" for muscle differentiation but when even a minor subset is active differentiation fails.

Twist and Id - inhibition within the extended bHLH/HLH/MEF2 network

Twist was first identified in Drosophila where it is essential for normal gastrulation [57, 58]. Although it is widely expressed in all mesodermal cells, its persistent expression becomes restricted to the muscle precursor cells before they start overt differentiation. Once embryonic muscle differentiation starts, twist is turned off in most cells except in some precursor cells that will proliferate and expand to give rise to adult muscles [59, 60]. Twist is a bHLH transcription factor [61, 62] that directly regulates muscle

differentiation since it activates DMEF, which is essential for muscle formation, in flies. In addition to DMEF activation, twist is shown to regulate transcription of rho and sna with other factors that bind to nearby enhancer sites [63]. However, it is interesting to note that twist alone does not show transactivational activity when a reporter containing multimerized twist binding site is used [64].

Vertebrate twist is cloned from mouse ([62], and our unpublished result), Xenopus [65], and chick [66]. Similar to Drosophila twist, mouse and Xenopus twist expression is restricted to the mesodermal lineage. In mouse, twist is not essential for gastrulation but is required for proper embryogenesis [67]. Murine twist is widely expressed in early mesoderm, including the somites, branchial arches, and limb buds, which includes all sites of skeletal muscle precursors. In the somites, which gives rise to both trunk and limb muscles, twist is initially expressed widely (at the epithelial somite stage) and as it matures, twist expression becomes localized to the sclerotome and dermomyotome. In older embryos (d15.5) restricted twist expression is still present in derivatives of these two compartments (our unpublished data). Notably, twist expression is excluded from the myotome and later in differentiated myotubes, with possible exception of satellite cells. However, our study described in chapter 2 found that there is overlap of twist and myf5 expression in young somites before the segregation of sclerotome and dermomyotome. This observation and the fact that twist and MRF's share a dimerization motif that would allow direct association of twist with MRFs led to a series of molecular characterization of MRF:twist interactions described in chapters 2,3, and 4.

Although it has not been rigorously demonstrated, it is likely that twist is expressed in the immediate precursors of myoblasts of mouse also. In the trunk, those cells in the dorsomedial lip, that express myf5 but have not yet turned on myogenin, are probably

still expressing twist. Our study shows that twist expression extends from dermomyotome to this region although it lacks single cell resolution to be completely convincing (unpublished data). Molecular interactions we describe in chapters 2 and 3 probably play pivotal roles in determining the fate of these cells: those that continue to express twist because twist successfully competed for E-proteins (and MEF2) take on the dermatomal or dermamyotomal fate, while those that express high levels of myf5 become myotomal by successfully turning on myogenin and MEF2. Another intriguing possibility of twist expression in muscle precursor cells is in the migrating limb muscle precursor cells (see Fig. 1). Although twist expression in these cells have not be proven by double labeling with Pax3 or c-met, twist is expressed in the domain of cells that do express Pax3 and c-met. It is interesting to note that in twist -/-mice, the forelimb bud fails to develop fully. It will be highly informative to test for Pax3, c-met, and MRF expression in the limb level somites of twist mutant animals to see if premature myogenesis (therefore no migration and proliferation) occurs.

In transfection assays murine twist is a powerful negative regulator of skeletal muscle differentiation [68-70] and, like MEF2, it appears to alter MyoD family function by multiple mechanisms. Murine twist is co-expressed within the domain of myf5 positive cells of the early (day 8.25) somite that does not yet express any myogenin ([69**, 70]chapter 2), which led us to conclude that direct molecular interactions between twist and components of the core myogenic network might occur and be relevant. Recent studies have shown that when twist is asked to interact with components of the myogenic network *in vitro*, it produces three distinct molecular effects. First, twist can compete with MRFs for available E-protein partners and hence inactivates DNA binding by the MRFs in a manner analogous to Id ([69], chapter 2). Id 1,2,3, and 4 proteins are negative regulators of bHLH family factors, especially the E-proteins. They contain an HLH dimerization motif but lack a basic region. When they form heterodimers with

bHLH proteins, the resulting complexes do not bind to DNA [71]. Second, twist:E heterodimers form an active DNA-binding complex with a E-box consensus unlike the preferred muscle E-box core, suggesting that it may activate or inactivate as yet unidentified target genes (chapter 2). Third, twist binds MEF2 in a manner that inhibits MEF2/MRF synergy [69].

Competition between MRFs and twist for E-proteins differs from competition by Id family members in an important way. Successful competition for E proteins by twist (or other non-MRF bHLHs) permits formation of an alternate DNA-binding factor, while competition by Id forms an inactive heteroduplex. This distinction suggests that the original model for inhibition of MRF action by Id family members should be adjusted, so that negative regulation of the MRFs can be distributed among both HLH and bHLH class regulators. In this case, we imagine that Ids may operate mainly in a buffering capacity, perhaps becoming especially important for fine-tuning the amount of available E protein at the time of a major shift in network composition. This view of Id family action seems attractive beyond the example of MRFs and twist, because the number of different E-protein-dependent bHLH regulators that we know about has continued to rise and their expression patterns and phenotypes argue that they must function, at least some cases, in the presence of Id expression.

The third mechanism of twist inhibition focuses on physical interactions between twist and MEF2. Spicer and Lassar recently showed that twist and MEF2 proteins can bind to each other [69**]. They therefore suggested that twist can preferentially inhibit myogenesis by a second competition mechanism in which MEF2 is titrated away from the MRFs. We have found that twist can also very efficiently inhibit muscle specific transcription by a MyoD~E47 forced dimer which is resistant to HLH and bHLH competition [72]. Curiously, MEF2 does not enhance myogenesis initiated by

MyoD~E47 as it does the E47 and MyoD monomer pair; so it seems unlikely that titration of either MEF2 or E proteins are the only mechanisms by which twist inhibits muscle differentiation. It is not yet clear what transcriptional enhancing or repressing actions can be stimulated by twist:MEF2 or twist:E protein complexes, and identifying target genes for their action (if any) will be crucial for sorting out the relative importance of the various twist inhibitory mechanisms.

The multiplicity of negative regulators of myogenesis, together with their patterns of expression, make an important prediction for genetic studies of their activity. For example, null mutation of Id regulators, twist or other bHLHs expressed in muscle precursors are not, by themselves, likely to lead to wholesale premature or ectopic myogenesis. Rather, elimination of multiple "negative regulators" including twist and multiple Ids from expression in muscle precursors will probably be required.

References

1. Davis, R.L., H. Weintraub, and A.B. Lassar, *Expression of a single transfected cdna converts fibroblasts to myoblasts.* Cell, 1987. 51 (6): p. 987-1000.

2. Sorrentino, V., R. Pepperkok, R.L. Davis, W. Ansorge, and L. Phillipson, *Cell proliferation inhibited by MyoD1 independently of myogenic differentiation.* Nature, 1990. 345: p. 813-815.

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

4. Ludolph, D.C. and S.F. Konieczy, *Transcription factor families: muscling in on the myogenic program.* J. FASEB, 1995. 9: p. 1595-1604.

5. Rudnicki, M.A., Schnegelsberg, P. N. J., Stead, R. H., Braun, T., Arnold, H. H., *MyoD or myf-5 is required for the formation of skeletal muscle.* Cell, 1993. 75: p. 1351-1359.

6. Buckingham, M., *Skeletal muscle development and the role of the myogenic regulatory factos.* Biochemical Society Transaction, 1995. 24: p. 506-509.

7. Weintraub, H., *The MyoD family and myogenesis: redundancy, networks, and thresholds.* Cell, 1993. 75: p. 1241-1244.

8. Megeney, L.A. and M.A. Rudnicki, *Determination versus differentiation and the MyoD family of transcription factors.* Biochem. Cell Biol., 1995. 73: p. 723-732.

9. Arnold, H.-H. and T. Braun, *Targeted inactivation of myogenic factor genes reveals their role during mouse myogenesis: a review.* Int. J. Dev. Biol., 1996. 40: p. 345-363.

10. Molkentin, J.D., B.L. Black, J.F. Martin, and E.N. Olson, *Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins.* Cell, 1995. 83: p. 1125-1136.

11. Kaushal, S., J.W. Schneider, B. Nadalginard, and V. Mahdavi, *Activation of the myogenic lineasge by mef2a, a factor that induces and cooperates with myod.* Science, 1994. 266: p. 1236-1240.

12. Cossu, G.K., R. Tajbakhsh, S. Didonna, S. Vivarelli, E. and Buckingham, M., *Activation of different myogenic pathways - myf-5 is induced by the neural tube and MyoD by the dorsal ectoderm in mouse paraxial mesoderm.* Development, 1996. 122: p. 429-437.

13. Cossu, G., S. Tajbakhsh, and M. Buckingham, *How is myogenesis initiated in the embryo.* Trends on Gene Therapy, 1996. 12 No. 6: p. 218-223.

14. Lassar, A.B. and A.E. Munsterberg, *The role of positive and negative signals in somite patterning.* Current Opinion in Neurobiology, 1996. 6: p. 57-63.

15. Molkentin, J.D.a.O., E. N., *Defining the regulatiory networks for muscle development.* Current Opinion in Genetics and Development, 1996. 6: p. 445-453.

16. Munsterberg, A.E., J. Kitajewski, D.A. Bumcrot, A.P. McMahon, and A.B. Lassar, *Combinatorial signaling by sonic hedgehog and Wnt family members induces myogenic bHLH gene expression in the somite.* Genes & Development, 1995. 9: p. 2911-2922.

17. Stern, H.M., A.M.C. Brown, and S.D. Hauschka, *Myogenesis in paraxial mesoderm - preferential induction by dorsal neural-tube and by cells expressing Wnt-1.* Development, 1995. 121 (11): p. 3675-3686.

18. Fan, C.M. and M. Tessier-Lavigne, *Patterning of mammalian somites by surface extoderm and notochord: evidence for sclerotome induction by a hedgehog homolog.* Cell, 1994. 79: p. 1175-1186.

19. Edmondson, D.G., G.E. Lyons, J.F. Martin, and E.N. Olson, *MEF2 gene expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis.* Development, 1994. 3: p. 265-274.

20. Molkentin, J.D., A.B. Firulli, B.L. Black, J.F. Martin, C.M. Hustad, N. Copeland, N. Jenkins, G. Lyons, and E.N. Olson, *MEF2B is a potent transactivator expressed in early myogenic lineages.* Molecular and Cellular Biology, 1996. 16 No. 7: p. 3814-3824.

21. Olson, E., H. Arnold, P. Rigby, and B. Wold, *Know your neighbors: three phenotypes in mull mutant of the myogenic bHLH gene mrf4.* Cell, 1996. 85: p. 1-4.

22. Pourquie, O., C.M. Fan, M. Coltey, E. Hirsinger, Y. Watanabe, and C. Breant, *Lateral and axial signals involved in avian somite patterning-a role for bmp4.* Cell, 1996. 84 (3): p. 461-471.

23. Zimmerman, L.B., J.M. Dejesusescobar, and R.M. Harland, *The spemann organizer signal noggin binds and inactivates bone morphogenetic protein-4.* Cell, 1996. 86 (4): p. 599-606.

24. Piccolo, S., Y. Sasai, B. Lu, and E.M. Derobertis, *Dorsoventral patterning in xenopus-inhibition of ventral signals by direct binding of chordin to bmp-4.* Cell, 1996. 86 (4): p. 589-598.

25. Yang, X.M., K. Vogan, P. Gros, and M. Park, *Expression of the met receptor tyrosine kinase in muscle progenitor cells in somites and limbs is absent in splotch mice.* Development, 1996. 122: p. 2163-2171.

26. Bladt, R., D. Riethmacher, S. Isenmann, A. Aguzzi, and C. Birchmeier, *Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud.* Nature, 1995. 375: p. 768-771.

27. Epstein, J.A., D.N. Shapiro, J. Cheng, P.Y.P. Lam, and R.L. Maas, *Pax3 modulates expression of the c-met receptor during limb muscle development.* PNAS, 1996. 93 (9): p. 4213-4218.

28. Daston, G., E. Lamar, M. Oliver, and M. Goulding, *Pax-3 is necessary for migration but not differentiation of limb muscle precursors in the mouse.* Development, 1996. 122: p. 1017-1027.

29. Olson, E.N., M. Perry, and R.A. Schulz, *Regulation of muscle differentiation by the MEF2 family of MADS box transcription factors.* Developmental Biology, 1995. 172: p. 2-14.

30. Edmondson, D.G., T.C. Cheng, P. Cserjesi, T. Chakraborty, and E.N. Olson, *Analysis of the myogenin promoter reveals an indirect pathway for postiive autoregulation mediated by the muscle-specific enhancer factor mef-2.* Mol. Cell, 1992. 12 (9): p. 3665-3677.

31. Yee, S.P. and P.W. Rigby, *The regulation of myogenin gene expression during embryonic development of the mouse.* Genes Dev., 1993. 7: p. 1277-1289.

32. Ma, P.C.M., M.A. Rould, H. Weintraub, and C.O. Pabco, *Crystal structure of MyoD bHLH domain-DNA complex: perspectives on DNA recognition and implications for transcriptional activation.* Cell, 1994. 77: p. 451-459.

33. Molkentin, J.D., B.L. Black, J.F. Martin, and E.N. Olson, *Mutational analysis of the DNA binding, dimerization, and transcriptonal activation domains of MEF2C.* Molecular & Cellular Biology, 1996. 16 No. 6: p. 2627-2636.

34. Donoviel, D.B., M.A. Shield, J.N. Buskin, H.S. Haugen, C.H. Clegg, and S.D. Hauschka, *Analysis of muscle creatine-kinase gene regulatory elements in skeletal and cardiac muscles of transgenic mice.* Molecular and cellular biology, 1996. 16: p. 1649-1658.

35. Nabeshima, Y., K. Hanaoka, M. Hayasaka, E. Esumi, S.W. Li, and I. Nonaka, *Myogenin gene distruption results in perinatal lethality because of severe muscle defect.* Nature, 1993. 364: p. 532-535.

36. Rawls, A., J.H. Morris, M. Rudnicki, T. Braun, H.H. Arnold, W.H. Klein, and E.N. Olson, *Myogenin functions do not overlap with those of MyoD or myf-5 during mouse embryogenesis.* Developmental Biology, 1995. 172 (1): p. 37-50.

37. Hasty, P., A. Bradley, J.H. Morris, D.G. Edmondson, J.M. Venuti, and E.N. Olson, *Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene.* Nature, 1993. 364: p. 501-506.

38. Kopan, R., *Notch signaling-aristocracy and democracy.* in press, 1996.

39. Kopan, R., J.S. Nye, and H. Weintraub, *The intracellular domain of mouse notch-aconstitutively activated repressor or myogenesis directed at the basic helix-loop-helix region of myod.* Development, 1994. 120 (9): p. 2385-2396.

40. Lindsell, C.E., C.J. Shawber, J. Boulter, and G. Weinmaster, *Jagged-a mammalian ligand that activates notch1.* Cell, 1995. 80 (6): p. 909-917.

41. Gu, W., J.W. Schneider, G. Condorelli, S. Kaushal, V. Mahdavi, and B. Nadal-Ginard, *Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation.* Cell, 1993. 72: p. 309-324.

42. Schneider, J.W., W. Gu, L. Zhu, V. Mahdavi, and B. Nadal-Ginard, *Reversal of terminal differentiation mediated by p107 in Rb -/- muscle cells.* Science, 1994. 264: p. 1467-1471.

43. Parker, S.B., G. Eichele, P. Zhang, A. Rawls, A.T. Sands, A. Bradley, E.N. Olson, J.W. Harper, and S.J. Elledge, *p53-independent expression of p21/Cip1 in muscle and other terminally differentiating cells.* Science, 1995. 267: p. 1024-1027.

44. Halevy, O., B.G. Novitch, D.B. Spicer, S.X. Skapek, J. Rhee, G. Hannon, D. Beach, and A.B. Lassar, *Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD.* Science, 1995. 267: p. 1018-1021.

45. Nevins, J.R., *E2F: a link between the Rb tomor suppressor protein and viral oncoproteins.* Science, 1992. 258: p. 424-429.

46. Weinberg, R.A., *The retinoblastoma protein and cell cycle control.* Cell, 1995. 81: p. 323-330.

47. Clarke, A.R., E.R. Maandag, M. van Roon, N.M.T. van der Lugt, M. van der Valk, M.L. Hooper, A. Berns, and H. te Riele, *Requirement for a functional Rb-1 gene in murine development.* Nature, 1992. 359: p. 328-330.

48. Jacks, T., A. Fazeli, E.M. Schmitt, R.T. Bronson, M.A. Goodell, and R.A. Weinberg, *Effects of an Rb mutation in the mouse.* Nature, 1992. 359: p. 295-300.

49. Lee, E.Y., C.Y. Chang, N. Hu, Y.C. Wang, C.C. Lai, K. Herrup, W.H. Lee, and A. Bradley, *Mice deficient for Rb are nonviable and show defects in neurogenesis and hematopoesis.* Nature, 1992. 359: p. 288-294.

50. Rao, S.S., C. Chu, and D.S. Kohtz, *Ectopic expression of cyclin D1 prevents activation of gene transcription by myogenic basic helix-loop-helix regulators.* Mol. Cell.Biol., 1994. 14: p. 5259-5267.

51. Skapek, S.X., J. Rhee, D.B. Spicer, and A. Lassar, *Inhibition of myogenic differentiation in proliferating myoblast by cyclin D1-dependent kinase.* Science, 267. 267: p. 1022-1024.

52. Pevereli, F.A.R., T. Ramqvist, R. Saffrich, R. Pepperkok, M.V. Barone, and L. Philipson, *Regulation of G1 progression by E2A and Id helix-loop-helix proteins.* EMBO, 1994. 13(18): p. 4291-4301.

53. Thayer, M.J., S.J. Tapscott, R.L. Davis, W.E. Wright, A.B. Lassar, and H. Weintraub, *Positive auto-regulation of the myogenic determination gene myod1.* Cell, 1989. 58 (2): p. 241-248.

54. George-Weinstein, M., *et al.*, *Skeletal myogenesis: The preferred pathway of chick embryo epiblast cells in vitro.* Developmental Biology, 1996. 173: p. 279-291.

55. Linjones, J. and S.D. Hauschka, *Myogenic determination factor expression in the developing avian limb bud-an rt-pcr analysis.* Developmental Biology, 1996. 174 (2): p. 407-422.

56. Tajbakhsh, S. and M.E. Buckingham, *Lineage restriction of the myogenic conversion factor myf-5 in the brain.* Development, 1995. 121 (12): p. 4077-4083.

57. Simpson, P., *Maternal-zygotic gene interactions during formation of the dorsoventral pattern in Drosophila embryos.* Genetics, 1983. 105: p. 615-632.

58. Nusslein-Volhard, C.W.E. and H. Kluding, *Mutations affecting the pattern of the larval cuticle in Drosophila melanogaster.* Wlhelm Roux's Arch. Dev. Bio., 1984. 193: p. 267-282.

59. Bate, M.R.E. and D.A. Currie, *Cells with persistent twist expression are the embryonic precursors of adult muscles in Drosophila.* Development, 1991. 113: p. 79-89.

60. Currie, D.B.M., *The development of adult abdominal muscles in Drosohila: myoblasts express twist and are associated with nerves.* Development, 1991. 113: p. 91-102.

61. Thisse, B.S.C., C. Gorostiza-Thisse, and F. Perrin-Schmitt, *Sequence of the twist gene and nuclear localization of its protein in endomesodermal cells of early Drosophila embryos.* EMBO, 1988. 7: p. 2175-2183.

62. Wolf, C.T., C. Stoetzel, B. Thisse, P. Gerlinger, and F. Perrin-Schmitt, *The M-twist gene of Mus is expressed in subsets of mesodermal cells and is closely related to the Xenopus X-twi and the Drosophila twist gene.* Dev. Bio., 1991. 143: p. 363-373.

63. Ip, Y., R. Park, D. Kosman, K. Yazdanbakhsh, and M. Levine, *dorsal-twist interactions establish snail expression in the presumptive mesoderm of the Drosophila embryo.* Genes & Development, 1992. 6: p. 1518-1530.

64. Szymanski, P. and M. Levine, *Multiple modes of dorsal-bHLH transcriptional synergy in the Drosophila embryo.* EMBO, 1995. 14: p. 2229-2238.

65. Hopwood, N.D., A. Pluck, and J.B. Gurdon, Cell, 1989. 59: p. 893-.

66. Spicer, D.B. and A.B. Lassar, unpublished results.

67. Chen, Z.F. and R.R. Behringer, *Twist is required in head mesenchyme for cranial neural tube morphogenesis.* Genes & Development, 1995. 9: p. 686-699.

68. Hebrock, M., K. Wertz, and E.M. Fuchtbauer, *M-Twist is an inhibitor of muscle differentiation.* Developmental Biology, 1994. 165: p. 537-544.

69. Spicer, D.B., J. Rhee, W.L. Cheung, and A.B. Lassar, *Inhibition of myogenic bHLH and MEF2 transciption factors by the bHLH protein twist.* Science, 1996. 272: p. 1476-1480.

70. Yun, K., *The murine twist bHLH regulator inhibits myogenesis by multiple molecular mechanisms.* Ph.D. thesis-California Institute of Technology, 1997.

71. Jen, Y., H. Weintraub, and R. Benezra, *Overexpression of Id protein inhibits the muscle differentiation program: in vivo association of Id with E2A proteins.* Genes & Development, 1992. 6: p. 1466-1479.

72. Neuhold, L.A. and B. Wold, *HLH forced dimers: tethering MyoD to E47 generates a dominant positive myogenic factor insulated from the negative regulator Id.* Cell, 1993. 74: p. 1033-1042.

73. Smith, T.H., A.M. Kachinsky, and J.B. Miller, *Somite subdomains muscle cell origins and the 4 muscle regulatory factor proteins.* Journal of Cell Biology, 1994. 127 (1): p. 95-105.

Model for axial and limb myogenesis in the mouse

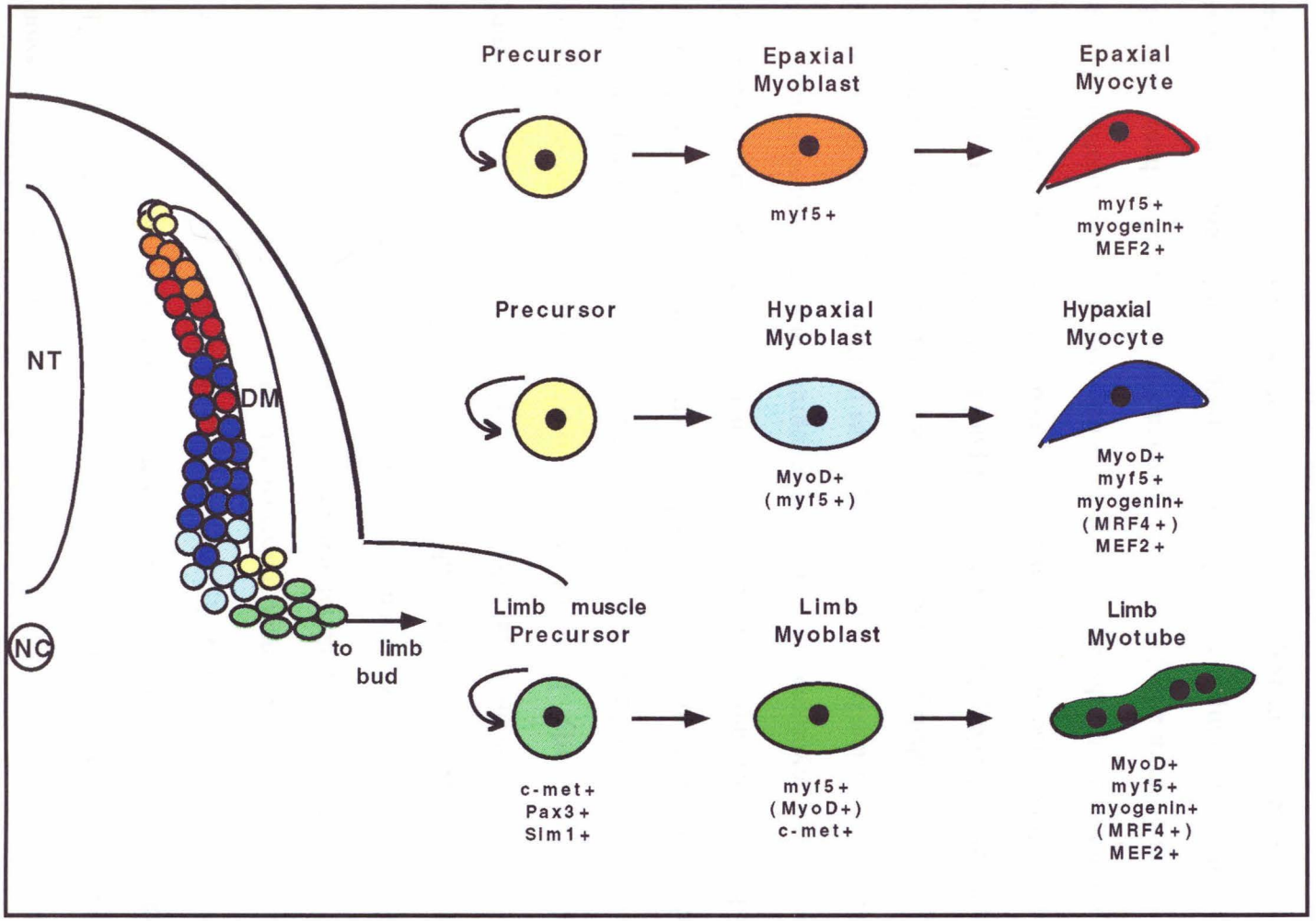


Figure 1. Model for trunk and limb myogenesis in the mouse.

Multiple pathways initiate trunk and limb skeletal myogenesis in the paraxial mesoderm of the mouse. The model pictured (and discussed in the text) is drawn from expression data for the MRFs [73], from phenotypes of c-met and Pax3 mutant mice [26, 27], from experimental data in mouse and in chicken that identify the sources of inducing and inhibitory signals [12-17, 22], and from phenotypic analysis of various MRF knockouts [8, 9, 21]. The schematic transverse section shown (left) is a forelimb level somite at around E10.5. DM, dermomyotome; NC, notochord; NT, neural tube. In the myogenic pathways shown (right), different MRFs (MyoD or myf5) are expressed first, reflecting the distinct inductive pathways that specify the cells for myogenesis. In some cases the other determination MRF (shown in parentheses in the second column) is expressed subsequently. However, the cellular progression is similar in each case and leads to the onset of expression of myogenin and MEF2 and then to terminal muscle differentiation and later, to MRF4 expression (see text for details). Considerable evidence supports at least three probably lineages as shown. First, the most dorsomedial myotomal compartment (progression from yellow-orange-red) begins to develop earliest in the embryo, expresses myf5 in response to signals from the neural tube that probably include Wnt and sonic hedgehog, and ultimately produces epaxial muscles of the deep back. A precursor pool for this sublineage is indicated in yellow in the transverse section and is positioned at the dorsomedial lip of the dermomyotome where the dermomyotome abuts the myotome. The location of these precursors is not certain and there is no known marker that identifies them. Second, the second myotomal compartment (blue-colored progression) begins later, and starts with MyoD expression in response to one or more signals from the dorsolateral ectoderm [12]. In this model, it is suggested that this first MyoD+ compartment later contributes to the hypaxial body wall muscle, which is unaffected in c-met mutants (see text). The immediate precursors of the MyoD-initiated myoblasts are suggested to

originate from dermomyotome adjacent to the ventrolateral myotome, as indicated by the lower group of yellow colored cells. Third, the limb muscle (green-colored progression) derives from cells specified as skeletal muscle precursors in the somite in response to opposing diffusible signals originating in lateral plate mesoderm (perhaps BMP4) and the neural tube [22]. These precursors are dependent on and marked by the expression of Pax3 and c-met for migration, but they do not express any MRFs until after they enter the limb bud where they ultimately differentiate into limb myoblasts and then limb myotubes.

Core skeletal myogenic network

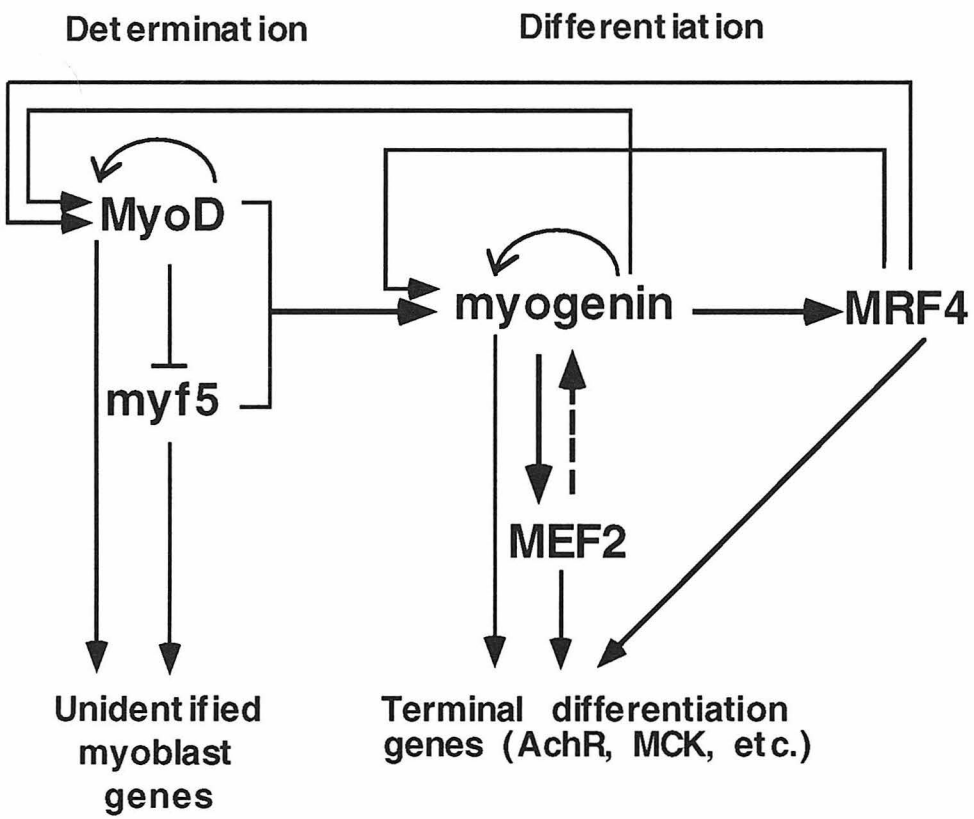


Figure 2A. Core skeletal myogenic network

(A) The core skeletal muscle regulatory network and its targets as defined by genetic relationships. Interactions shown are based on gain-of-function and loss-of-function analysis. Loss-of-function studies in knockout mice have shown that MyoD and myf5 are jointly epistatic to myogenin, and that myogenin is epistatic to MRF4. The dotted arrow from MEF2 to myogenin indicates that MEF2 can help to positively regulate myogenin in some gain-of-function assays but that MEF2 expression is not sufficient to turn on myogenin by itself. Some autoregulatory and cross-regulatory relationships (represented by solid arrows or bars for activatory or inhibitory relationships, respectively) have been detected only by gain-of-function experiments in cell culture and may not be active in vivo.

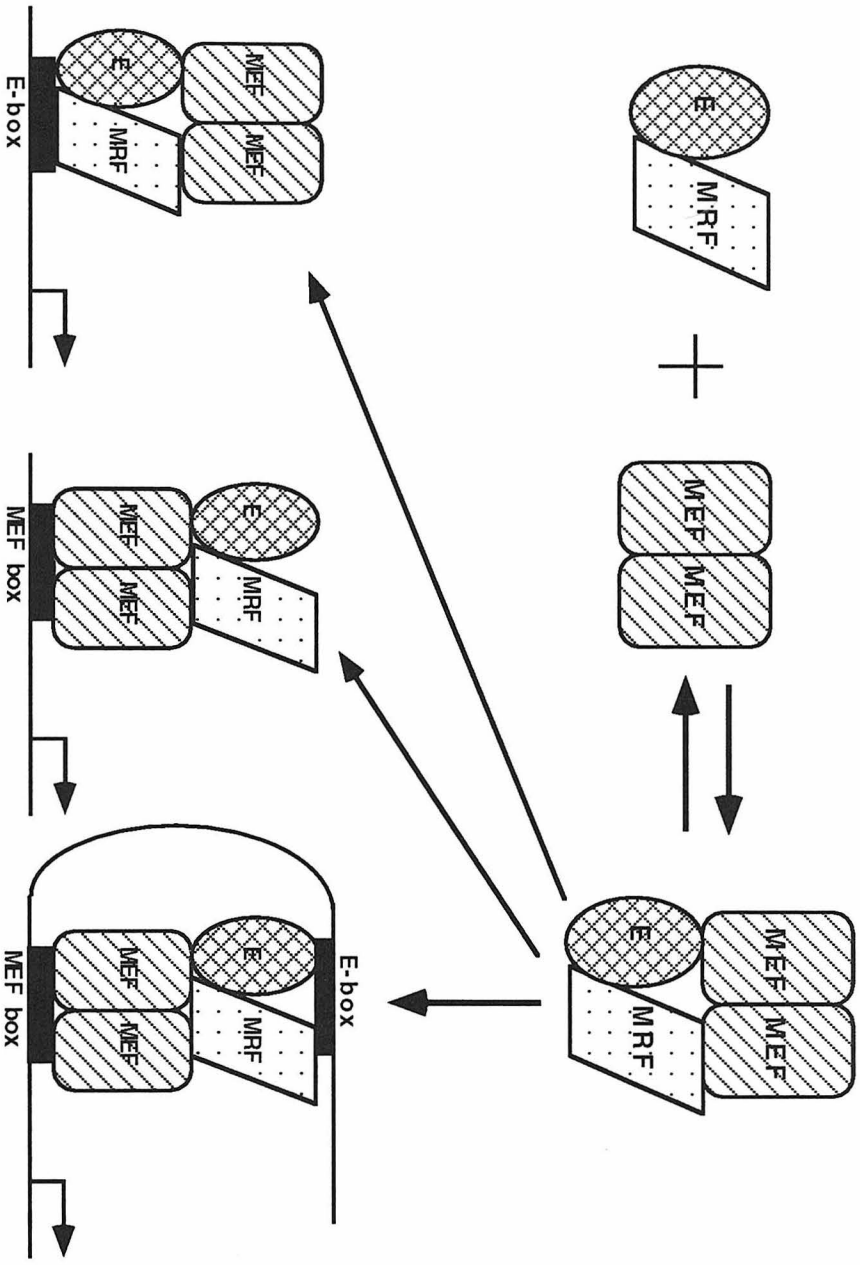

E
MRF
MEF
MEF
E-box
MEF box

Figure 2B. A model for physical interactions between MEF2 factors and MRF:E factors and some ways in which these complexes might act on target genes, as proposed by Molkentin et al.. MRF:E dimers can physically associate with MEF2 and it is proposed that both factors are brought in this manner to occupy either E-box sites (through the MRF:E DNA binding domain) or MEF2 sites (through the MEF2 DNA binding domain) or both sites. Note that in skeletal muscle, where both MEF2 and MRF are expressed, this model suggests a way in which MEF2 factors (which are not themselves highly muscle-specific) may engage a strongly muscle-specific regulator (MRF:E) at an MEF2 site. Single line arrows represent active gene promoters.

Major Muscle Phenotypes of MRF Knockout Mice

Developmental Stage	MyoD$^{-/-}$	Myf5$^{-/-}$	Myf5$^{-/-}$ MyoD$^{-/-}$	myogenin$^{-/-}$	MRF4$^{-/-}$*
Myotome before E-9.5	Normal	No myocytes No myogenin	No myoblasts	Anatomically wildtype	Reduced early myotomal myogenesis
Myotome after E10.5	Normal	"Recovering" myotome	Few myoblasts	Severe differentiation defect	"Recovering" myotome
Newborn	Grossly normal Increased Myf5	Grossly normal	Few cells Increased fat	Severe differentiation defect	Mainly normal

Table 1. Major phenotypes of MRF-knockout mice.S

See references [8, 9, 21] and references therein. * All three MRF4-knockout allelels are affected to variable extents by disrupted expression from the closely linked myf5 gene. This makes it uncertain what fraction of the early myotomal deficit is due to myf5 and what fraction, if any, is due to myotomal MRF4 itself.

Chapter 2

Twist suppresses myogenesis through multiple mechanisms

Contents of this chapter and

chapter 3 are submitted together as a single publication

to Mechanisms of Development

Multiple Mechanisms of bHLH regulator Twist in Mouse Somitogenesis

Kyuson Yun, Lawrence Schaufler°, and Barbara Wold*

Division of Biology 156-29

California Institute of Technology,

Pasadena, CA 91125

*To whom correspondence should be addressed

°Present address: Dept. of Biochemistry, Box 357350, Univ. of Washington,

Seattle WA 98105

Running title: A dual mechanism for twist

Key words: HLH, MyoD, MRF, myogenesis, somite, twist

SUMMARY

Twist is a powerful negative regulator of MRF dependent myogenesis in cultured mammalian cells. In this work we focus on the developmental relevance of myogenic inhibition by twist and on its mechanistic basis. An expression study found that there is a significant domain of coexpression for twist and myf-5 RNAs in somites of E8.5 mouse embryos. This spatiotemporal overlap is important because it suggests that molecular interactions between twist and MRFs, whether direct or indirect, could occur within individual myogenic precursor cells of the paraxial mesoderm. In vitro DNA binding studies then showed that either intact twist or the twist bHLH domain alone can efficiently dimerize with E-proteins and can also inhibit MyoD:E DNA binding to a muscle class E-box target site, indicating that twist can compete for limiting E proteins in a manner analogous to Id. Unlike the HLH inhibitor Id, however, twist also formed sequence specific DNA binding complexes with E-protein partners, and these bound preferentially to a non-muscle class E-box sequence, CATATG. We therefore suggest that twist inhibits myogenesis by a dual mechanism in which the ability of twist-E dimers to recognize a novel class of E-boxes is used to regulate twist target genes that help specify cell phenotypes (a precursor state or a sclerotomal/dermatomal fate) that are non permissive for muscle differentiation.

INTRODUCTION

Twist encodes a basic-helix-loop-helix (bHLH) class regulatory protein that was first identified in *Drosophila* where it is essential for early mesoderm specification and gastrulation [1-4]. Later in *Drosophila* development, twist is selectively expressed in progenitors of skeletal muscle, but upon muscle differentiation it is terminally downregulated [5, 6]. In the mouse, twist expression is also initiated broadly in early embryonic mesoderm and is subsequently modulated in the paraxial mesoderm as the pluripotential somite is subdivided into sclerotome, dermamyotome and myotome. As in *Drosophila*, twist somitic expression is terminally downregulated in differentiating myocytes of the myotomes [7-9]. This expression pattern in the mouse and its apparent parallel in *Drosophila*, as well as recent observations that twist can dominantly inhibit muscle differentiation in a C2C12 cultured cell model [9], suggest that mouse twist may play a role in the pathway that leads to myogenesis in the developing somite.

Although the molecular properties of twist have not previously been described, its primary structure and similarity to other bHLH proteins suggest that functions for twist are likely to depend on the identity, concentration and relative affinities of other HLH and bHLH proteins co-expressed with twist in specific cell types. Twist would be expected to dimerize with itself or with other bHLH proteins to form sequence-specific DNA-binding factors; these might then act as positive or negative regulators of transcription, depending on the DNA target site and its sequence context. Accordingly, it has been shown in *Drosophila* that twist can act as a positive and negative regulator of downstream genes [10, 11]. Twist might also indirectly influence the action of other bHLH proteins if it competes effectively for enabling partners such as E proteins or for the occupancy of similar DNA binding sites.

Finally, twist might interact with non-bHLH accessory factors that enhance MRF (muscle regulatory factor) action such as MEF2 [12].

To better constrain the possible influences of twist in pre-myogenic and myogenic cells in the mouse, we studied its expression during somitogenesis with particular attention to early somites in which myogenic precursors are first specified and segregated. The observation of a substantial domain of co-expression for twist and myf-5 RNAs raises the possibility that molecular interactions may occur between these regulators within individual cells of the early somite. This led us to begin developing a molecular framework for interpreting twist cellular phenotypes that is based on twist dimerization and DNA-binding properties together with its effects on MRF factors. Results of in vitro DNA binding studies together with transfection analyses indicate that twist can inhibit MRF-dependent muscle differentiation by two mechanisms that can be dissected experimentally; one requires only the bHLH domain and resembles Id inhibition (in this chapter) while the other requires additional twist domains (see chapter 2). Analysis of twist interactions with a novel class of nonmuscle E-box DNA binding site suggests a basis for the second mechanism in which genes containing such twist binding sites would act in specifying non-muscle phenotypes. This is discussed in the context of HLH/bHLH networks and their roles in helping to specify distinct cell fates.

MATERIALS AND METHODS

In situ hybridization

S^{35} in situ hybridization was performed on E8.5 embryos with antisense twist probe made from the pBtwi3' plasmid, which contains the XbaI/EcoRI fragment from twist 3' UTR cloned into pBSII/KS+ plasmid, linearized with XbaI and transcribed with T3 RNA polymerase (probe made from the whole coding sequence gives identical pattern) according to the method of Lyons et al. [13]. S^{35} in situ on parallel sections with myf-5 probe (made from the full length cDNA clone (pBSMyf5s) linearized with SalI, transcribed with T7 RNA polymerase, and hydrolyzed to ~ 300bp fragments) was done to compare with twist-labeled sections. Paraffin embedded parallel sections were 6μm thick. They were photographed on Axiophot under dark-field and phase optics using 20X objective.

In vitro transcription and translation

In vitro transcription reactions were performed on linearized DNA templates using T3 or T7 RNA polymerases (using the enzyme supplier's buffer and recommended conditions for in vitro synthesis of capped RNA transcripts, Promega or BMB). The full length and truncated twist plasmids were linearized with BssHII and transcribed with T3 RNA polymerase; MyoD plasmid was linearized with BamHI and transcribed with T7 RNA polymerase; E32.5 was digested with EcoRI and transcribed with T3 RNA polymerase; E12 was digested with BamHI and transcribed with T3 RNA polymerase; Id was linearized with EcoRI and transcribed with T3 RNA polymerase. The transcription products were phenol/Sevag extracted, ethanol precipitated and resuspended in 10 mM Tris (pH7.5), 1 mM EDTA. 1 μg of above RNA was used in

rabbit reticulocyte lysate system for in vitro translation, following the manufacturer's instructions (Promega).

Electrophoretic mobility shift assay

The MCK12 target DNA binding site (GATCCCCCCAACACCTGCTGCCTGA) is taken from the MEF-1 binding site of the MCK enhancer. The mutant competitor, MCK34, has a mutated "E-box" (GATCCCCCCAACACGGTAACCCTGA) [14]. The twist binding site, TWIE1/2, was designed in the context of MCK12 oligo where the core "E-box" sequence was changed from CACCTG to CATATG (Weintraub & Blackwell, personal communication). Twist, Id, MyoD and E-proteins synthesized in reticulocyte lysates were individually quantified using a Phosphorimager from a 10% SDS/polyacrylamide gel. The number of methionine residues in each molecule, determined from the primary sequence information, was used to normalize the samples. Lysates programmed for each regulator were mixed, brought to constant lysate volume with mock-translated lysate from the same lot, and allowed to interact with each other at 37° for 15 minutes before ^{32}P end-labeled probe was added to the reaction mixture. The molecules were then allowed to bind the labeled DNA for 20 minutes at room temperature in our standard reaction condition: 20 mM HEPES (pH7.6), 50mM KCl, 1mM EDTA, 1mM DTT, 5% glycerol, and 1 mg of poly-(dIdC). We tested the pH of the binding buffer (pH7.0, MOPS and pH7.6, HEPES) and gel-running buffer (pH7.0, MOPS and pH8.3, .5X TBE) and the temperatures at which the gel was run (4° and R.T), and found no significant difference. Thus, all experiments shown were performed under our standard condition and the complexes were resolved on 5% polyacrylamide/.5X TBE gels.

Transfections

All HLH and bHLH regulators were cloned into the eukaryotic expression vector pECE [15]. This plasmid provided the SV40 enhancer/promoter and 3' RNA processing sites. Supercoiled DNA was purified by Maxi prep (Promega) followed by a single cesium chloride banding procedure. The promoter dosage was normalized by adding pECE vector. In each experiment, 5 ug of reporter E1-117MCK-CAT plasmid [16] was co-precipitated with 5 ug of pSVLacZ and the regulators (5 ug of MyoD plasmid DNA and equimolar amounts of other regulators were used for each 10 cm plate). C3H10T1/2 cells were seeded on 10 cm plates containing Dulbecco's modified Eagle's medium and 10% fetal bovine serum (growth medium). Transfections were done as previously described [17] with 8-10 hours of incubation in 30 μM chloroquine plus precipitate. Cultures to be assayed in differentiation-promoting conditions were cultured in growth medium for 24 hours after removing chloroquine and precipitate. They were then transferred to 2% horse serum containing media (differentiation medium) for 72 hours and assayed for CAT activity as described by Gorman et al. [18].

Plasmids

The twist coding sequence previously cloned in the lab (Glackin et al., unpublished results) was modified by the introduction of a consensus Kozak sequence to facilitate in vitro translation and cloned into EcoRI and SacI sites of Bluescript II/KS+ (Stratagene). The E32.5 clone is a truncated form of E2-2/ITF2 [19].

RESULTS

Twist expression in somites

Previous studies of twist expression in the mouse somite have focused mainly on its quantitatively dominant expression in the sclerotome and on the notable absence of twist from the established myotome and on the widespread expression of twist in early mesoderm [7, 9]. This mutually exclusive expression of twist and muscle specific RNAs within patterned somites would seem to support the idea that twist can promote alternate nonmuscle fates (sclerotomal or dermatomal) or perhaps inhibit myogenesis by cell-cell interactions; however, it does not provide a clear basis for considering cell autonomous regulatory and molecular interactions between twist and myogenic regulators, although the data are clearly consistent with twist playing some active role in defining nonmyotomal somitic cell types (sclerotome and dermatome). We therefore performed a twist/myf-5 co-expression study that focused on younger somites where prior studies have shown that there is a substantial domain of myf-5 positive cells that have not yet differentiated into myocytes [20]. In situ hybridizations of adjacent sections in Figure 1 show that in somites of day E8.5 embryos, twist and myf-5 are coexpressed over the major domain of myf-5 expression. Although in situ hybridizations lack single-cell resolution, it seems very likely that the domain of overlap reflects co-expression within individual cells. This suggested to us that molecular and regulatory interactions between twist and MRFs are likely to be biologically relevant in the axial myogenic lineage.

Twist:E-protein heteromers form and bind to a twist-preferred E-box sequence

Genetic and molecular studies of *Drosophila* twist have identified twist binding sites in the regulatory regions of downstream target genes [11], but no direct target genes for twist have been identified in vertebrates. An initial test of DNA binding to the

Drosophila twist consensus sequence failed in EMSA assay using rabbit reticulocyte lysate products programmed with murine twist or murine twist together with members of the E-protein family (data not shown). We therefore began to characterize murine twist DNA binding by creating a modified oligonucleotide with a change in the E-box sequence core to "CATATG", identified as the optimal target sequence for bacterially-expressed Xenopus twist in a SAAB site selection experiment (H. Weintraub and K. Blackwell, personal communication). Although programmed reticulocyte lysates containing murine twist alone did not bind to this altered target sequence, substantial binding was observed when an E-protein family member was either co-incubated (Fig. 2A) or cotranslated with twist. For example, either full-length twist or a truncated twist protein (containing only the bHLH region and a small carboxy-terminal region) was sufficient to dimerize with E32.5 and bind DNA. The DNA-binding form is most likely a heterodimer, based on the observation of a single, more rapidly migrating complex when the truncated twist was used (compare lanes 3 and 4). The possibility that this band represents a single preferred species of a symmetric, higher order oligomer of twist with E-protein is not formally excluded. E32.5 alone also bound to this sequence, but these homomeric complexes disappeared in favor of heteromeric complexes when twist was included, suggesting that heterodimerization with twist is favored over homodimerization.

Multiple E-proteins have been identified in mammals [21]. They are obvious candidates for enabling partners of twist, but it is uncertain whether all could perform this function. Preliminary expression surveys at the RNA level found that all four available mouse E-protein genes are detectably expressed in mouse embryos at E8.5-E10.5 (our unpublished data). We therefore tested E12, E47 [22], HEB[23], and E32.5/E2-2[19] to determine which could cooperate with twist to bind the target DNA in an EMSA assay. Each formed a sequence-specific DNA binding complex with

twist, as shown for E12 in Fig. 2B. Competition with a 50X molar excess of the unlabeled target sequence completely eliminated DNA binding while a 50X molar excess of a mutant oligo did not. No E-protein family member was significantly more efficient as a twist partner than another, and binding to the target site was usually more efficient using lysates programmed with twist plus E-protein as compared to E-protein alone.

Twist represses myogenesis initiated by MRF's

Functional assays for the effects of twist expression were performed in C3H10T1/2 cells because, within the limitations of cultured cell lines, these cells most closely approximate multipotential somitic mesoderm of the developing mouse. They have been shown to yield differentiated muscle, cartilage, or fat cells following experimental stimulation [24, 25]. Transfection of 10T1/2 cells with any one of the four MRF bHLH factors (MyoD, myf-5, myogenin, or MRF4/herculin/Myf6), coupled with removal of growth factors, yields differentiated myocytes (reviewed in [26]). 10T1/2 cells were transfected with a MyoD expression vector and a muscle differentiation reporter gene, MCK-CAT [16]. High level MCK-CAT expression is typically observed in differentiated myocytes but absent or low in 10T1/2 host cells or in myoblasts, respectively, prior to muscle differentiation. The transfections were performed in the presence of the twist expression vector or a vector control that lacked a twist insert. The result of the co-transfection experiment was a strikingly effective inhibition of muscle differentiation by twist (Fig. 3A, compare MyoD plus vector with MyoD plus twist). Similar results were obtained when muscle differentiation was monitored by immunostaining for the expression of endogenous myosin heavy chain (MHC) or by measuring the activity of a 4R-CAT reporter gene which contains four copies of the MCK muscle specific E-box in front of tk-CAT reporter (data not shown). Twist was also found to inhibit myogenesis initiated by

myf-5, MyoD, myogenin, or combinations of these (Figure 3B and data not shown). The absolute levels of MCK-CAT activation achieved by each of the MRF's varied significantly, but in every case, twist co-expression reduced MCK-CAT activity to the assay background level. These observations are consistent with Hebrok et al. (1994) and with our unpublished data that an already committed myoblast cell line, C2C12, produces cell lines that are defective in their ability to differentiate when they express a stably transfected twist vector.

Twist competes for enabling E-protein partners through the bHLH region

One mechanism by which twist could inhibit myogenesis would be through HLH:HLH dimerization that would directly or indirectly inactivate MyoD:E-protein complexes. We therefore tested the effect of twist on the formation and DNA-binding activity of MyoD:E-protein complexes in vitro. Using the products of programmed rabbit reticulocyte lysates, we compared the relative abilities of Id, a known competitor, and twist to inhibit the binding of the MyoD:E12 dimer to a muscle-class E-box target DNA site. In this assay, the target oligonucleotide sequence was derived from the right E-box of the E1 enhancer [14]. As expected, MyoD:E12 heteromers formed and bound the target DNA with high efficiency, (Fig. 5 lanes 7 &15), and neither Id nor twist reacted detectably with this muscle class target DNA alone or in the presence of E12 (Fig. 5 lanes 4, 5 and 13,14). The DNA binding by the MyoD:E12 complex was eliminated in a dominant negative manner by Id (lanes 8 to 11). Twist proved to be nearly as efficient as Id on a equimolar basis in eliminating the muscle-specific DNA binding of the MyoD:E12 complex (lanes 16 to 19). The truncated twist containing the bHLH region was sufficient to inhibit MyoD:E12 binding (data not shown).

We next tested whether the truncated twist is sufficient to inhibit MyoD initiated myogenesis in vivo. Truncated twist ("twi-bHLH": residues 116-206), as well as the minimal bHLH domain of twist (residues 106-184), inhibited myogenic differentiation in C3H10T1/2 cells with only slightly reduced efficiency relative to full-length twist (Fig. 5 and data not shown). This supports the idea that one mode of twist inhibition involves bHLH domain competition that ultimately renders MRF's inactive.

The inhibition of MyoD:E12 complex DNA binding observed in Fig. 4 could be due to the interaction of twist with E12 and/or MyoD. If inhibition was primarily due to twist:E12 dimerization at the expense of MyoD:E12 complex formation, then adding additional E12 would be expected to restore MyoD:E12 heterodimers. To test this, the indicated amounts of MyoD, twist and E12 were co-incubated with target muscle E-box-containing DNA for 15 minutes, and then additional E12 programmed lysate or control lysate was added followed by a further 15 minute equilibration. The MyoD:E12 DNA-binding complex was recovered to its original level when excess E12 was added (Fig. 6, lane 8). This is consistent with formation of twist:E12 complexes as the primary inhibitory action and is also consistent with the observation that twist:E12 complexes form readily and bind an alternative target E-box site (Fig. 2A, B). Similar add-back experiments with MyoD revealed only minimal recovery of the DNA-binding complex, suggesting that twist:MyoD is not the limiting complex, although there may be an association with MyoD that is quantitatively minor relative to twist:E.

DISCUSSION

We have examined molecular and functional interactions of murine twist with bHLH and HLH regulators of the MyoD, E-protein, and Id families. Much prior molecular

and genetic evidence argues that interactions among members of the latter bHLH/HLH groups are important in controlling the transition from pre-myogenic somitic mesoderm to myotomal myocytes [26-29]. The role of twist in myogenesis has been much less clear, but it is a candidate for multiple functions that include defining the number and/or phenotype of precursor cells; actively driving cells of the somite toward non-muscle fates; or repressing myogenesis in precursor cells or emerging non-muscle derivatives. Results from this work showed that *in vitro*, twist has the capacity to act in a dual fashion, negatively regulating MRF bHLH DNA-binding activity while simultaneously promoting an alternative DNA-binding complex. Supporting observations from transfection experiments in cultured cells suggest that these properties help to explain the potent inhibitory effect of twist on muscle differentiation observed in these assays. Expression studies identified a substantial domain of overlap for myf-5 and twist in early somites, and this provided the first evidence that cell autonomous interactions between twist and MRF regulators may also occur during development. A working model for twist action in the somite integrates expression pattern, molecular mechanism, and functional effects from transfection studies. In this model, the dual inhibitory action of twist obligatorily links the suppression of one cell phenotype (differentiating skeletal muscle) with the expression of an alternative phenotype (somitic precursor or, later, sclerotomal or dermatomal cell type).

Murine twist was found to interact with all four known mammalian E-protein species to form heteromeric sequence-specific DNA-binding factors, most probably heterodimers. The fact that both MyoD family members and twist seem to favor heteromeric interactions with E-proteins suggests that competition for limiting E-proteins will occur if all three species (MyoD or myf-5, twist, and an E-protein) are co-expressed. Evidence supporting the possibility of such competition was obtained

from experiments in which twist inhibited DNA binding by MyoD:E-protein complexes in a dose-dependent manner, very similar to Id. A truncated twist protein that retained little other than the bHLH region was also capable of competition in vitro and myogenic inhibition in transfection assays. Interestingly, twist and Id share an otherwise uncommon primary sequence feature (PTLP) within the amino-terminal portion of the loop in their HLH domains. Prior site directed mutagenesis within the Id loop has shown that this motif is essential for competitive inhibition of MyoD by Id [30]. Moreover, SCL/tal1 is another bHLH protein with a similar loop feature (PTHP), and its ectopic expression in cultured myogenic cells can partially suppress muscle differentiation. It binds to E2-2 with high affinity, suggesting that it would likely inhibit MRF:E-protein DNA binding as twist does [31]. However, SCL/tal1 function and expression are normally associated only with hematopoetic lineages, and there is presently no evidence to suggest a physiological role in the myogenic lineage. It will now be interesting to test whether the PTLP loop residues identify a special subset of HLH and bHLH proteins that interact with E-proteins with especially high affinity relative to the affinity of E-proteins for MRFs or other bHLH partners.

DNA binding by twist complexes to a non-muscle class of synthetic E-box-containing oligonucleotides strongly suggests that in some biological settings twist homomers and/or twist:E-protein heteromers will form and act as DNA-binding regulatory factors. Although in vitro DNA binding by twist:E-protein complexes was robust over a range of pH and salt conditions initially surveyed (Materials and Methods), no physiological target gene of vertebrate twist has yet been identified. For this reason, it is not certain whether binding by twist-containing factors will enhance transcription (as is the case for the genes of *Drosophila* [10, 11, 32]), repress transcription, or perhaps do both depending on the identity of the oligomerization partner and/or the context of the binding site. We have tested for the ability of twist alone or twist plus

E12 to activate a reporter gene containing four multimerized twist E-boxes in the same sequence context as the in vitro DNA binding oligonucleotide (see Materials and Methods) and saw no significant effect (data not shown). This negative result is identical to that obtained from similar studies of twist activity in Drosophila. There, twist is clearly a significant regulator in the context of native target genes such *sna* and *rho* [11], but multimerizing the Drosophila twist binding site in front of a LacZ reporter construct failed to demonstrate activity [33]. By analogy, it seems quite possible that mammalian twist will also require factors that bind at nearby sites in native enhancers or silencers to affect transcription. These issues will ultimately be resolved with the identification and characterization of physiological targets of murine twist.

A simple view of the *in vitro* DNA site preferences reported here for twist:E dimers is that they argue for similar preferential interactions with non-canonical muscle-class E-boxes in vivo. Verification will require the identification of direct mammalian targets of twist. The synthetic twist-class E-box core sequence we used in this work was originally identified as the preferred binding site for recombinant Xenopus twist (H. Weintraub and K. Blackwell, personal communication), and it is therefore quite possible that other sequences might be even more strongly preferred by murine twist:E-protein or twist:twist complexes. Nevertheless, the site used here is clearly preferred by twist over muscle E-boxes, and it provides a useful starting point for scanning regulatory regions of candidate twist target genes.

Our expression studies focused on the early somite and showed that twist RNA is present broadly prior to subdivision into dermamyotome and sclerotome. This is consistent with a function for twist in precursors of both muscle and non-muscle somitic derivatives. In slightly more mature somites, where myf-5 expression is

established, the twist domain overlaps the myf-5 domain. The latter observation provided the rationale for considering molecular interactions within a bHLH/HLH network that contain both classes of regulators. Subsequently, as the somite matures, changes are observed in twist expression such that the sclerotome and dermatome clearly express relatively high levels of twist, while differentiated cells of the myotome express none [7-9]. The strong exclusion of twist expression from the myotome relative to sclerotome and dermatome is striking. It clearly suggests that should a cell in the sclerotome, for example, begin to inappropriately express MRFs, it would still probably not differentiate as muscle. However the existing expression data neither rule in nor rule out the possibility that in these growing, patterned somites, twist and MRFs might be transiently co-expressed in a specific transitory cellular compartment that feeds the expanding myotome. Such a compartment need not comprise a large spatiotemporal domain because relevant cells may pass rather quickly from twist-positive/MRF-negative status through a twist-positive/MRF-positive intermediate state to a final MRF-positive/twist-negative state. Because co-expression of twist and MRFs may define cells on the cusp of committing to the myogenic fate, a detailed examination of possible coexpression in the advanced somite will be of interest.

The quantitative shifts and domain distinctions in twist expression throughout development of the axial mesoderm are reminiscent of a similarly complex pattern seen in *Drosophila* development, where these shifts reflect context dependent functions for twist. At the molecular level, this is consistent with a regulator whose action is strongly dependent on interaction with non-twist class cofactors in target genes or on the potential to dimerize with multiple partners. We conclude that twist is a strong candidate bHLH regulator to participate in the regulation of early transitory steps of myogenesis; that its down-regulation is a likely pre-condition for

execution of muscle differentiation, and as discussed below, that it may also be relevant for controlling the phenotype of myf-5-expressing cells that have been observed at non-myotomal sites. For example, myf-5-expressing cells have previously been noted in the presegmental plate mesoderm [20, 34] and also in the sclerotomes of day 9 embryos [35], but neither is site appropriate for myogenesis. It is not yet known whether these myf-5 positive cells express twist, but they are positioned within domains of cells that do. Co-expression of twist and other negative regulators such as Id and I-mfa in those cells may explain the failure of those cells to differentiate into muscle.

Recently, a twist null mouse was generated, and its initial characterization reported [36]. Embryos homozygous for a mutation in the twist gene died by embryonic day 11.5, and the most profound effects were in the developing neural tube. However, the phenotype was pleiotropic, and somites were also significantly affected. In the somites, cell numbers were substantially reduced relative to wild-type and significant cell death was observed, suggesting trophic and/or cell proliferative effects for twist. Within the myotomal domain, the time of onset of myogenin expression appeared grossly normal, but the number of myogenin positive cells seemed reduced. This phenotype may be a milder version of the situation in *Drosophila* in which expression of the MyoD homologue called nautilus is absent in twist mutant embryos, a phenotype that has been attributed to failure to establish or maintain myogenic precursor cells [37] . Contrary to what one might expect if twist were a significant negative regulator of myogenesis, no ectopic or premature myogenesis is observed in twist mutant embryos. In addition to the trophic and/or proliferative effect twist might have on somitic cells as discussed above, another possible explanation of this observation lies in the view that negative regulation of myogenesis is a function that is distributed among multiple HLH and bHLH class regulators. Hence in these

embryos, other HLH and bHLH regulators that contribute to negative regulation of myogenesis would prevent improper myogenesis.

When twist was allowed to interact with MRF and E-proteins of the myogenic bHLH network, we found evidence that it could produce two effects that could be dissected experimentally; formation of an active DNA-binding complex with a non-muscle sequence preference and inactivation of DNA binding by MyoD due to competition for E-protein partners. This dual capacity is inherently context dependent, since it arises from interactions of twist with several other bHLH regulators in the system. The presence of multiple negative regulators of myogenesis such as Id and twist predicts that disruption of either one alone will not lead to wholesale premature or ectopic myogenesis. Rather, elimination in the somitic domain of several negative regulators including twist and Id would be required. The proposition that multiple negative HLH/bHLH regulators act in a single HLH/bHLH regulated developmental pathway has a precedent in the *Drosophila* eye, where it was recently shown that both *hairy* and *emc* are coexpressed anterior to the morphogenetic furrow. Elimination of either negative regulator individually has no phenotype in the eye, but elimination of both causes premature progress of the furrow and associated differentiation [38]. In the mouse somite, we do not yet know whether additional competitive or supporting contributions will come from other recently described bHLH molecules such as paraxis, scleraxis, dermo-1 and bHLH-EC2 [39-41], whose domains of expression partly overlap with that of twist.

Acknowledgments

We thank Drs. T. Kadesch and C. Murre for E2-2/ITF2 and E32.5 clones respectively; Drs. K. Blackwell and H. Weintraub for sharing unpublished data; Dr. G. Lyons for counsel on in situ hybridization techniques; and Drs. P.Sternberg, K.Zinn, P. Mueller and J. Miner, and members of Wold group for their critical comments on the manuscript. K.Y. was supported by an NIH predoctoral Trainee Grant. This work was supported by a grant to B.W. from the NIH (No. AR40708).

REFERENCES

Bain, G., Gruenwald, S. and Murre, C. (1993). E2A and E2-2 are subunits of B-cell specific E2-box DNA binding proteins. *Mol & Cell. Bio.* **13**, 3522-3529.

Bate, M. R., E.; and Currie, D.A. (1991). Cells with persistent twist expression are the embryonic precursors of adult muscles in Drosophila. *Development* **113**, 79-89.

Brown, N. L., Sattler, C., Paddock, S. and Carroll, S. B. (1995). Hairy and emc negatively regulate morphogenetic furrow progression in the Drosophila eye. *Cell* **80**, 879-887.

Burgess, R., Cserjesi, P., Ligon, K. and Olson, E. (1995). Paraxis: a basic helix-loop-helix protein expressed in paraxial mesoderm and developing somites. *Developmental Biology* **168**, 296-306.

Chen, Z. F. and Behringer, R. R. (1995). Twist is required in head mesenchyme for cranial neural tube morphogenesis. *Genes & Development* **9**, 686-699.

Cserjesi, P., Brown, D., Ligon, K., Lyons, G., Copeland, N., Gilbert, D., Jenkins, N. and Olson, E. (1995). Scleraxis: a basic helix-loop-helix protein that prefigures skeletal formation during mouse embryogenesis. *Development* **121**, 1099-1110.

Currie, D. B., M. (1991). The development of adult abdominal muscles in *Drosophila*: myoblasts express *twist* and are associated with nerves. *Development* **113**, 91- 102.

Ellis, l., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A. and Rutter, W. J. (1986). Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose.

Fan, C.-M. T.-L., Marc. (1994). Patterning of mammalian somites by surface ectoderm and notochord: evidence for sclerotome induction by a hedgehog homolog. *Cell* **79**, 1175-1186.

Goldfarb, A. and Lewandowska, K. (1995). Inhibition of cellular-differentiation by the SCL/TAL oncoprotein- transcriptional repression by an Id-like mechanism. *Blood* **85**, 465-471.

Hasty, P., Bradley, A., Morris, J. H., Edmondson, D. G., Venuti, J. M., Olson, E. N. and Klein, W. H. (1993). Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature* **364**, 501-506.

Hebrok, M. W., Karin; Fuchtbauer, Ernst-Martin. (1994). M-twist Is an Inhibitor of Muscle Differentiation. *Developmental Biology* **165**, 537-544.

Henthorn, P., Kiledjian, M. and Kadesch, T. (1990). Two distinct transcription factors that bind the immunoglobulin enhancer microE5/kappa 2 motif. *Science* **247**, 467-470.

Hu, J. S., Olson, E. N. and Kingston, R. E. (1992). HEB, a helix-loop-helix protein related to E2A and ITF2 that can modulate the DNA-binding ability of myogenic regulatory factors. *Mol. Cell. Bio.* **12**, 1031-1042.

Ip, Y., Park, R., Kosman, D., Yazdanbakhsh, K. and Levine, M. (1992). *dorsal-twist* interactions esgtablish *snail* expression in the presumptive mesoderm of the *Drosophila* embryo. *Genes & Development* **6**, 1518-1530.

Johnson, J. E., Wold, B. J. and Hauschka, S. D. (1989). Muscle creatine kinase sequence elements regulating skeletal and cardiac muscle expression in transgenic mice. *Mol. Cell. Biol.* **9**, 3393-3399.

Kopan, R. N., J.S.; Weintraub, H. (1994). The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. *Development* **120**, 2385-2396.

Lassar, A. B., Buskin, J. N., Lockshon, D., Davis, R. L., Apone, S., Hauschka, S. D. and Weintraub, H. (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.

Leptin, M. (1991). twist and snail as positive and negative regulators during Drosophila development. *Genes & Development* **5**, 1568-1576.

Leptin, M. R., S. (1994). Autonomy and non-autonomy in *Drosophila* mesoderm determination and morphogenesis. *Development* **120**, 853-859.

Lyons, G. E., Ontell, M., Cox, R., Sassoon, D. and Buckingham, M. (1990). The expression of myosin genes in developing skeletal muscle in the mouse embryo. *J. Cell Biol.* **111**, 1465-1476.

McGuire, P. G. and Alexander, S. M. (1992). Urokinase expression during the epithelial-mesenchymal transformation of the avian somite. *Developmental Dynamics* **194**, 193-197.

Michelson, A. M., Abmayr, S. M., Bate, M., Arias, A. M. and Maniatis, T. (1990). Expression of a MyoD family member prefigures muscle pattern in Drosophila embryos. *Genes & Development* **4**, 2086-2097.

Miner, J. H. and Wold, B. J. (1991). c-*myc* inhibition of MyoD and myogenin-initiated myogenic differentiation. *Mol. Cell. Biol.* **11**, 2842-2851.

Molkentin, J. D., Black, B. L., Martin, J. F. and Olson, E. N. (1995). Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. *Cell* **83**, 1125-1136.

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

Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Shaowei, L., Nonaka, I. and Nabeshima, Y.-I. (1993). Myogenin gene disruption results in perinatal lethality because of severe muscle defect. *Nature* **364**, 532-535.

Nusslein-Volhard, C. W., E.; Kluding, H. (1984). Mutations affecting the pattern of the larval cuticle in Drosophila melanogaster. Zygotic loci on the second chromosome. *Wilhelm Roux's Arch. Dev. Biol* **193**, 267-282.

Ott, M.-O., Bober, E., Lyons, G., Arnold, H. and Buckingham, M. (1991). Early expression of the myogenic regulatory gene, *myf-5*, in precursor cells of skeletal muscle in the mouse embryo. *Development* **111**, 1097-1107.

Pesce, S. and Benezra, R. (1993). The loop region of the helix-loop-helix protein Id1 is critical for its dominant negative activity. *Molecular and Cellular Biol.* **13**, 7874-7880.

Quertermous, E. H., H; Blanar, MA; Quertermous, T. (1994). Cloning and characterization of a basic helix-loop-helix protein expressed in early mesoderm and the developing somites. *Proc. Natl. Acad. Sci* **91**, 7066-7070.

Rudnicki, M. A., Schneglesberg, P. N. J., Stead, R. H., Braun, T., Arnold, H.-H. and Jaenisch, R. (1993). MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell* **75**, 1351-1359.

Sager, R. L., P. (1982). Preadipocyte determination either by insulin or by 5-azacytidine. *Proc. Natn. Acad. Sci* **79**, 480-484.

Simpson, P. (1983). Maternal-zygotic gene interactions during formation of the Dorsoventral pattern in Drosophila embryos. *Genetics* **105**, 615-632.

Smith, T. H., Kachinsky, A. M. and Miller, J. B. (1994). Somite subdomains, muscle cell origins, and the four muscle regulatory Proteins. *J. Cell Biol.* **127**, 95-105.

Szymanski, P. and Levine, M. (1995). Multiple modes of dorsal-bHLH transcriptional synergy in the Drosophila embryo. *EMBO* **14**, 2229-2238.

Taylor, M. V., Beatty, K. E., Hunter, H. K. and Baylies, M. K. (1995). Drosophila MEF2 is regulated by twist and is expressed in both the primordia and differentiated cells of the embryonic somatic visceral and heart musculature. *Mechanisms of Development* **50**, 29-41.

Taylor, S. M. and Jones, P. A. (1979). Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. *Cell* **17**, 771-779.

Thisse, B. S., C; Gorostiza-Thisse, C; Perrin-Schmitt, F. (1988). Sequence of the *twist* gene and nuclear localization of its protein in endomesodermal cells of early Drosophila embryos. *EMBO J* **7**, 2175-2183.

Weintraub, H. (1993). The MyoD family and myogenesis: redundancy, networks, and thresholds. *Cell* **75**, 1241-1244.

Wolf, C. T., C; Stoetzel, C; Thisse, B; Gerlinger, P; Perrin-Schmitt, F. (1991). The m-twist gene of Mus is expressed in subsets of mesodermal cells and is closely related to the Xenopus X-twi and the Drosophila twist gene. *Dev Biol* **143**, 363-373.

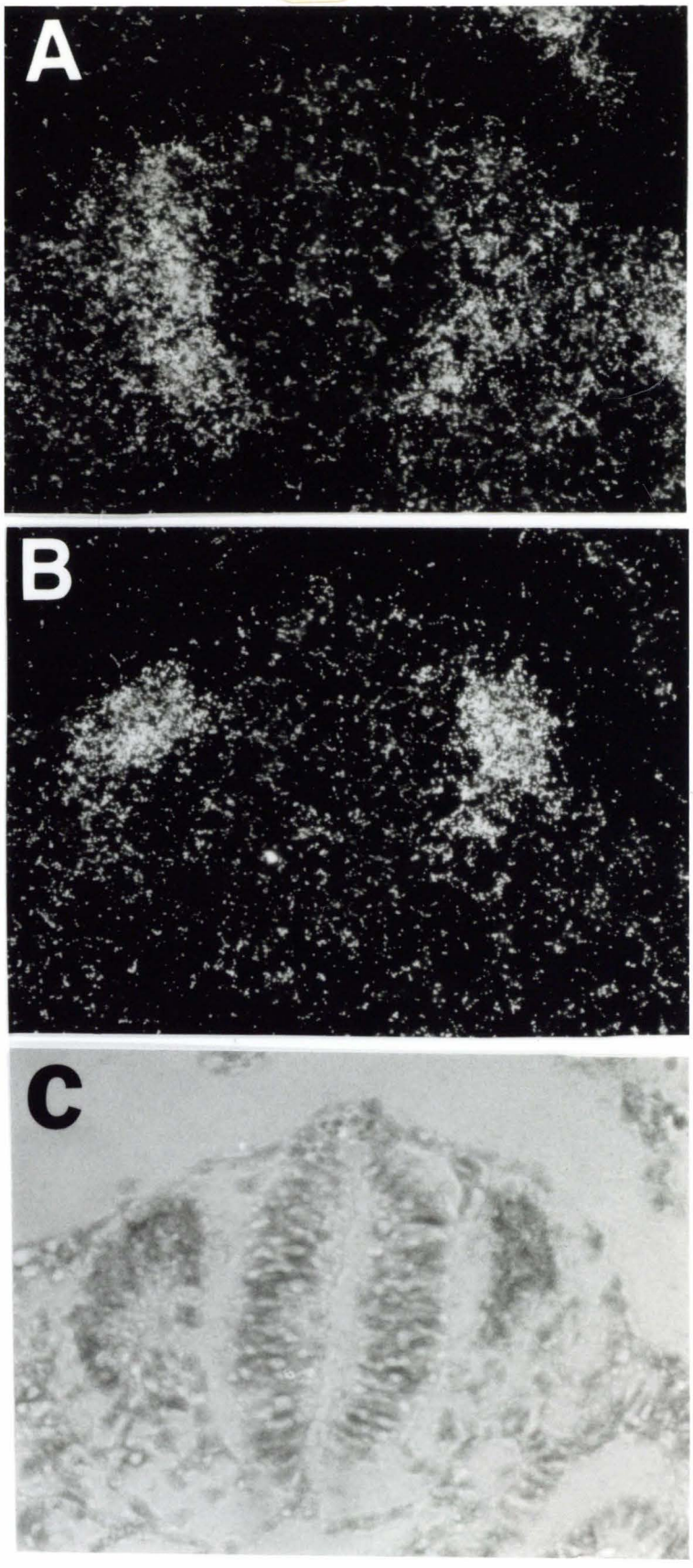

A
B
C

Figure 1. Expression of twist in early mouse somite. Transverse sections of 8.5d embryos probed with S^{35}-labeled twist (A) and myf-5 (B) probes on parallel sections. An epithelial stage somite from a 8.5d embryo probed for twist shows an even twist expression in the somite. Adjacent section probed with myf-5 shows strong myf-5 expression in the dorsal domain of the somites, a sub-domain of twist expression. (C) phase-contrast photo image of same section shown in (B).

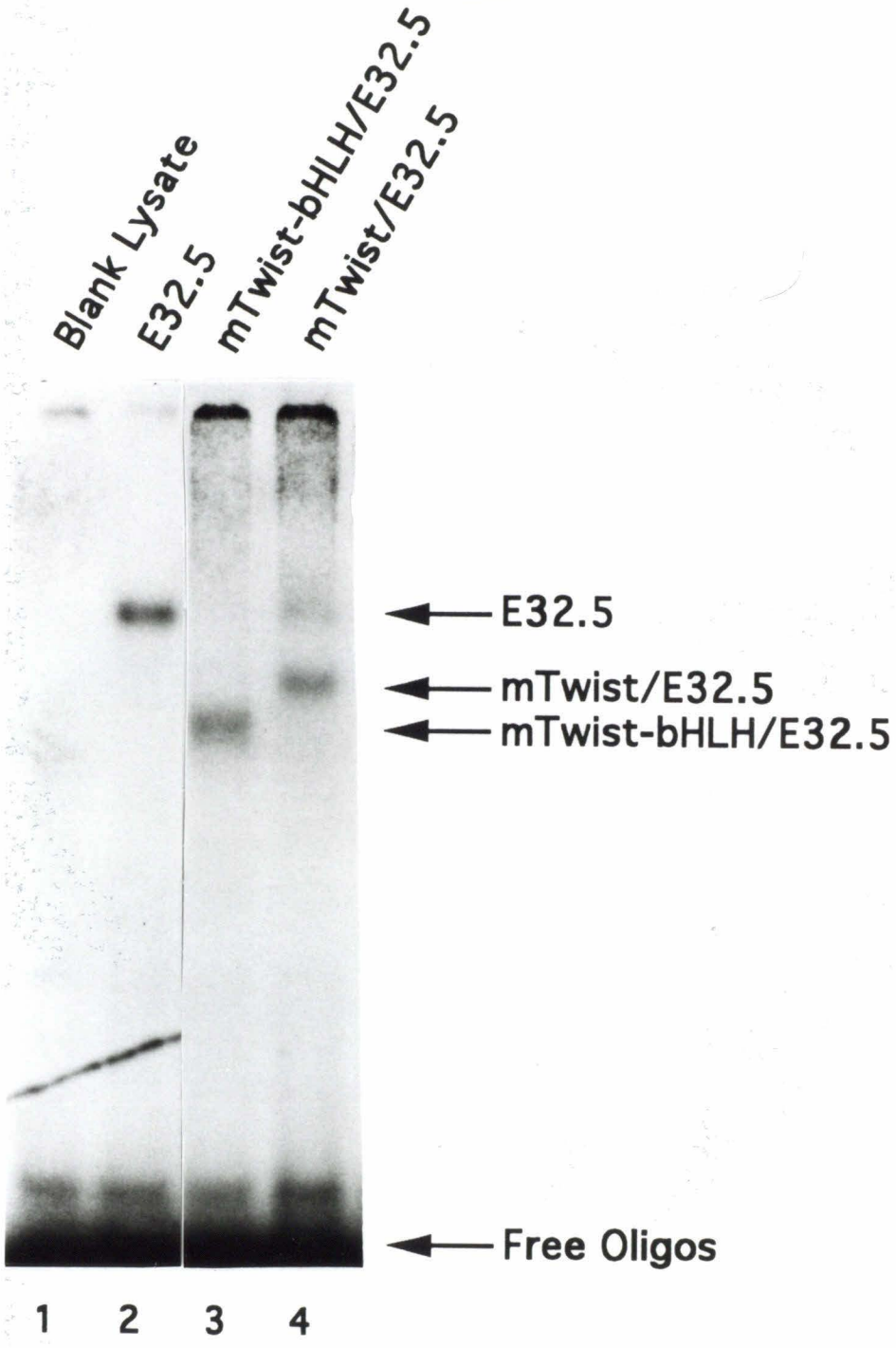

Blank Lysate
E32.5
mTwist-bHLH/E32.5
mTwist/E32.5
E32.5
mTwist/E32.5
mTwist-bHLH/E32.5
Free Oligos
1
2
3
4

Figure 2A. In vitro DNA binding assays, full-length twist and twist-bHLH are sequence-specific DNA-binding proteins. E32.5 is a truncated cDNA containing the bHLH region of the E2-2 gene, a member of the E-protein family. Twist is capable of interacting with various members of the E-protein family, including E12, E47, HEB, and E2-2/ITF2/E32.5, and binds DNA. The faster migrating species in this experiment is the heteromer between truncated twist and E32.5, while the slower migrating species represents the full-length twist and E32.5 heteromer.

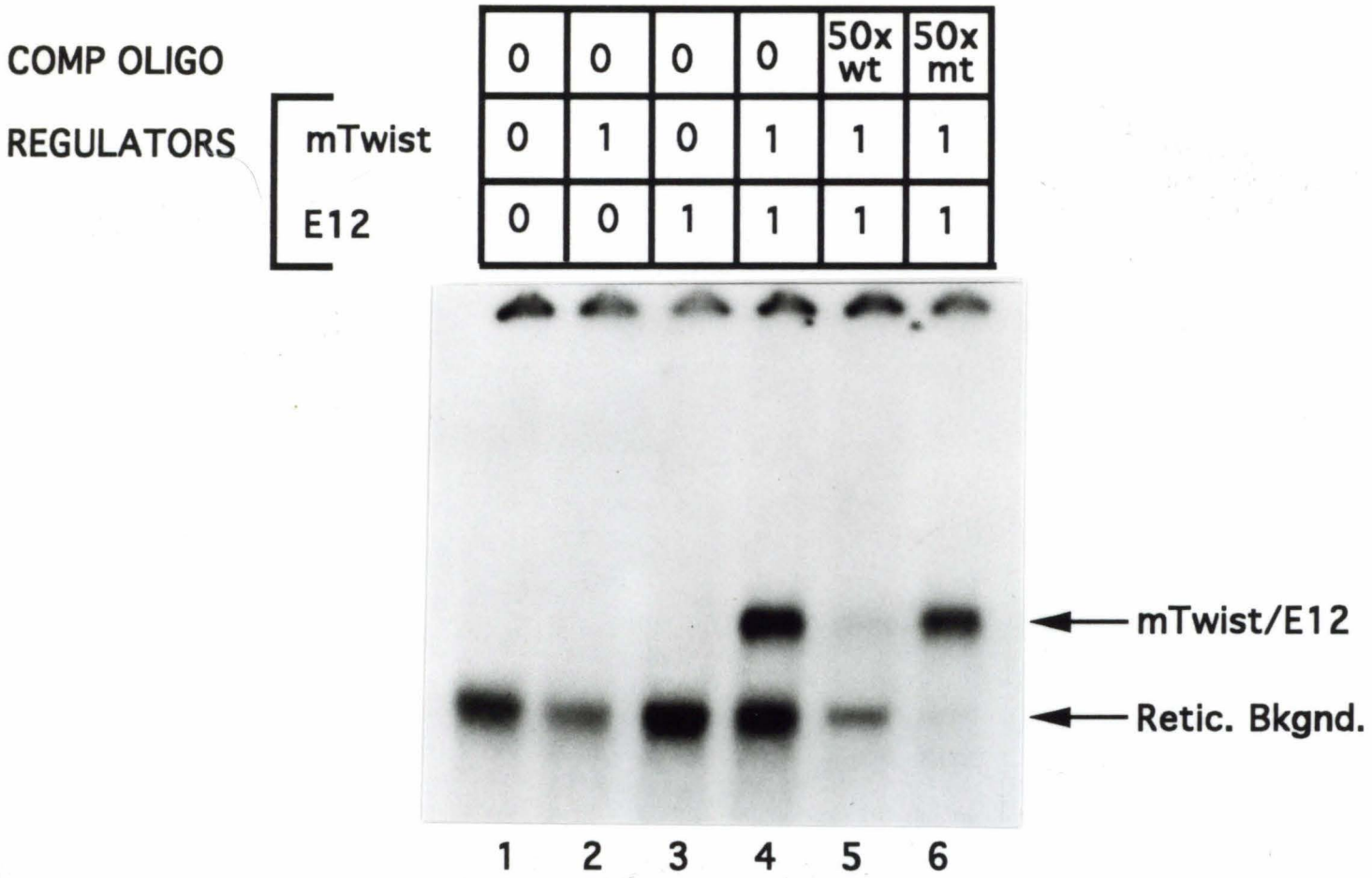

COMP OLIGO
REGULATORS
mTwist
E12
0 0 0 0 50x wt 50x mt
0 1 0 1 1 1
0 0 1 1 1 1
mTwist/E12
Retic. Bkgnd.
1 2 3 4 5 6

Figure 2B. Twist:E12 forms a sequence-specific DNA-binding complex that prefers a CATATG E-box binding site in vitro DNA binding competition assays. In the presence of 50X molar amounts of unlabeled competing sequence (TWI E1/2), the signal from the protein-bound target sequence (^{32}P-labeled) is diminished. However, in the presence of excess amounts of a mutant oligonucleotide (MCK34), the signal remains intact.

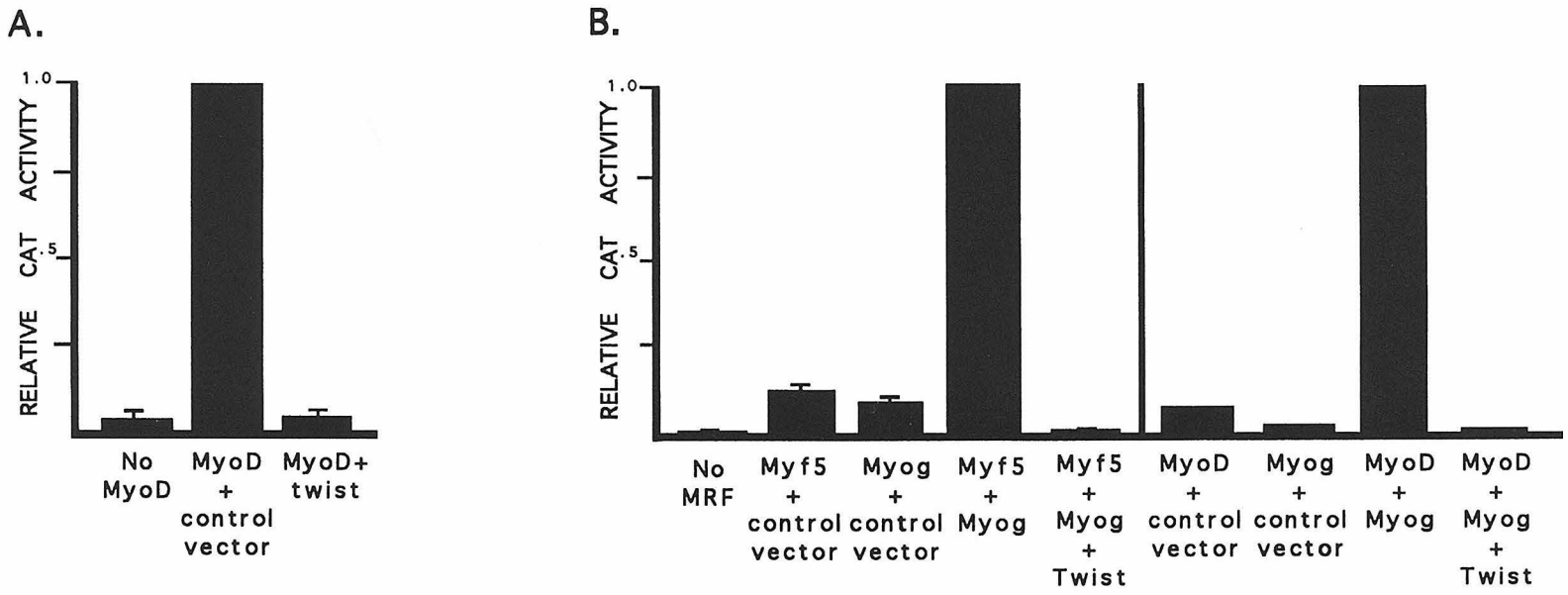

A.
RELATIVE CAT ACTIVITY
1.0
.5
No MyoD
MyoD + control vector
MyoD+ twist
B.
RELATIVE CAT ACTIVITY
1.0
.5
No MRF
Myf5 + control vector
Myog + control vector
Myf5 + Myog
Myf5 + Myog + Twist
MyoD + control vector
Myog + control vector
MyoD + Myog
MyoD + Myog + Twist

Figure 3A. Twist inhibits MyoD-initiated muscle differentiation in 10T1/2 cells. Equimolar amounts of expression plasmids containing the coding regions of MyoD or MyoD and twist were transfected into C3H10T1/2 cells. Following transfer to low serum, differentiation-promoting medium, cells were harvested and assayed for activity from the MCK-CAT reporter. CAT activity was quantified using a Phosphorimager. When MyoD is co-transfected with the reporter, the CAT activity level rises, but when twist is co-transfected with MyoD and the reporter, the CAT activity drops to the background level. CAT activity levels from different experiments were averaged following normalization to MyoD (n= 6).

Figure 3B. Combinations of MRF's were transfected into 10T1/2 cells, similar to panel A. Even in the presence of myogenin, in addition to MyoD or myf-5, twist represses the myogenic reporter. CAT activity levels in the two experiments were normalized to Myf-5+myogenin and MyoD+myogenin, respectively.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
E12	0	1	0	0	1	0	1	1	1	1	1	0	1	0	1	1	1	1	1
MyoD	0	0	1	0	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1
Id	0	0	0	1	1	1	0	1	2	5	10	0	0	0	0	0	0	0	0
Twist	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	2	5	10

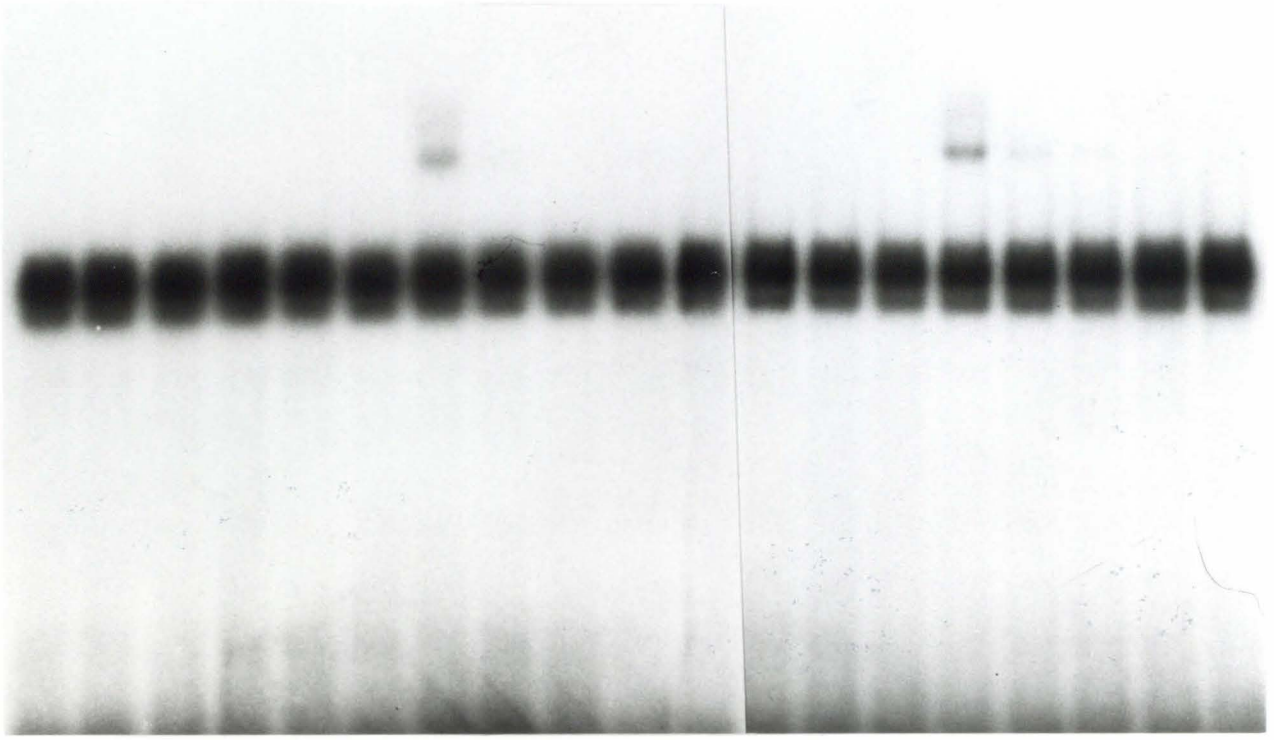


Figure 4. Side-by-side comparison between Id and twist in competition for available E12 in a DNA binding assay. In vitro translation products of programmed rabbit reticulocyte lysates were normalized to each other by the number of ^{35}S-labeled methionines expected in each translation product. In an in vitro DNA binding assay using the MCK E-box sequence, twist and Id inhibits the DNA-binding activity of the MyoD:E12 complex in a dose-sensitive manner.

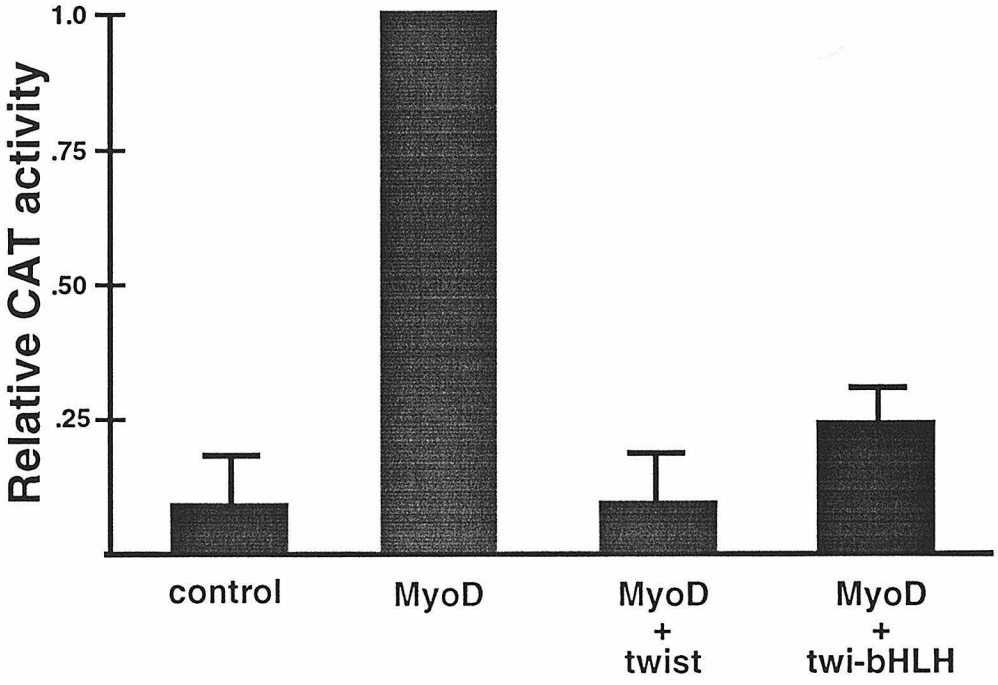
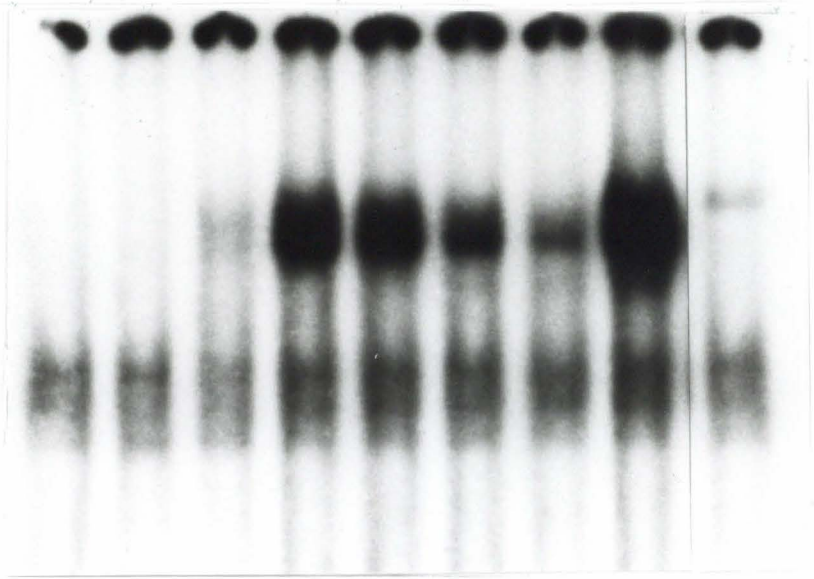

Relative CAT activity
1.0
.75
.50
.25
control
MyoD
MyoD + twist
MyoD + twi-bHLH

Figure 5. The twist bHLH region is sufficient to repress MyoD activity. Equimolar amounts of expression plasmid containing MyoD and twist, full-length or truncated, were co-transfected with the MCK-CAT reporter construct. The experimental paradigm is identical to that for Fig. 4. The truncated twist molecule is similar to full-length twist in its ability to inhibit MyoD from activating its downstream reporter. CAT activity levels are normalized to that of MyoD (n=3).

+ E12	0	0	0	0	0	0	0	4	4
mTwist	0	0	0	0	1	4	8	4	0
E12	0	1	0	1	1	1	1	1	1
MyoD	0	0	1	1	1	1	1	1	0



E12
E12/MyoD
Retic. Bkgnd.

1 2 3 4 5 6 7 8 9

Figure 6. Twist inhibits MyoD:E12 DNA-binding activity in vitro. Using the molar ratio amounts of regulators indicated above the lanes, we tested whether twist can interact with MyoD:E12 at the protein level. MyoD and E12 bind efficiently to the labeled target sequence, the MCK oligo. With increasing molar amounts of twist added, there is a dose-sensitive diminution of the MyoD:E12 DNA-binding complex. This complex is restored when additional amount of E12 is added after initial competition with twist has taken place (compare lane 8 with lane 6).

Chapter 3

Twist inhibits myogenesis in the absence of bHLH competition through a mechanism that requires the N-terminus

Contents of this chapter and those of chapter 2

are submitted together for publication

SUMMARY

Data presented in chapter 2 show that twist and MRF may directly or indirectly interact with each other in physiologically relevant settings during mouse embryogenesis. In accordance with the expectation from its expression pattern, twist is an inhibitor of myogenic differentiation. In addition to bHLH competition for available E-proteins, twist forms active DNA binding complexes with E-proteins that prefer non-muscle class E-box site. From these observations, we suspect that twist regulates myogenesis as a direct transcription factor. In this chapter, we further dissect the mechanisms of twist inhibition by using both wildtype MyoD and a tethered dimer between MyoD and E47 (MyoD~E47), which forms intramolecular dimer that is insensitive to bHLH competition. First, we show that twist prevents onset of myogenin expression in MyoD transfected cells. This marks an early key step in the myogenic program targeted by twist suppression. Second, we show that although DNA binding ability of MyoD~E47 stays intact in the presence of twist, co-expression of twist prevents MyoD~E47 from activating muscle differentiation reporter. Interestingly, a truncated form of twist missing the N-terminus cannot inhibit the tethered dimer, although it contains the domains necessary for MEF2 interaction (Spicer *et al.*, 1996) MEF2 has been shown to interact with both MRF:E and twist:E complexes (Molkentin *et al.*, 1995; Spicer *et al.*, 1996). Twist inhibition of MyoD and MyoD~E47 cannot be abrogated by addition of exogenous MEF2. From these observations we suggest the existence of another mechanism of twist inhibition and postulate that this mechanism involves regulation of downstream target gene expression by twist and/or twist interaction with other co-factors, in addition to MEF2, in MRF transfected cells.

INTRODUCTION

Twist is a member of the basic helix-loop-helix family of transcription factors (Thisse, 1988; Wolf, 1991). This large family of molecules is largely divided into three subgroups depending on the expression pattern and molecular characteristics of each molecule. The first subgroup (group I) of factors includes the widely expressed E-proteins that form efficient homo- and heterodimers. The group I factors can form partners with the "tissue-specific" factors (group II) that include the myogenic regulatory factors (MRF) and twist, among many others. These regulators do not form efficient homodimers and seem to rely mostly on the E-proteins for active heterodimer formation. The third group consists of dominant negative regulators that lack the basic region and hence act as dominant negative "sponges" that compete for available E-proteins, represented by Id 1,2,3, and 4 and emc among others. (For details and references, see chapter 1)

Inherent in the design of these molecules are several means of modulating their activities post-translationally. First, because these molecules can physically interact with each other at the protein:protein level, the activity of a given molecule depends on the relative concentration and availability of other bHLH regulators that are present. The dominant negative regulators of the third group demonstrate this point most clearly. Id inhibits MyoD initiated myogenesis by efficiently sequestering the available E-proteins into inactive complex (Benezra *et al.*, 1990). Neuhold and Wold showed that this inhibition of a bHLH factor can be bypassed by tethering its E-protein partner through a flexible linker (Neuhold and Wold, 1993). Second, because the DNA binding specificity is determined by both basic regions, a given molecule can bind to different target sites depending on the partner choice (Murre, 1989; Davis *et al.*, 1990; Blackwell *et al.*, 1990). A good example of this is shown by the E-proteins. E12/E47 can form homodimers and bind to immunoglobulin enhancer elements in the

hematopoetic cells, and they can also form heteromers with MRFs to bind to the muscle class E-box sites in the MCK enhancer (Henthorn *et al.*, 1990; Murre *et al.*, 1989; Lassar *et al.*, 1991). Third, at least some of the bHLH regulators are subject to phosphorylational control. Olson and colleagues have suggested that PKC (protein kinase C) can inactive myogenin by phorphorylating a key residue (Thr 115) essential for DNA binding (Li *et al.*, 1992). However, not all MRF's are under the same kind of regulation since MRF4 is active in the presence of PKC (Hardy et al, 92). And lastly, these molecules can share common cofactors (for example, MEF2), and therefore competition for available cofactors is another means of modulating their activities.

The results in Chapter 2 showed that twist inhibits myogenesis initiated by MyoD through at least two of the mechanisms discussed above. In addition, Spicer and his colleagues recently published their work that shows that twist can also compete for available MEF2 (myogenic co-factor) (Spicer *et al.*, 1996). MEF2 is a MADS box transcription factor that enhances MRF activity by directly associating with MRF:E complex. (See chapter 1 for more detail) Apparently, twist:E complex can also bind MEF2 and this complex formation is much more efficient when twist forms heteromers with E-protein (Spicer *et al.*, 1996). In this chapter, we explore the relative importance of twist:E:MEF2 interaction in overall twist activity by challenging twist with a tethered dimer between MyoD and E47 (MyoD~E47).

MyoD~E47 forms intramolecular dimer through a flexible linker region and hence is insensitive to local concentrations of other bHLH molecules (Neuhold and Wold, 1993). It has been shown to be resistant to Id inhibition and much more active, especially in myoblasts, than wildtype MyoD (Neuhold and Wold, 1993). Using this molecule and truncated twist as molecular tools, we explore the details of twist

inhibitory mechanism and in the process, uncover a new mechanism that probably involves transcriptional regulation by twist.

MATERIALS AND METHODS

RNAse protection

The RNA samples from C3H10T1/2 cells transfected with bHLH regulators (as described in chapter 2) were collected in growth and differentiation conditions and assayed for expression of myogenin. 6 μg of total RNA was used per lane with ^{32}P-labeled myogenin and GAP-DH probes simultaneously. RNAse protection was done as previously described (Miner and Wold, 1991).

RT-PCR

RNA samples from transfected cells were harvested in growth and differentiation conditions, as described in chapter 2. 100 ng of total RNA was used per reaction for exogenous twist and 1 ng of total RNA was used for GAP-DH. See Patapoutian et al. 1995 for details of the reaction conditions, including the primer sequences used to amplify twist and GAP-DH (Patapoutian *et al.*, 1995). Oligonucleotide primers used for amplifying MEF2C transcripts are described in (Martin *et al.*, 1993).

Plasmids

MyoD~E47 plasmid was designed and characterized in our laboratory and described in detail in Neuhold and Wold (1993). See chapter 2 Materials and Methods section for other plasmids. MEF2C expression plasmid is from Dr. Eric Olson's laboratory and it contains CMV promoter fused to MEF2C coding region (see Molkentin et al. 1995).

Transfection and CAT assays

See chapter 2 for details.

RESULTS

Twist blocks activation of myogenin.

Thus far, the analysis of twist inhibition has focused on the endpoint of muscle differentiation, scored by the expression of terminal differentiation markers such as MCK (muscle creatine kinase) or MHC (myosin heavy chain). An earlier and apparently key step for the expression of terminal differentiation genes is the high level expression of myogenin, which has been shown to play an essential role in myocyte differentiation in vivo (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). In cell culture, all established myogenic lines examined to date express at least one MRF family member in the proliferating, non-differentiated myoblast state (usually MyoD or myf-5). Upon differentiation, these lines express at least two MRFs, with myogenin most often being induced prior to full myocyte differentiation. Similarly, MyoD-transfected 10T1/2 cells induce expression of endogenous myogenin after exogenous MyoD is expressed. From 10T1/2 cells transiently transfected with MyoD, the status of endogenous myogenin in the presence and absence of co-transfected twist was determined by an RNAse protection assay. In the presence of transfected twist, exogenous MyoD failed to activate endogenous myogenin (Fig. 1). This defines an early position in the myogenic differentiation hierarchy at which twist can inhibit muscle differentiation.

Twist prevents the activation of myogenin by MyoD, indicating an early point in myogenic differentiation that twist affects. When we asked whether the negative effects of twist on muscle differentiation could be bypassed by providing exogenous myogenin (see chapter 2 figure 3B) either alone or in combination with myf-5 or MyoD, twist still inhibited MCK-CAT activity. In those experiments, the combination of either MyoD or myf-5 with myogenin gave synergistic increases in MCK-CAT reporter activity, but even these high levels of myogenic report were completely suppressed by added twist.

From these results, we conclude that the presence of twist very efficiently suppresses the combined myogenic activity of all MRF's, and that repression of myogenin expression is not the only restriction point in MyoD plus twist transfected cells.

MyoD~E47 binds DNA in the presence of twist

Previous studies have shown that this tethered heterodimer, which consists of MyoD and E47 monomers joined via a flexible glycine rich linker peptide, apparently folds efficiently into functional DNA binding dimer that is highly resistant to inhibition by Id monomers in bandshift assays (Neuhold and Wold, 1993). A similar result to that found previously for Id was obtained when the tethered dimer was co-incubated with twist (Fig. 2A). Thus twist cannot override the tether in vitro DNA binding assays. Moreover, twist does not inhibit MCK E-box oligonucleotide binding by the tethered dimer due to any other interaction, such as formation of a higher order, non-DNA binding complex. These results, taken together with the earlier E-protein add-back results, argue that the predominant basis for inhibition of MyoD:E protein binding to their muscle class E-box target in vitro is the interaction of twist with E-proteins. If there are interactions between MyoD and twist, they appear to be comparatively minor.

The MyoD~E47 forced dimer is inhibited by full length twist.

Although the bHLH domain of twist is sufficient to inhibit myogenesis, the interaction of twist:E protein heteromers with an alternative class of non-muscle E-box suggests a second possible mechanism of action beyond E protein titration. We therefore asked whether twist could inhibit myogenesis driven by the tethered MyoD~E47 heterodimer. If HLH competition were the sole mechanism of twist inhibition, then we would expect that twist would be unable to inhibit the action of the tethered dimer in cells, just as it was unable to inhibit DNA binding by the tethered dimer in vitro. Conversely, if twist also acts by activating or repressing its own set of target genes, which then inhibit

muscle differentiation, we would expect twist to inhibit the tethered dimer in vivo. As shown in figure 2, twist proved to be a robust inhibitor of MyoD~E47. This result contrasts with a similar experiment performed with Id. In that case, the tethered dimer is relatively insensitive to elevated Id levels (Neuhold and Wold, 1993). The truncated twist molecule missing the amino-terminal half is incapable of this inhibition in the same assay (Fig. 3). These observations strengthen the idea that in addition to inhibition through bHLH domain interactions, full length twist can also suppress myogenesis through another mechanism that requires the N-terminus.

Twist abolishes MEF2 enhancement

MEF2 is a MADS-box family transcription factor that synergizes with MRF's to enhance the myogenesis (Kaushal *et al.*, 1994; Olson *et al.*, 1995; Molkentin *et al.*, 1995)[7, 8]. Although MEF2 alone cannot initiate myogenesis, its DNA binding site is present in many muscle specific promoters. Olson and his colleagues have shown that MEF2 can directly associate with MRF:E factors to form a higher order complex, and the transactivational activity of this complex is 5-10 folds higher than that of MyoD:E complex (Kaushal *et al.*, 1994; Molkentin *et al.*, 1995). Recently, Spicer et al. have suggested that MEF2:MRF interaction is the major target of twist inhibition. They show physical association between MEF2 and twist:E complex. This complex formation is E-protein concentration sensitive and likely to form only on twist:E heterodimer. They propose that titration of available MEF2 is the main mechanism behind twist inhibition of myogenesis (Spicer *et al.*, 1996).

When twist was co-transfected with MEF2C and MyoD, the muscle reporter activity again fell to basal level (Fig. 4A). This suggested that MEF2 was not the limiting factors that could be supplemented to overcome twist inhibition. We then tested whether ectopic expression of both myogenin and MEF2 was sufficient to overcome

twist inhibition by co-transfecting twist. Under this condition, where the myogenic regulatory molecules should be present in abundance, twist still suppressed the expression of the muscle reporter, MCK-CAT that contains both MEF2 binding site and muscle class E-box (Fig. 4B). Recently, Spicer and his colleagues have shown that twist can directly interact with MEF2 factors and therefore can act as a competitor for MRF:E:MEF2 complex formation. Even so, in the experiment where both myogenin and MyoD are co-transfected with MEF2 and twist, we would expect some MRF:E:MEF2 complexes to form and be active since both myogenin and MyoD can interact with MEF2 (Molkentin *et al.*, 1995). However, we observe a complete shut-down of the MCK-CAT reporter expression in the presence of twist. Without measuring the actual protein levels from the transfected cells and the relative affinities of twist and MRF for MEF2, it cannot be proven that not all MEF2 are locked up in complex with twist; however, it is reasonable to assume that twist and MRF affinities for MEF2 are within the same order of magnitude since the interaction domains lie within the conserved region. These observations suggest that although competition for available MEF2 is a viable mechanism for twist function, it is not likely to be the major one, as tested further below.

Just as we have shown that twist competes for available E-proteins by demonstrating that addition of excess E-proteins relieved twist inhibition in DNA binding assays, we asked whether addition of increasing amounts of MEF2 can rescue myogenesis. Preliminary results indicate that increasing amounts of MEF2 expression vector cannot rescue the activity of the myogenic reporter from twist inhibition (data not shown). If the main mode of twist function is titration of MEF2 away from MRF, we would expect that large excess of MEF2 would enable both twi:E:MEF2 and MRF:E:MEF complex formation. However, this is not the case, and this observation is consistent with the fact that the truncated twist containing the necessary domains of interaction

with MEF2 (HLH and small carboxy terminal end) is not able to inhibit MyoD~E47 tethered dimer.

Our preliminary data (described below) indicate that the relative importance of MEF2:MyoD interaction in overall myogenesis is highly context dependent. When MyoD~E47 tethered dimer is transfected with MEF2, there is little enhancement of the myogenic reporter MCK-CAT (data not shown). This may be due to the saturated reporter activity in the experimental paradigm used, since MyoD~E47 is about an order of magnitude more efficient at activating MCK-CAT reporter than MyoD ((Neuhold and Wold, 1993), this work). However, when 4RCAT reporter, containing four copies of the MCK E1 enhancer E-box in tandem, is used with either MyoD or MyoD~E47, again there is no significant enhancement of the reporter activity provided by added MEF2 (data not shown). This result suggests that the reported MyoD:MEF2 interaction on 4RCAT reporter (Molkentin *et al.*, 1995) may be significant in the context of the minimal bHLH region of MyoD that was used in the original study by Molkentin et al. (1995), but not when full length MyoD of MyoD~E47 is used. When twist is co-transfected in these settings where MEF2 does not seem to play a role, twist still inhibits the MRF activity (data not shown). This observation indicates that although MEF2 competition by twist is a viable mechanism, there has to be another mechanism since twist inhibits MEF2-independent activation of a myogenic reporter. This may very well be twist:E:MEF2 binding to an unknown target site and regulating the expression of downstream genes. Results in this chapter suggest that twist can inhibit myogenic factors in the absence of E-protein and MEF2 competitions and that twist inhibition cannot be abrogated even when early differentiation regulators myogenin and MEF2 are ectopically expressed.

DISCUSSION

Twist binds DNA in a sequence specific manner, and it seems likely that regulation of one or more downstream target genes will be a mechanism of twist action. Support for this idea comes from several observations. Full length twist can inhibit the tethered dimer MyoD~E47 while the truncated molecule including the bHLH domain cannot (Fig 2 &3). Furthermore, Spicer et al. showed that a mutant form of twist lacking the basic region (Twi-ΔB, Spicer et al., 1996) fails to inhibit either the MCK reporter or the MEF2 reporter, suggesting that DNA-binding domain is essential for twist function. They further showed that the basic region is not necessary for interaction with MEF2, further supporting the idea that MEF2 titration is not the main mechanism. Since the truncated twist containing the bHLH region and the small carboxy-terminal region, the domains necessary for MEF2 interaction, is not capable of inhibiting the tethered dimer when the full length twist can, we suggest that MEF2 competition and bHLH competition only explain a part of full twist inhibition. Although none of these experiments directly prove that twist regulates downstream gene expression, together they strongly suggest that twist may be involved in transcriptional regulation of unknown target genes.

We show that one of the key events in early myogenesis, onset of myogenin expression, is blocked in twist expressing cells (Fig. 1). However, this does not seem to be the only restriction point since co-transfection of MyoD, myogenin and twist cannot rescue the cells from twist inhibition (Chapter 2, figure 3B). A recent report by Olson's group shows that MEF2 family of transcription factors may play an important role in muscle differentiation. They showed that MEF2 interacts with MRF's at the protein level and cooperatively activates downstream gene transcription (Molkentin *et al.*, 1995). In supporting the physiological relevance of these observations, there are

many muscle specific terminal differentiation genes whose regulatory regions contain E-boxes adjacent to MEF2 binding sites, including the MCK promoter, and some without the muscle class E-boxes. Lassar and colleagues recently proposed that the major mode of twist inhibition of MyoD is the formation of twist:E:MEF2 complex, and that twist competition for MEF2 is efficient only when twist forms heterodimeric complexes with E-proteins. When twist is co-transfected with MyoD or MyoD~E47 and MEF2C, with or without the added E-protein, MCK-CAT reporter level drops to background ((Spicer *et al.*, 1996), Fig. 4 and 5). Providing MyoD, myogenin and MEF2, therefore forcing all known early myogenic regulators, is still insufficient to bypass twist block (Fig. 4B). We have shown that MyoD~E47 forms intramolecular dimers that bind DNA in the presence of twist (chapter 2), and it is therefore reasonable to assume that MyoD~E47:MEF2 complex formation would occur normally in vivo in the presence of twist. However, contrary to a simple expectation from the protein:protein competition model proposed by Lassar's group, twist can still inhibit the tethered dimer and MEF2C. This observation and the fact that N-terminus of twist is required for full inhibitory effect of twist (Fig. 3), suggest that protein:protein competition tells only a part of the story and that downstream target regulation may play a more important role.

Although the molecular homology extends for MEF2 and MyoD family members from Drosophila to mouse, it is obvious that the regulatory circuitry is different. In Drosophila, twist directly regulates the expression of MEF2, the first of the myogenic regulatory molecules expressed in that system (Lilly *et al.*, 1995; Taylor *et al.*, 1995). In Drosophila, high level twist expression is required to determine the myogenic lineage and to activate DMEF2 and is suggested to play a myogenic determination function (Taylor *et al.*, 1995). Interestingly, Drosophila twist, which has very divergent amino- and carboxy-terminal regions from murine twist, can substitute in MEF2 competition

experiments. We have proposed that amino-terminus of twist contains a functional domain that is important for full inhibitory effect of twist and Spicer et al. have shown that carboxy-terminus of twist is necessary for interaction with MEF2. It is therefore intriguing that Drosophila twist could substitute for murine twist in MEF2 interaction study. It will be interesting to test whether Drosophila twist can inhibit MyoD~E47 tethered dimer in the presence or absence of MEF2. Both Drosophila and murine twist failed to show transactivational activity from artificial reporter constructs containing multimerized twist binding sites fused to tk-CAT reporter (Szymanski and Levine, 1995). It is possible that murine twist mainly functions as a repressor in most settings especially since another murine bHLH regulator, dermo-1 which shares homology with twist in the G-rich regions N-terminus to the bHLH domain as well as the highly conserved C-terminus domain immediately following the bHLH domain, also represses myogenesis and also does not show transactivational activity (Li *et al.*, 1995).

DNA binding by twist complexes to a non-muscle class of synthetic E-box-containing oligonucleotides strongly suggests that in some biological settings twist homomers and/or twist:E-protein heteromers will form and act as DNA-binding regulatory factors. This idea is further supported by the MyoD~E47 tethered dimer experiments using the truncated twist. The truncated twist, containing both the bHLH domain and the small carboxy-terminal region (regions involved in MEF2 interaction, Spicer et al.), fails to inhibit MyoD~E47 even though it should be able to interact with MEF2. Furthermore, the difference between the truncated twist and the full length twist in their abilities to suppress MyoD~E47 points to the N-terminus of twist containing an important functional domain. It is unclear whether this domain contains a transcriptional regulatory domain; however, it is obvious that it alone cannot function since twist-Δbasic cannot inhibit MyoD plus E-protein (Spicer *et al.*, 1996). Whether this domain contains the activator or repressor domain or whether it is necessary for proper folding

of twist for its optimal activity is not known. Hence, we suggest that in addition to protein:protein competitions observed in chapter 2 (bHLH competition) and by Spicer et al. (MEF2 competition), twist is likely to regulate downstream gene expressions that are incompatible with myogenic differentiation.

One obvious candidate set of target genes are the four Id family members; their upregulation by twist would be consistent with overlap in their expression domains in the embryo (Wang *et al.*, 1992; Evans and O'Brien, 1993) and their expression would help to suppress muscle differentiation. In 10T1/2 cell lines stably transfected with twist, we found there was no induction of any of the four known Id family regulators at the RNA level as monitored by a sensitive RT-PCR assay (data not shown). Id genes are not, therefore, likely to be major targets of twist and their upregulation cannot explain the twist mediated blockade to muscle differentiation.

A simple view of the *in vitro* DNA site preferences reported here for twist:E dimers is that they argue for similar preferential interactions with non-canonical muscle-class E-boxes in vivo. Verification will require the identification of direct mammalian targets of twist. The synthetic twist-class E-box core sequence we used in this work was originally identified as the preferred binding site for recombinant Xenopus twist (H. Weintraub and K. Blackwell, personal communication), and it is therefore quite possible that other sequences might be even more strongly preferred by murine twist:E-protein or twist:twist complexes. Nevertheless, the site used here is strongly preferred by twist over muscle E-boxes, and it provides a useful starting point for scanning regulatory regions of candidate twist target genes.

REFERENCES

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

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

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

Evans, S. and O'Brien, T. (1993). Expression of the helix-loop-helix factor Id during mouse embryonic development. *Dev. Biol.* **159**, 485-499.

Hasty, P., Bradley, A., Morris, J. H., Edmondson, D. G., Venuti, J. M., Olson, E. N. and Klein, W. H. (1993). Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature* **364**, 501-506.

Henthorn, P., Kiledjian, M. and Kadesch, T. (1990). Two distinct transcription factors that bind the immunoglobulin enhancer microE5/kappa 2 motif. *Science* **247**, 467-470.

Kaushal, S., Schneider, J. W., Nadal-Ginard, B. and Mahdavi, V. (1994). Activation of the myogenic lineage by MEF2A, a factor that induces and cooperates with MyoD. *Science* **266**, 1236-1240.

Lassar, A. B., Davis, R. L., Wright, W. E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D. and Weintraub, H. (1991). Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. *Cell* **66**, 305-315.

Li, L., Cserjesi, P. and Olson, E. N. (1995). Dermo-1: a novel twist-related bHLH protein expressed in the developing dermis. *Dev. Bio.* **172**, 280-292.

Li, L., Zhou, J., James, G., Heller-Harrison, R., Czech, M. P. and Olson, E. N. (1992). FGF inactivates myogenic helix-loop-helix proteins through phosphorylation of a conserved protein kinase C site in their DNA-binding domains. *Cell* **71**, 1181-1194.

Lilly, B., Zhao, B., Ranganayakulu, G., Paterson, B. M., Schulz, R. A. and Olson, E. N. (1995). Requirement of MADS domain transcription factor D-MEF2 for muscle formation in Drosophila. *Science* **267**, 688-693.

Martin, J., Schwarz, J. J. and Olson, E. N. (1993). MEF-2C: a tissue-restricted member of the MEF-2 family of transcription factors. *PNAS* **90**, 5282-5286.

Molkentin, J. D., Black, B. L., Martin, J. F. and Olson, E. N. (1995). Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. *Cell* **83**, 1125-1136.

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

Murre, C. M. P. S. V., H.; Caudy, M.; Jan, L.Y.; Jan, Y.N.; Cabrera, Carlos V.; Buskin, Jean N.; Hauschka, Sptephen D.; Lassar, Andrew B.; Weintraub, Harold; Baltimore, David. (1989). Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell* **58**, 537-544.

Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Shaowei, L., Nonaka, I. and Nabeshima, Y.-I. (1993). Myogenin gene disruption results in perinatal lethality because of severe muscle defect. *Nature* **364**, 532-535.

Neuhold, L. A. and Wold, B. (1993). HLH forced dimers: tethering MyoD and E47 generates a dominant positive myogenic factor insulated from negative regulation by Id. *Cell* **74**, 1033-1042.

Olson, E. N., Perry, M. and Schulz, R. A. (1995). Regulation of muscle differentiation by the MEF2 family of MADS box transcription factors. *Developmental Biology* **172**, 2-14.

Patapoutian, A., Yoon, J., Miner, J. H., Wang, S., Stark, K. and Wold, B. (1995). Disruption of the mouse MRF4 gene identifies multiple waves of myogenesis in the myotome. *Development* **121**, 3347-3358.

Spicer, D. B., Rhee, J., Cheung, W. L. and Lassar, A. B. (1996). Inhibition of myogenic bHLH and MEF2 transcription factors by the bHLH protein twist. *Science* **272**, 1476-1480.

Szymanski, P. and Levine, M. (1995). Multiple modes of dorsal-bHLH transcriptional synergy in the Drosophila embryo. *EMBO* **14**, 2229-2238.

Taylor, M. V., Beatty, K. E., Hunter, H. K. and Baylies, M. K. (1995). Drosophila MEF2 is regulated by twist and is expressed in both the primordia and differentiated cells of the embryonic somatic visceral and heart musculature. *Mechanisms of Development* **50**, 29-41.

Thisse, B. S., C; Gorostiza-Thisse, C; Perrin-Schmitt, F. (1988). Sequence of the *twist* gene and nuclear localization of its protein in endomesodermal cells of early Drosophila embryos. *EMBO J* **7**, 2175-2183.

Wang, Y., Benezra, R. and Sassoon, D. A. (1992). Id expression during mouse development: a role in morphogenesis. *Developmental Dynamics* **194**, 222-230.

Wolf, C. T., C; Stoetzel, C; Thisse, B; Gerlinger, P; Perrin-Schmitt, F. (1991). The m-twist gene of Mus is expressed in subsets of mesodermal cells and is closely related to the Xenopus X-twi and the Drosophila twist gene. *Dev Biol* **143**, 363-373.

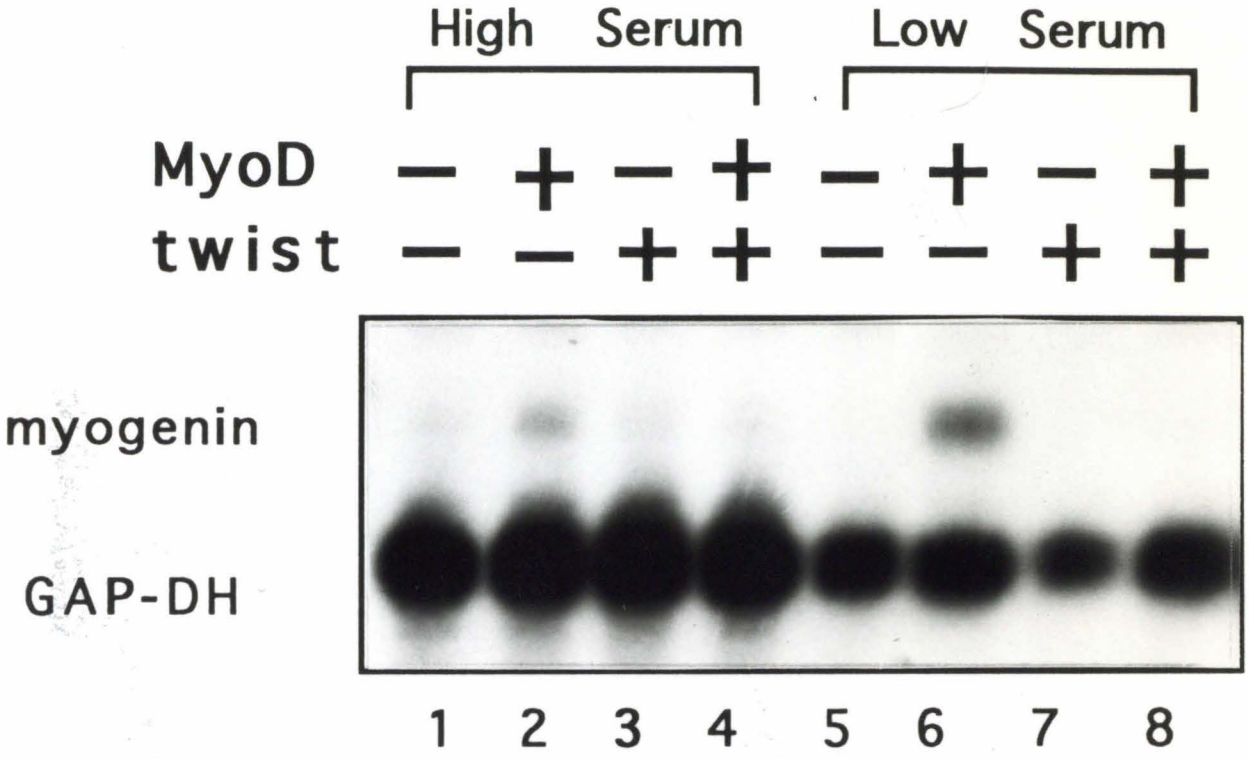

High Serum
Low Serum
MyoD
twist
myogenin
GAP-DH
1 2 3 4 5 6 7 8

Figure 1. MyoD fails to activate endogenous myogenin in twist-expressing cells. RNA from C3H10T1/2 cells transfected with MyoD were collected in high and low serum conditions and assayed for expression of myogenin in the presence and absence of co-transfected twist. As seen in lanes 2 and 6, MyoD activates endogenous myogenin shortly after it is expressed and myogenin message accumulates in low serum conditions. However, when twist is co-transfected, no endogenous myogenin is expressed. GAP-DH is used as an internal control in this assay.

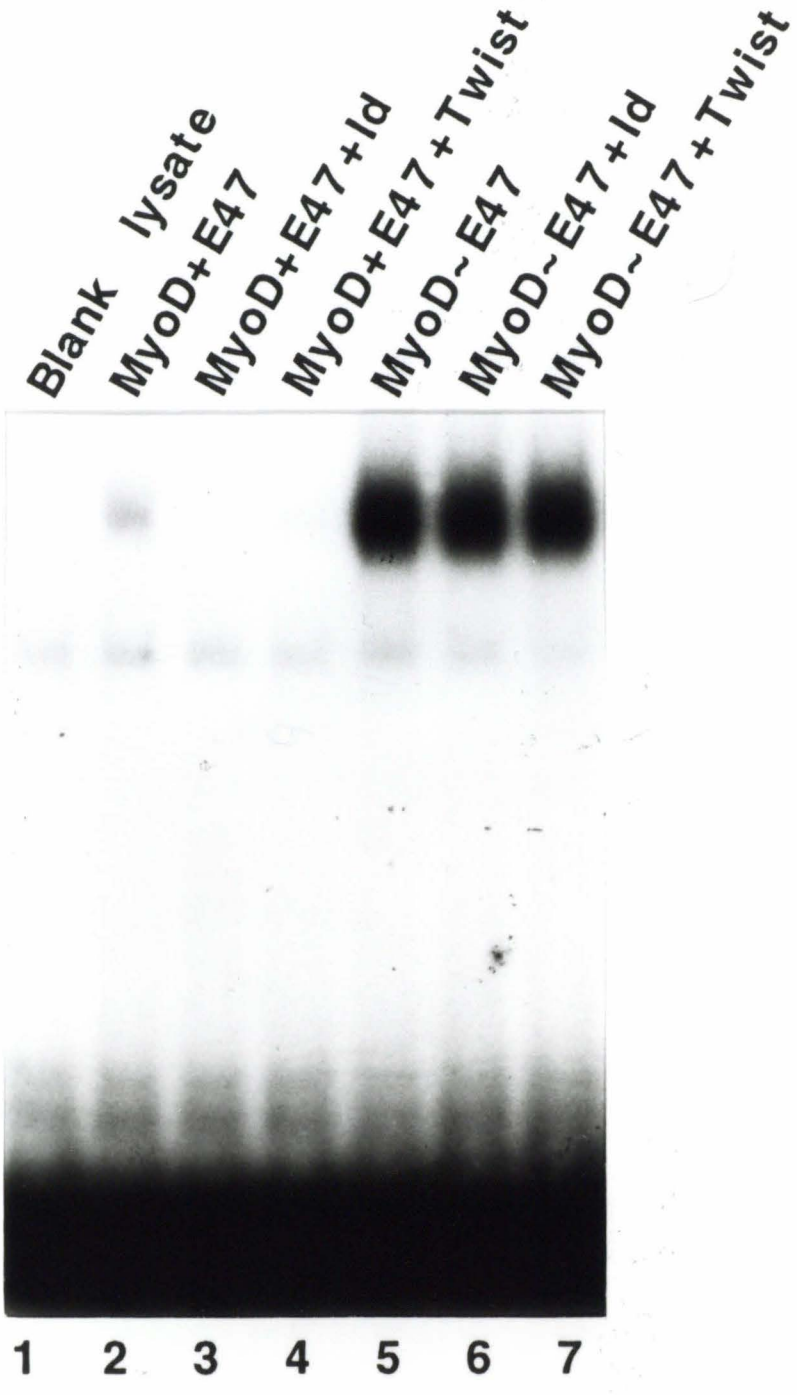
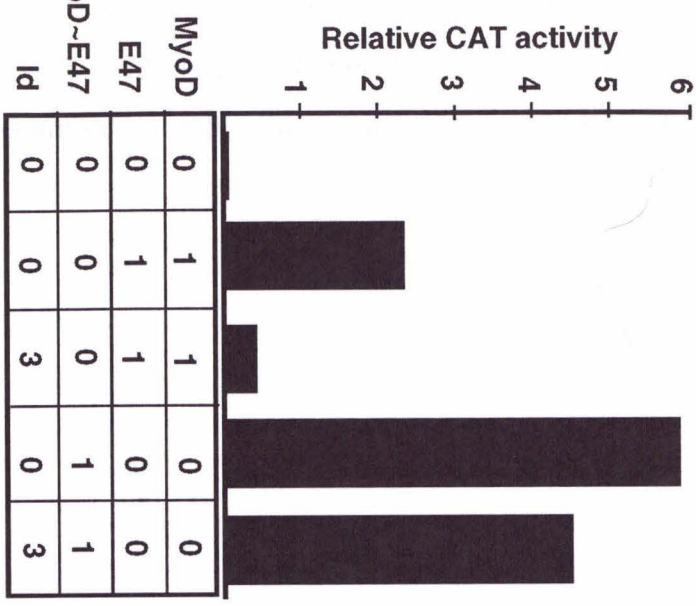

Blank lysate
MyoD+E47
MyoD+E47+Id
MyoD+E47+Twist
MyoD-E47
MyoD-E47+Id
MyoD-E47+Twist
1
2
3
4
5
6
7

Figure 2A. Tethered dimer between MyoD and E47 (MyoD~E47) is resistant to twist competition in vitro bandshift experiments. MyoD~E47 binds to its target sequence (MCK12) avidly, in the presence (10X molar excess) or absence of twist. This suggests that in vitro tethered molecule forms intramolecular dimers that are not inhibited by twist, either by HLH competition or by higher order non-DNA binding complex formation.


Relative CAT activity
1
2
3
4
5
6
MyoD
E47
MyoD~E47
Id
0 0 0 0 0
0 1 0 0 0
0 1 1 0 0
0 1 0 1 0
0 3 0 1 1

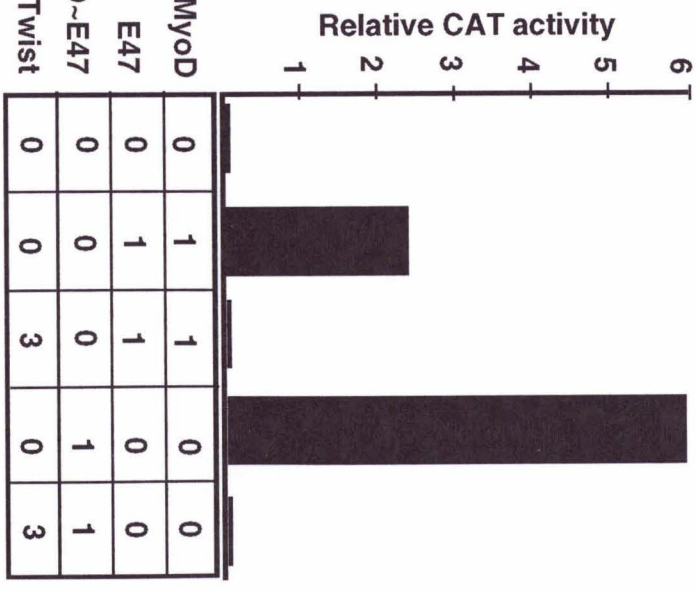

Relative CAT activity
1
2
3
4
5
6
MyoD
E47
MyoD~E47
Twist
0 0 0 0 0
0 1 0 0 0
0 1 1 0 0
0 0 0 1 0
0 3 0 1 1

Figure 2B. Myogenic program initiated by the tethered dimer is inhibited by twist in transfected cells. Although tethered dimer seems to retain its DNA binding capacity in the presence of twist (Fig. 1), twist blocks myogenesis initiated by MyoD~E47 in transfected 10T1/2 cells, suggesting an additional mechanism besides HLH competition for twist. This is contrasted with Id, whose action only involves HLH competition.

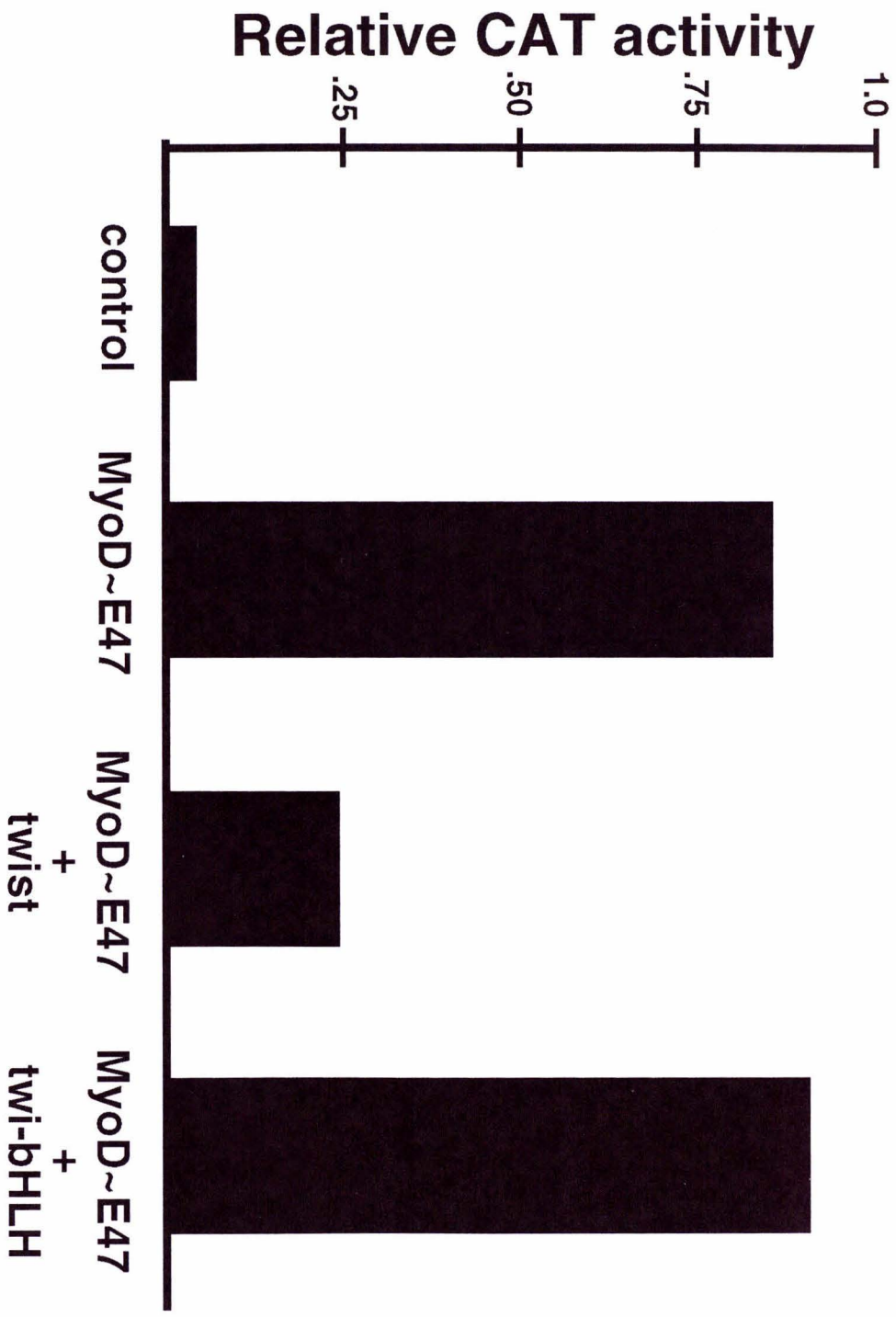

Relative CAT activity
.25
.50
.75
1.0
control
MyoD~E47
MyoD~E47 + twist
MyoD~E47 + twi-bHLH

Figure 3. Although twist full length molecule can inhibit MyoD~E47, truncated twist cannot. When truncated twist containing the bHLH region and its small COOH-terminus (domains necessary for MEF2 interaction) is co-transfected with the tethered dimer, MCK-CAT reporter level remains high. This result is consistent with the in vitro DNA binding assay using the tethered dimer which shows that the tethered dimer is insensitive to bHLH competition by twist. Furthermore, it shows that bHLH competition is not the major mechanism of full length twist inhibition. In addition, it identifies a functional domain in the amino-terminus of twist, which shows no homology to any known functional domains.

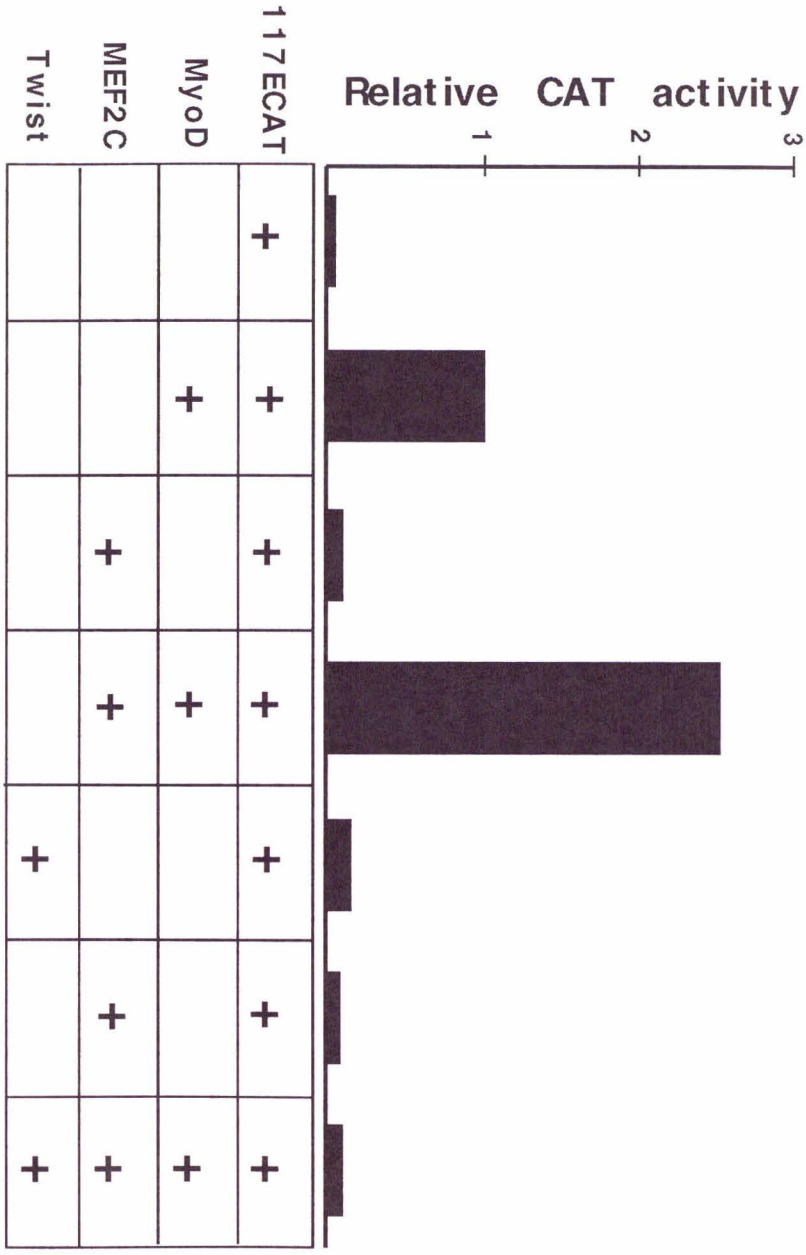

Relative CAT activity
1
2
3
117ECAT
MyoD
MEF2C
Twist
+

Figure 4A. MEF2 enhancement of myogenesis is abolished by twist co-expression. MEF2 is shown to cooperate with the bHLH myogenic regulatory factors (Kaushal *et al.*, 1994; Molkentin *et al.*, 1995) to enhance muscle specific gene expression and this interaction is thought to play a significant role in myogenesis. Since twist can inhibit all forms of bHLH MRF's we tested whether twist inhibition is targeted at MEF2 titration. Ectopic expression of MyoD and MEF2 cannot bypass twist inhibition of myogenesis.

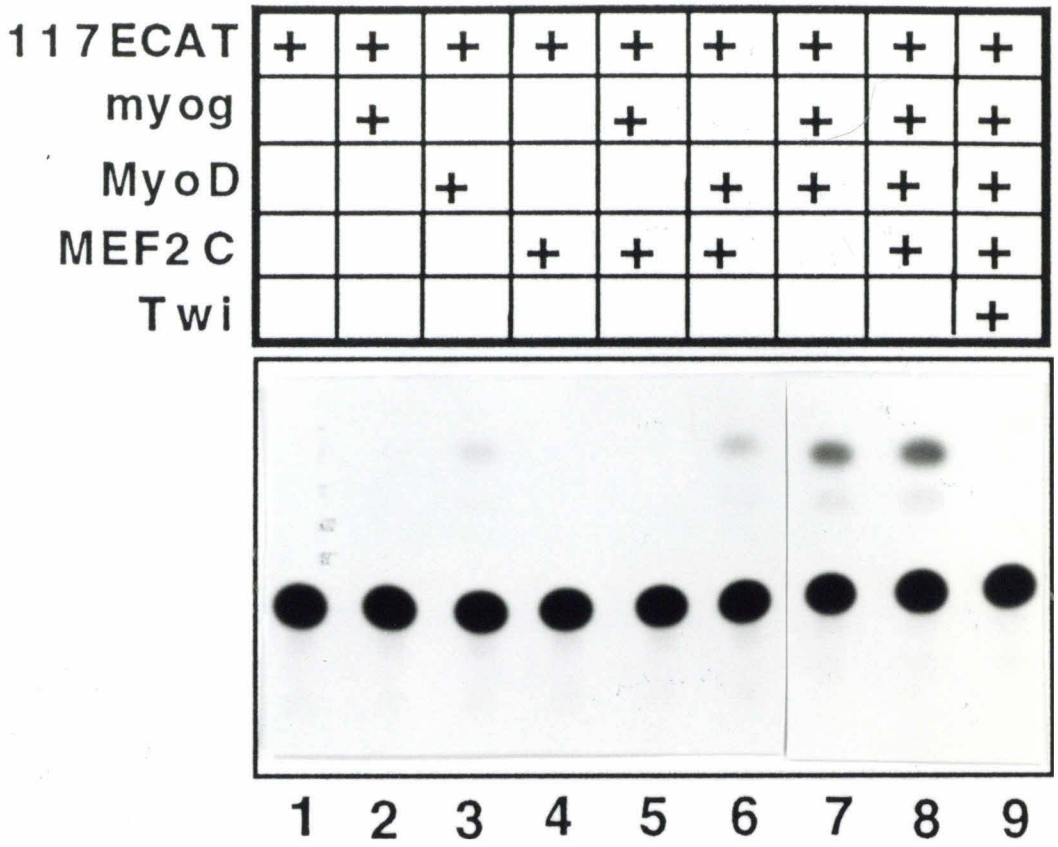

117ECAT + + + + + + + + +
myog + + + + + +
MyoD + + + + + +
MEF2C + + + + + +
Twi +
1 2 3 4 5 6 7 8 9

Figure 4B. Excess of MRF cannot abrogate twist inhibition.

Providing excess MRFs by co-transfecting MyoD and myogenin with MEF2 and twist (MyoD and myogenin both have been shown to interact with MEF2) is not sufficient to override twist inhibition. This observation suggests that competition for MEF2 by twist: to be a minor component of overall twist inhibition.

Chapter 4

In the presence of twist, growth arrest can be induced by MyoD but myogenic differentiation is blocked

Manuscript prepared for submission to

Mechanisms of Development

SUMMARY

Twist inhibits myogenesis by multiple mechanisms. Positive regulators of G1-S cell cycle progression are negative regulators of muscle differentiation, and conversely, negative regulators of G1-S progression (CDI's) can act as positive regulators of muscle differentiation. In this study, we examine whether this potent effect of twist expression is targeted at cell cycle progression. Cells expressing MyoD plus p21 or p27 withdraw from the cell cycle in the presence or absence of twist . This observation argues that twist affects only differentiation function directly and does not act in myogenesis by promoting proliferation. Twist also apparently leaves intact the growth arrest activity of MyoD. In support of this conclusion, twist expressed from the myogenin promoter can inhibit "determined" myoblasts, 10T1/2 cells expressing MyoD, from activating differentiation markers. These observations, together with those presented in chapter 3, suggest that twist inhibition is targeted at late as well as early events of myogenic program.

INTRODUCTION

Twist is a member of the basic helix-loop-helix family of transcription factors expressed early in mouse development (Thisse, 1988; Wolf, 1991; Fuchtbauer, 1995; Stoetzel *et al.*, 1995), our unpublished data). It is widely expressed in early mesoderm, soon after gastrulation, and is subsequently expressed at high levels in the developing somites, branchial arches and limb buds, among other mesenchymal sites. In the developing somites, twist expression is at first widespread, but as the somite matures, twist becomes localized to sclerotome and dermomyotome and is excluded from the myotome ((Wolf, 1991; Hebrok, 1994; Stoetzel *et al.*, 1995), our unpublished data). Consistent with this expression pattern, in vitro molecular and tissue culture studies have shown that twist inhibits myogenic differentiation by interfering with MyoD function ((Hebrok, 1994; Spicer *et al.*, 1996), chapters 2 & 3). Hence, although there is a transient co-expression of twist and myf5 in the developing somite before the epithelial somite segregates into dermomyotome and sclerotome (chapter 2), twist expression is strongly downregulated once the muscle differentiation begins. Previously, we suggested a working model in which cells co-expressing twist and myf5 may be in transition from the undetermined somitic state to a myogenic fate and that direct or indirect physical interaction between twist and myf5 plays a significant role in determining which cells progress to differentiate as muscle and when they do so (Chapter 2). In this model, those cells that continue to express myf5 and downregulate twist become postmitotic myocytes of the myotome in this model while those that maintain twist expression either remain in the progenitor state for a more extended period or become sclerotomal or dermamyotomal cells. This model is consistent with high level twist expression in the neighboring nonmyotomal compartments.

Myogenic differentiation has been studied extensively in the vertebrate system through both in vivo embryonic analysis and in tissue culture studies. MRF's (bHLH family transcription factors) and MEF2 family (MADS domain factors) members are thought to play central roles in specifying muscle by cross-activating each other and by directing the expression of muscle specific genes. Mutational analysis and embryonic expression pattern studies of MRF's (MyoD, myogenin, myf5, and MRF4) have confirmed their importance in determination and differentiation in vivo. In C3H10T1/2 fibroblast cells, among many other permissive cells, ectopic expression of MRF's leads to activation of other MRF's and terminal differentiation markers upon serum withdrawal. Recent studies demonstrate that in addition to MRF's, MEF2 family members play an important role in muscle differentiation. Molkentin et al. (1996) have shown that MRF's and MEF2 can physically associate with each other to form highly active complexes that stimulate transcription from endogenous and artificial muscle specific enhancer/promoter cis regulatory elements. (see Chapter 1 for details and references)

In vitro molecular characterization and tissue culture studies of bHLH interactions between the MRF's and twist showed that twist can apparently inhibit MRF action by multiple mechanisms. The first is similar to the dominant negative regulator Id, and twist competes efficiently for the available E-proteins thereby preventing active MRF:E-protein complex formation. A second mechanism of inhibition involves disruption of the positive interaction between MEF2 with MRFs (Spicer *et al.*, 1996). A third mechanism of myogenic repression is suggested by the capacity of twist:E protein heterodimers to bind DNA target sites and by the ability of twist to inhibit muscle gene transcription that depends on the transfected MyoD~E47 tethered dimer. These observations are consistent with twist acting as a DNA binding regulator of as yet unidentified target genes whose expression is incompatible with execution of muscle differentiation.

We have shown that in MyoD and twist co-transfected cells, a key differentiation factor, myogenin, is not activated (Chapter 3). In vivo knockout studies have shown that myogenin is required for most muscle differentiation in vivo (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). Consistent with this conclusion, endogenous myogenin expression is activated by ectopic expression of other MRFs in transfected cells. Therefore, suppression of myogenin cross-activation by MyoD provides a simple and direct explanation for twist inhibition. The fact that myogenin expression is blocked points out that an early differentiation step in myogenic differentiation is suppressed in the presence of twist. Although the exact timing of myogenin expression seems to vary slightly depending on the cells tested, myogenin expression approximately coincides with cell cycle arrest . Therefore, an obvious candidate function that might be altered, either directly or indirectly, by twist early in the myogenic program is the cell cycle progression machinery.

It has been shown that terminal skeletal muscle differentiation and proliferation are opposing phenotypes. When myoblasts differentiate, they exit from the cell cycle and express various muscle-specific genes. They do not re-enter the cell cycle. Accordingly, MyoD expression induces growth arrest and many cell cycle regulatory molecules have been shown to either directly interact with MyoD or indirectly affect the myogenic differentiation (see reviews (Lassar *et al.*, 1994; Harper and Elledge, 1996). For example, p21 and p27 CDI's (cyclin dependent kinase inhibitors) are expressed in the myotome and they are induced upon MyoD expression in transfected 10T1/2 cells (Nguyen *et al.*,), Palmer et al, submitted). p21 or p27 co-transfection with MyoD enhances myogenic differentiation by withdrawing cells out of cycle ((Halevy *et al.*, 1995) this work). Conversely, co-transfection of MyoD and cyclin D1, which pushes cells through G1, inhibits myogenic differentiation ((Skapek *et al.*, 1995). Also,

MyoD is shown to interact with Rb family members (Gu *et al.*, 1993) and Rb-/- mice studies show that Rb function is required in differentiated myotubes to prevent further DNA synthesis (Fuchtbauer, 1995).

MATERIALS AND METHODS

Plasmids

MyoD, twist, p21, and p27 coding regions were cloned into pECE vector which provides the SV40 promoter and polyA site. Construction and characterization of MyoD~E47 are described in Neuhold & Wold, 1992. MEF2C expression plasmid was a gift from E. Olson's laboratory. myog-twi plasmid was constructed by fusing the coding region of twist to 1.5kb myogenin regulatory region described in Cheng et al. 1993.

Tissue Culture and CAT assay

C3H10T1/2 cells were seeded at low density in 15%FBS/DMEM in 6cm plastic dishes. In each transfection, 1 μg of MyoD expression plasmid and equivalent molar amount of twist expression plasmid in same vector background were used, along with 1-2 μg of p27 or p21 expression plasmids. The mixture of DNA with carrier (total of 15 μg per plate) was precipitated using calcium phosphate method as described. Transfected cells were treated with 30μM chloroquine overnight and changed to fresh medium. Cells were allowed to recover for one day and switched to differentiation medium (2% horse serum in DMEM) for 3 days before they were harvested for CAT assay.

Tissue culture and Immunostaining

10T1/2 cells were transfected as described above except that cells were harvested in growth serum 24-36 hours after recovery period. BrdU was added to 10 μM in growth serum 10 to 12 hours prior to harvest time. At the time of harvest, cells were rinsed in PBS and fixed in ice cold 100% ethanol. They were re-hydrated in PBS and stained for CD8 cell surface marker, either conjugated to FITC primary antibody (from DAKO) or with unconjugated anti-CD8 from Sigma and subsequently labeled with FITC

through biotinylated secondary and avidin D conjugated with FITC. Cells were fixed again in ethanol and rehydrated. For BrdU antibody labeling, the cells were treated with 2M HCl/PBS for 5-10 minutes at R.T. and rinse thoroughly in PBS. Rat anti-BrdU antibody () was used at 1:20 dilution and secondary antibody conjugated to TRITC was used to visualize BrdU positive cells. Photograph was taken under the Olympus microscope using appropriate filters. Cells were counted by random choice of fields and equivalent number of total transfected cells were counted within each experiment.

LIVE/DEAD staining

To differentially label dead cells from live cells, we used LIVE/DEAD eukolight viability/cytotoxicity kit from Molecular Probes according to the manufacturer's instructions. Briefly, the transfected cells were rinsed in PBS and 10 µl of calcein stock solution and 5 µl of ethidium homodimer were diluted in 10 ml of PBS. Cells were covered with 5 ml of staining solution for 15 minutes in 37 C. For double labeling of transfected cells with dead cells, cells were labeled with anti-CD8 first (see above) and then labeled with ethidium homodimer. Staining solution was rinsed off and the cells were covered with PBS and checked under fluorescence microscopy. The cells were then re-cultured by adding growth medium.

RT-PCR

RT-PCR analysis was done essentially as described by Patapoutian et al . Exogenous twist expression was monitored by 5' twist internal primer with 3' primer from the SV40 polyA tail region. 100ng of total RNA was used in each reaction. GAP-DH samples were taken out after 16 cycles and myogenin and exogenous twist samples were taken out after 27 cycles of amplification. PCR products were separated on a 6%^ denaturing acrylamide gel.

RESULTS

Growth arrested cells expressing MyoD and p21 or p27 cannot differentiate in the presence of twist.

During normal skeletal muscle differentiation in vivo or in cell culture, myoblasts withdraw permanently from the cell cycle before they differentiate. MyoD has been shown to inhibit growth arrest and to induce terminal differentiation in transfected cell. Accordingly, MyoD induces the expression of p21(Nguyen *et al.*,) and co-transfection of MyoD with p21 or p27 substantially enhances muscle specific gene expression (Li *et al.*, 1992), Palmer et al., submitted, figure 1) Hence, one of the ways in which twist could inhibit myogenesis initiated by MyoD may be through forcing MyoD expressing cells through continuous cell cycle. Therefore, we first asked whether twist inhibition of myogenesis can be bypassed by ectopic expression of p21 or p27 with MyoD. As shown in figure 1, although co-transfection of p21 or p27 with MyoD enhances the CAT activity from the MCK reporter by 5-10 fold, co-expression of twist brings the reporter activity to background level. This result suggests that twist is either capable of pushing cells through cycle in the presence of MyoD and p21 or p27, or that twist is capable of blocking muscle differentiation even in growth arrested cells.

Twist expressing cells can withdraw from cell cycle.

To discriminate between these possibilities, cells with the pertinent regulators, alone and in combination, were evaluated for their cell cycling status by labelling with BrdU. If twist forces cell cycle progression, then we would expect that the fraction of cells in S-phase would be elevated in all cases where twist is successfully repressing MyoD initiated differentiation. A functionally neutral cell surface marker (CD8) was used to identify transfected cells by co-transfection with the regulators, and duplicate plates of each precipitate were made to make parallel comparisons between MCK-CAT activity

and BrdU incorporation. The cell cycle status of MyoD transfected cells was first tested in the presence of p27 and/or twist at low cell density in the presence of high serum (15%FBS), conditions that highly promote cell cycle progression. A second condition was high cell density in which contact inhibition promotes cell cycle arrest and, in the presence of MyoD, differentiation. As expected and shown in Fig 2 and Table 1, MyoD alone did not induce significant cell cycle withdrawal in transfected cells at low cell density in the presence of high serum, and this correlates with very low MCK-CAT reporter activity in the companion sample. However, the inclusion of p27 resulted in reduction of BrdU+ cells, indicating that some of these transfected cells are no longer in S phase. This correlated with correspondingly elevated activity from the muscle reporter gene. Interestingly, when twist was included, there was no significant increase in the percentage of cells in S-phase (BrdU positive). In fact, the S-phase labelling index dropped when twist was included. We verified that the reason for reduced S-phase labelling in twist transfected samples was not an increase in cell death due to apoptosis by using a reagent that selectively labels dead cells in parallel with vital labeling of transfected cells by anti-CD8 antibody (see Materials and Methods for details, data not shown). Counts of CD8 positive cells from these transfections revealed that live transfected cells were present in comparable levels in the presence or absence of twist. We therefore conclude that the myogenic suppressing action of twist does not involve forced cell cycle progression through S-phase in this test system. A further implication is that twist can suppress expression of muscle differentiation genes in cells that are mitotically quiescent.

Concurrent expression of twist under myogenin control blocks myogenic reporter activity.

Since the above data suggested that twist inhibition can act after cell cycle withdrawal, we expressed twist later in the myogenic program, using the myogenin promoter. This

promoter region has been characterized by (Cheng *et al.*, 1993; Yee and Rigby, 1993) and is shown to be active in tissue culture cells as well as in transgenic embryos. Twist expression from this promoter is dependent on MyoD and it mimics endogenous myogenin expression (Fig. 3). Myogenin is a differentiation class MRF expressed after the determination step; therefore, the expectation in this experiment is that exogenous MyoD is expressed initially following transfection, an that it has had sufficient time to activate other known and unknown myogenic events that precede myogenin (and therefore myog-twi) expression. Allowing the early myogenic program to proceed in the absence of twist interference, until the onset of endogenous myogenin, was not sufficient to lock the transfected cells into muscle differentiation pathway since myogenin promoter driven twist expression results in the block of MCK reporter expression (Fig 3).

DISCUSSION

We and others have shown that twist expression is inhibitory to muscle differentiation. Twist can do this using at least three distinct mechanisms of inhibition. One mechanism involves the bHLH competition and protein:protein interaction with E-proteins (Chapter 1 and (Spicer *et al.*, 1996)). The second mechanism, shown by Spicer and colleagues, is mediated by twist's ability to sequester MEF2 family members by direct protein:protein interaction. The third mechanism requires the N-terminus of twist and does not rely on bHLH competition (Chapter 2). In this study, we examined the effects of twist expression on several major, early steps in myogenesis to identify the target points of twist inhibition.

Twist is widely expressed in proliferating mesodermal cells in the developing embryo, including the myogenic precursor cells, and its expression is excluded from the myotome as myocytes differentiate (Wolf, 1991; Fuchtbauer, 1995; Stoetzel *et al.*, 1995), our unpublished data). In *Drosophila*, twist-expressing myogenic precursors in late stage embryos are capable of dividing and expanding before they differentiate to form the adult muscles (Currie, 1991). Therefore, it seemed possible that twist may be inhibiting muscle differentiation by interacting directly or indirectly with the cell cycle machinery in the transfected cells to force cycling. We have tested this hypothesis by antibody staining of transfected cells labeled with BrdU (Materials and Methods for details). The results of our study show that MyoD plus p21 or p27 transfected cells are capable of withdraw from the cell cycle in the presence of twist. Consistent with enhanced myogenic report by MCK-CAT reporter (Fig. 1), co-expression of p27 results in significant shift in the number of cells that are arrested in the cell cycle. Addition of twist to these cells has no positive effect on their ability to withdraw from cell cycle (Fig 2 and Table 1). In fact, co-expression of MyoD, p27, and twist seems

to withdraw more cells out of cell cycle than MyoD and p27 in both low and high density culture conditions. Mutational analysis revealed that MyoD's ability to induce growth arrest and differentiation are separable functions (Sorrentino *et al.*, 1990; Crescenzi *et al.*, 1990). Therefore, it is plausible that in the presence of twist, which can interact with MEF2 and might also bind other myogenic co-factors, more MyoD becomes available for its cell cycle withdrawal function. In this scenario, twist titrates other cofactors that would normally interact with MyoD to initiate differentiation. This explanation assumes that MyoD interacts with different molecules for the differentiation and cell cycle arrest functions and that twist has significant affinity for myogenic co-factors. At this time, these seem reasonable possibilities.

The experiments reported here make it clear that twist inhibition of myogenesis initiated by MyoD does not operate by blocking cell cycle withdrawal in 10T1/2 cells. Instead, it acts through mechanisms suppress terminal differentiation gene expression. This highlights previously reported parallel pathways activated by MyoD expression: one that leads to growth arrest and the other leading to muscle differentiation (Sorrentino *et al.*, 1990; Crescenzi *et al.*, 1990). Twist inhibition of MyoD function therefore seems to affect only one of the two pathways. This observation also points out that at least one important step (Cell cycle withdrawal) toward terminal differentiation can take place in the presence of twist.

Interestingly, expressing twist relatively late in the myogenic program still inhibits muscle differentiation. Although myogenin knock-in into myf5 locus experiment suggests that MRFs can substitute for each other functionally in the developing embryo, single mutant analysis of individual MRFs show different phenotypes (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). In particular, myogenin knockout animals clearly distinguish myogenin as a differentiation factor normally required in myoblasts.

clearly distinguish myogenin as a differentiation factor normally required in myoblasts. The results from myog-twi experiment support this observation (Fig. 3). Ectopic expression of MyoD in 10T1/2 cells could "determine" these cells, as it does in vivo, but did not immediately activate terminal differentiation markers. Concurrent expression of twist and myogenin in these cells blocks muscle differentiation, similar to those undifferentiated myoblasts in myogenin -/- mice. It has been observed that these mutant myoblasts can differentiate once they are removed from the animal and cultured (Nabeshima *et al.*, 1993). Therefore, it will be interesting to test if different culture conditions could alleviate twist inhibition in myog-twi expressing 10T1/2 cells.

Twist inhibition is a powerful block that can occur late in the myogenic program and it occurs in growth arrested cells. Since these cells do not go through apoptosis, as do Rb -/- cells (Schneider *et al.*, 1994). However, it is unclear exactly what the status of these cells is. We have monitored markers of sclerotomal or dermatomal fates in twist expressing cells, but observed no induction of such markers (Pax1,3,7,9, Id1,2,3,4, shh, sim-1). This is not to say that the fates of these cells have assumed the precursor-like phenotype or some other cell phenotype. The markers used may simply fail to detect the twist dependent phenotype.

Although much has been learned about the early myogenic determination steps in the last several years, it seems likely that there may be unidentified steps in the process. Despite the general knowledge that ectopic expression of MRF's can convert many cells into muscle, there are "non-permissive" cells that do not convert. For example, expression of MyoD in CV1 cells result in growth arrest but these cells do not differentiate (Crescenzi *et al.*, 1990). Many rhabdomyosarcoma tumor cell lines express high levels of MyoD but fail to differentiate. These observations suggest that either these cells express high levels of negative regulators such as twist or Id or that

they may have problems in other differentiation steps. The differentiation defective phenotype of these rhabdomyosarcoma cells can be complemented when 10T1/2 fibroblast are fused to these cells. Naive 10T/12 cells do express low levels of endogenous twist (our unpublished data) and it will be interesting to see whether high level twist expressing fibroblast cells can still rescue the muscle differentiation phenotype when fused to these cells. It is interesting to note that the C2C12 myogenic cell line is one of very few cell lines that do not express twist (our unpublished data), consistent with twist having an inhibitory effect on myogenesis, and when twist is stably transfected into these cells, they fail to differentiate ((Hebrok, 1994), our unpublished results).

REFERENCES

Cheng, T.-C., Wallace, M. C., Merlie, J. P. and Olson, E. N. (1993). Separable regulatory elements governing myogenin transcription in mouse embryogenesis. *Science* **261**, 215-218.

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

Currie, D. B., M. (1991). The development of adult abdominal muscles in *Drosophila*: myoblasts express *twist* and are associated with nerves. *Development* **113**, 91- 102.

Fuchtbauer, E. M. (1995). *Dev. Dyn.* **204**, 316-.

Gu, W., Schneider, J. W., Condorelli, G., Kaushal, S., Mahadavi, V. and Nadal-Ginard, B. (1993). Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. *Cell* **72**, 309-324.

Halevy, O., Novitch, B. G., Spicer, D. B., Skapek, S. X., Rhee, J., Hannon, G., Beach, D. and Lassar, A. B. (1995). Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science* **267**, 1018-1021.

Harper, J. W. and Elledge, S. J. (1996). Cdk inhibitors in development and cancer. *Current Opinion in Genetics & Development* **6**, 56-64.

Hasty, P., Bradley, A., Morris, J. H., Edmondson, D. G., Venuti, J. M., Olson, E. N. and Klein, W. H. (1993). Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature* **364**, 501-506.

Hebrok, M. W., Karin; Fuchtbauer, Ernst-Martin. (1994). M-twist is an inhibitor of muscle differentiation. *Developmental Biology* **165**, 537-544.

Lassar, A. B., Skapek, S. X. and Novitch, B. (1994). Regulatory mechanisms that coordinate skeletal muscle differentiation and cell cycle withdrawal. *Current Opinion in Cell Biology* **6**, 788-794.

Li, L., Zhou, J., James, G., Heller-Harrison, R., Czech, M. P. and Olson, E. N. (1992). FGF inactivates myogenic helix-loop-helix proteins through phosphorylation of a conserved protein kinase C site in their DNA-binding domains. *Cell* **71**, 1181-1194.

Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Shaowei, L., Nonaka, I. and Nabeshima, Y.-I. (1993). Myogenin gene disruption results in perinatal lethality because of severe muscle defect. *Nature* **364**, 532-535.

Nguyen, H. T., Bodmer, R., Abmayr, S. M., McDermott, J. C. and Spoerel, N. A. Schneider, J. W., Gu, W., Zhu, L., Mahdavi, V. and Nadal-Ginard, B. (1994). Reversal of terminal differentiation mediated by p107 in Rb -/- muscle cells. *Science* **264**, 1467-1471.

Skapek, S. X., Rhee, J., Spicer, D. B. and Lassar, A. B. (1995). Inhibition of myogenic differentiation in proliferating myoblasts by cyclin D1-dependent kinase. *Science* **267**, 1022-1024.

Sorrentino, V., R., P., Davis, R. L., Ansorge, W. and Philipson, L. (1990). Cell proliferation inhibited by MyoD1 indpendently of myogenic differentiation. *Nature* **345**, 813-815.

Spicer, D. B., Rhee, J., Cheung, W. L. and Lassar, A. B. (1996). Inhibition of myogenic bHLH and MEF2 transcription factors by the bHLH protein twist. *Science* **272**, 1476-1480.

Stoetzel, C., Weber, B., Bourgeois, P., Bolcato-Bellemin, A. L. and Perrin-Schmitt, F. (1995). Dorso-ventral and rostro-caudal sequential expression of M-twist in the postimplantation murine embryo. *Mechanisms of Development* **51**, 251-263.

Thisse, B. S., C; Gorostiza-Thisse, C; Perrin-Schmitt, F. (1988). Sequence of the *twist* gene and nuclear localization of its protein in endomesodermal cells of early Drosophila embryos. *EMBO J* **7**, 2175-2183.

Wolf, C. T., C; Stoetzel, C; Thisse, B; Gerlinger, P; Perrin-Schmitt, F. (1991). The m-twist gene of Mus is expressed in subsets of mesodermal cells and is closely related to the Xenopus X-twi and the Drosophila twist gene. *Dev Biol* **143**, 363-373.

Yee, S.-P. and Rigby, P. W. J. (1993). The regulation of myogenin gene expression during the embryonic development of the mouse. *Genes & Development* **7**, 1277-1289.

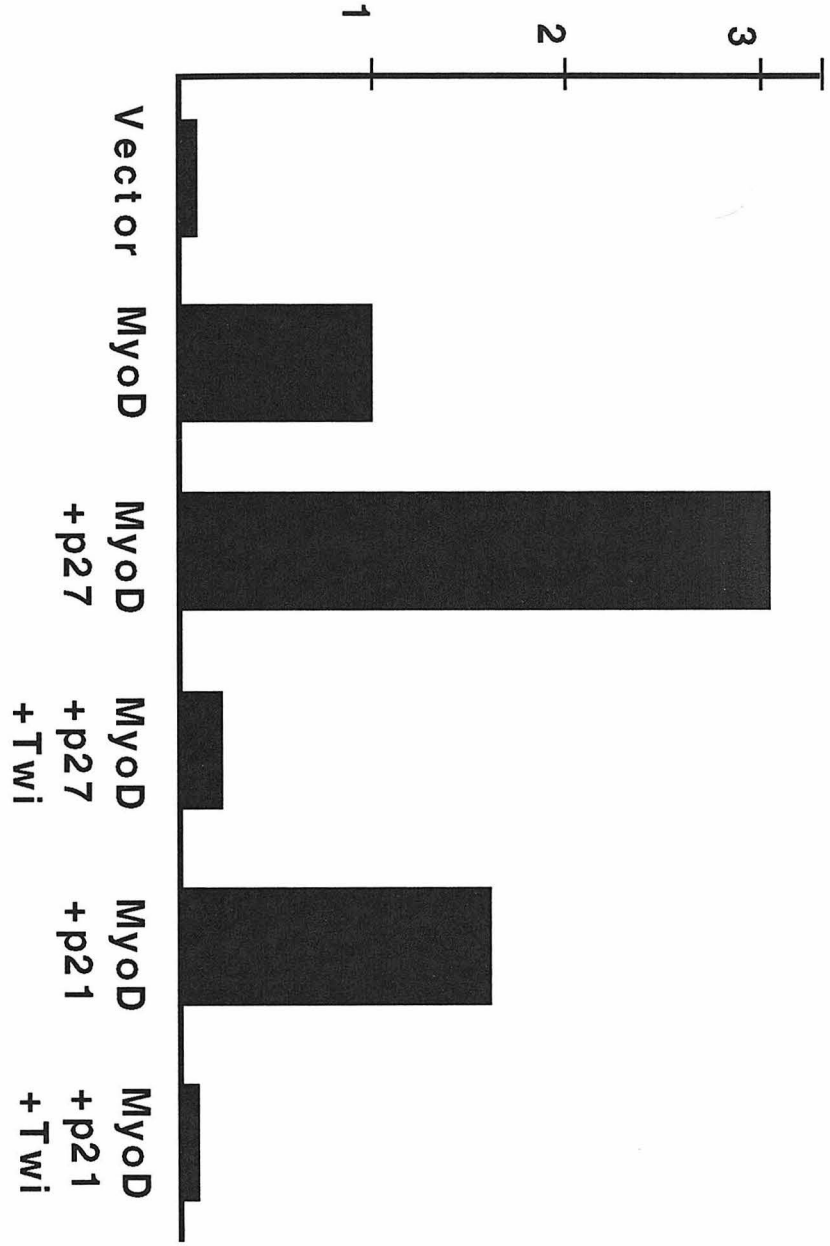

1
2
3
Vector
MyoD
MyoD +p27
MyoD +p27 +Twi
MyoD +p21
MyoD +p21 +Twi

Fig. 1 Twist inhibits myogenesis even in the presence of cell cycle inhibitors p21 and p27 with MyoD. p21 and p27 CDI's enhance myogenic differentiation when co-transfected with MyoD by arresting cell cycle. When twist is co-transfected with these regulators, the MCK-CAT reporter levels falls back to basal level.

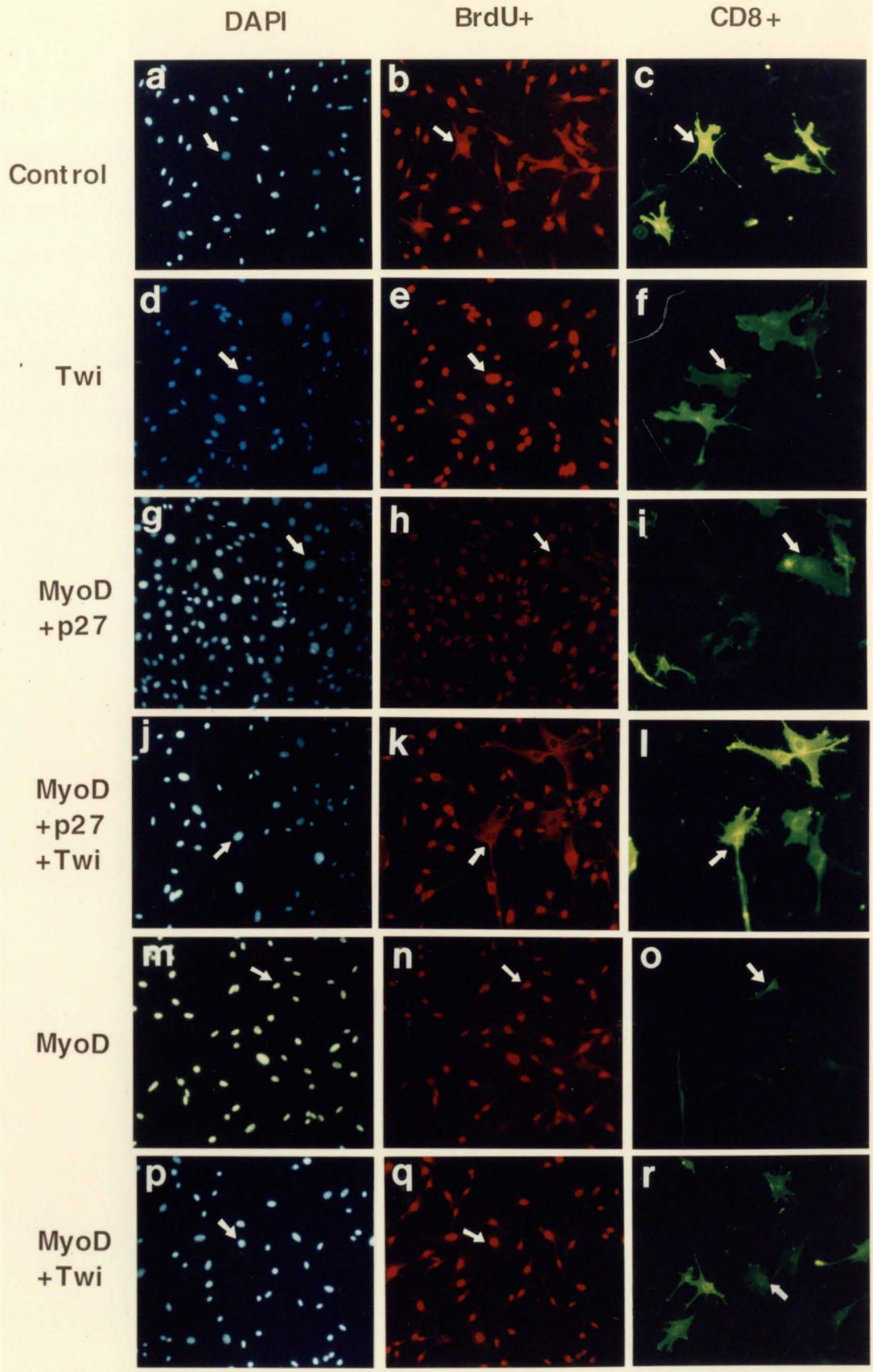

DAPI
BrdU+
CD8+
Control
Twi
MyoD
+p27
MyoD
+p27
+Twi
MyoD
MyoD
+Twi
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r

Fig. 2 Immunohistostaining of transfected 10T1/2 cells labeled with BrdU for approximately 10 hours under low density condition show that twist does not alter the cell cycle status of MyoD transfected cells. C3H10T1/2 cells were transfected with either CD8 transfection marker alone(A-C) or with MyoD (D-F), MyoD+p27 (G-I), or MyoD+p27+twist (J-L). Majority of the cells that are transfected with CD8 alone or CD8+MyoD label for BrdU, suggesting that they are still cycling. However, most of the cells transfected with MyoD+p27 or MyoD+p27+twist do not label for BrdU, suggesting that they are arrested.

	Low density plate			Confluent plate		
	CD8+	CD8+BrdU+	$\frac{CD8+BrdU+}{CD8+}$	CD8+	CD8+BrdU+	$\frac{CD8+BrdU+}{CD8+}$
Control	224	196	88%	225	102	45%
Twist	203	166	82%	218	102	47%
MyoD	228	170	75%	204	69	34%
MyoD+Twist	210	175	83%	196	46	23%
MyoD+p27	210	96	46%	220	38	17%
MyoD+p27+Twist	218	57	26%	213	17	8%

Table 1. Transfected 10T1/2 cells were stained for CD8 (to identify transfected cells) and BrdU (to mark cells in S-phase) in either low density or contact inhibited high density conditions (both high serum condition). The cell numbers indicate total numbers of positive staining cells counted from randomly chosen fields in each plate.

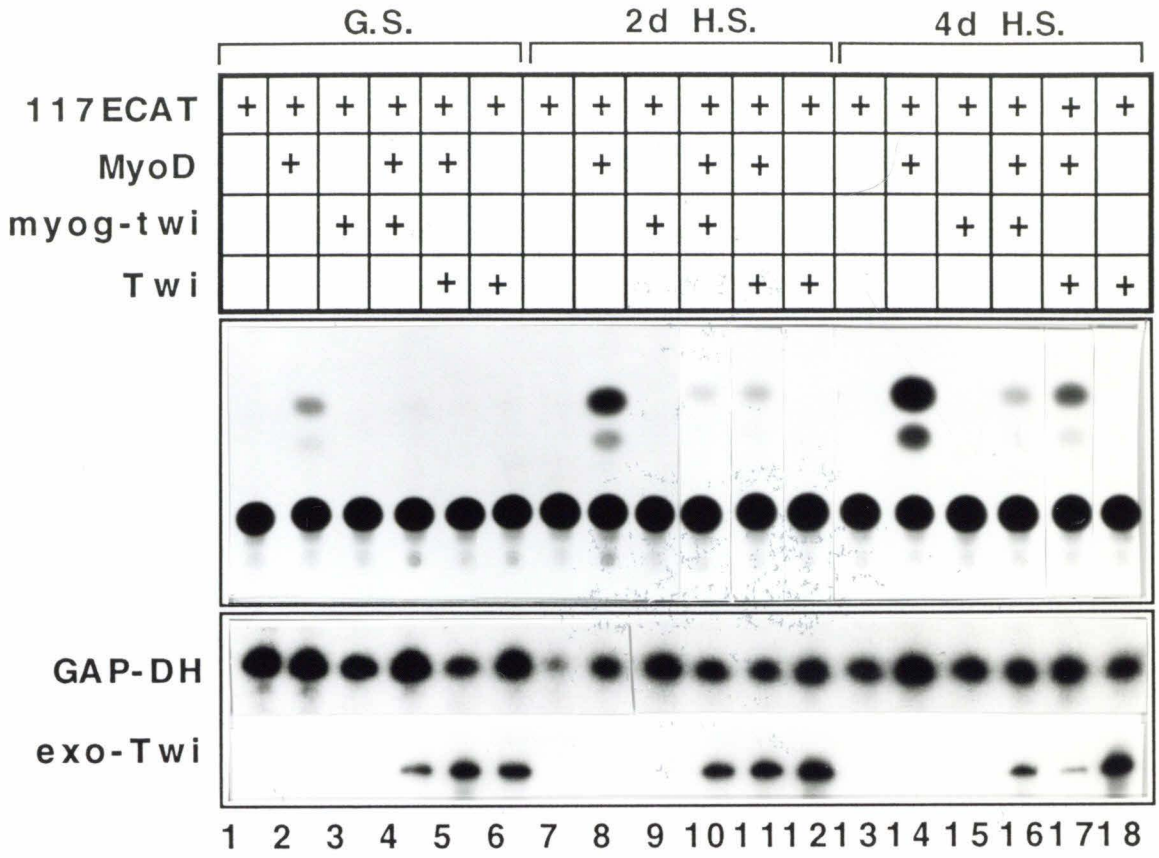

G.S.
2d H.S.
4d H.S.
117ECAT
MyoD
myog-twi
Twi
GAP-DH
exo-Twi
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Fig. 3 (top) When twist is expressed later in the myogenic program, from the myogenin promoter, muscle differentiation is still inhibited. This suggests that twist is capable of blocking determined myoblast from becoming fully differentiated. (bottom) RT-PCR analysis of transfected 10T1/2 cells show that myog-twi construct expression depends on MyoD expression and that it copies endogenous myogenin expression.

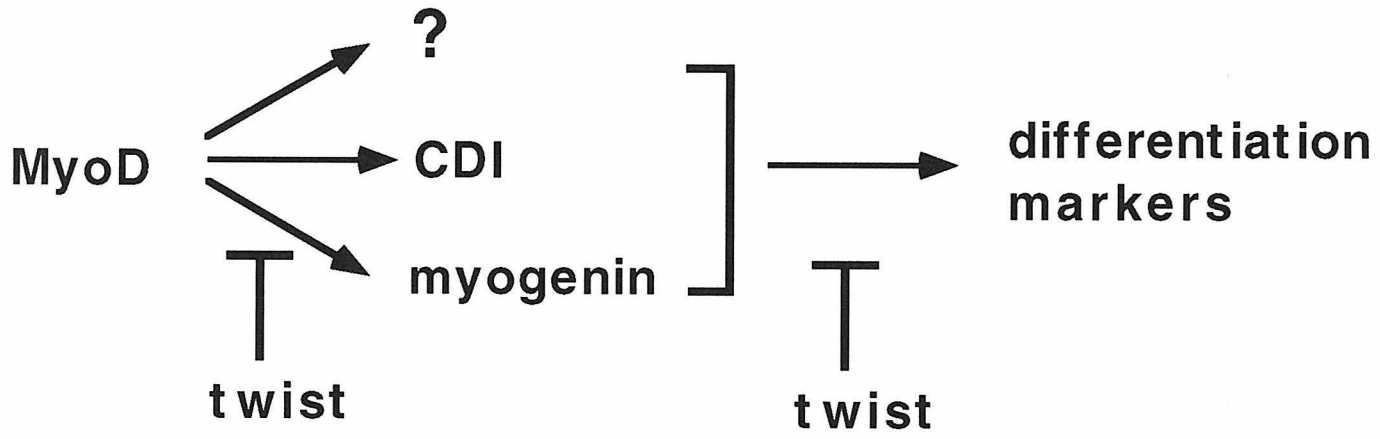

?
MyoD
CDI
myogenin
twist
differentiation
markers
twist

Fig.4 Simplified schematics of twist target points in myogenic differentiation. Twist can act early when co-transfected with MyoD by preventing onset of myogenin expression. In addition, twist can function after the cell cycle arrest and early myogenic determination steps have taken place when expressed from the myogenin promoter to prevent terminal differentiation.

Appendix A

Yeast Two Hybrid Screen for Twist Interacting Proteins

SUMMARY

Twist is a basic helix-loop-helix transcription factor that has been shown to interact with other bHLH molecules as well as with a MADS-box factor MEF2. Since the identity of the partner in different cellular settings may define the activity of twist, a yeast two hybrid screen was performed from mouse E9.5 and E10.5 cDNA fusion libraries. Of the 30 positive clones identified, five had open reading frames. These clones encode twist itself, an E-protein, and three unknown proteins. The results of this screen suggest that twist can form homodimers and that it can interact with non-bHLH factors. The E-protein isolated from this screen might be a new alternatively spliced variant of E2-5 gene or it may be a novel E-protein. The characterization of the unknowns might reveal some important co-factors that modulate twist and other bHLH factor functions.

INTRODUCTION

Twist is a member of the basic helix-loop-helix (bHLH) family of transcription factors. This family of transcription factors can be largely subdivided into three groups: 1) widely expressed E-proteins including E12, E47, E2-2, HEB that form homo- and hetero-dimers 2) tissue specific factors such as the MyoD family, members of the achaete scute complex, and early mesodermal regulator such as twist, among others, and that preferentially form heterodimers with the E-protein family members, 3) dominant negative factors that do not contain the basic region for contacting DNA, such as Id family members and emc that form inactive complexes with either E-proteins or tissue specific factors.

What is inherent in the molecular design of the bHLH family and in the leucine-zipper family of transcription factors is the inevitable combinatorial effects depending on partner availability and choice among the factors. The Id family members and twist have been shown to compete for available E-proteins with MyoD when they are co-transfected into fibroblast cells. Hence, they prevent MyoD function post-translationally via protein:protein competition when there is limiting amounts of E-proteins. When exogenous E-proteins are highly expressed, Id is not an efficient negative regulator of MyoD since the titrating effect of Id is not significant in that setting.

The helix-loop-helix region is mainly required for dimerization and the basic region for DNA binding. Hence, depending on the partner, a given bHLH factors may bind to different target sequences and regulate different downstream gene expressions. Tissue specific factors such as MyoD interacts with E-proteins mostly to form MyoD:E-protein

complexes. However, in some settings, they may directly interact with each other and form tissue specific dimer complexes. This dimer would be expected to behave completely differently from either twist:E or dermo-1:E complexes such that in a minimal theoretical setting where there are only three factors present, one would expect six different species of complexes whose target site preference (and possibly the activity on the promoter, depending on the neighboring factors) may be different.

In addition to the interactions among bHLH regulators themselves, recent studies have revealed that many non-bHLH transcription factors as well as cellular factors can interact with bHLH factors. Most relevant in the myogenic lineage is the demonstrated interaction between MyoD, myogenin, and twist with MEF2 family members. Although these molecules do not share the same dimerization motif, they do interact with each other physically and bind DNA cooperatively. On the other hand, E1A and Rb also interact with the bHLH domain of MyoD. Consistent with these observations, recent screens using bHLH regulators in yeast two hybrid screen turned up a large number of unknown molecules that do not contain a bHLH domain but do interact with various bHLH factors in a sequence specific manner. These cofactors add yet another level of complexity in understanding the consequence of a bHLH factor expression in a given setting: there may be a competition for available co-factors in addition to the bHLH rivalry. Depending on the co-factor, the target site preference or transcriptional activity of a given complex is likely to vary.

In the developing mouse embryo, many bHLH factors have been identified to date that over-lap in their expression patterns. One can only imagine the complex traffic control problems these nuclei must face to prevent complete chaos. The goal of the experiments described in this chapter was to identify potential partners of twist in the developing embryo using the yeast two hybrid screen. The importance of identifying

the twist partners is obvious since the downstream target gene identification depends directly on identifying the proper partner(s) of twist.

MATERIALS AND METHODS

Library screen

Yeast two hybrid library screen kit modified by Stanley Hollenberg had been used successfully with E12 bHLH domain as a bait by E. Olson's group (Cserjesi *et al.*, 1995; Burgess *et al.*, 1995). LexA fusion vector BTM116 was constructed by Paul Bartel, and it contains the TRP1 gene and a polylinker downstream of the lexA coding sequence. It does not contain a nuclear localization signal in order to reduce the background; hence, only the interacting cDNA partners will transport the lexA fusion protein into the nucleus. Truncated twist containing the bHLH domain and the following COOH-terminus end was fused to lexA sequence at the SmaI site and this fusion junction was verified to be in-frame by sequencing.

The cDNA library used in the screen was also from Hollenberg's laboratory. A mixture of E9.5 and E10.5 mouse embryonic cDNA libraries fused to VP16 activation domain was used in the screen. The library plasmid contains the LEU2 gene and NLS-VP16-linker unit driven by the ADH promoter. The insert size ranges from 350 - 700 nucleotides and the inserts were PCR amplified to increase the amount of cDNA. By the time of this screen, they had been amplified three times. Inserts from this library were sequenced directly using either M13 universal primer (bottom strand) or by YTH1 primer (5' GAG TTT GAG CAG ATG TTT A 3', top strand).

L40 yeast strain (MATa his3D200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)4-HIS3 URA3::(lexAop)8-lacZ gal4?? gal80??) was used as the host strain for the screen. The expression of both HIS3 and lacZ are driven by minimal GAL1 promoter fused to multimerized lexA binding sites. Hence, only when the lexA binding site is occupied (lexA binding domain from the bait fusion protein) and activational domain present

(cDNA library fusion protein), these transformants can grow on His- plates and express lacZ.

Competent L40 cells were made and transformed with the bait twist fusion protein and grown on a minimal plate (YD + Ade(20mg/ml), His(20mg/ml), Leu(30mg/ml), Lys(30mg/ml), Ura(20mg/ml): note that this is different from the recipe recommended by the Hollenberg protocol. Standard LiAc transformation was used, as described in the Hollenberg's kit, to introduce the bait expression plasmid into L40. Single colony was purified and its DNA extracted and sequenced to confirm that a proper bait plasmid was carried in L40/lexA-twi. For the large scale library transformation, L40/lexA-twi was grown in minimal media over night and then in rich media(YPD) for 3-4 hours until OD of ~.6. Again, the cells were prepared for LiAc transformation following the protocol and the mixture of E9.5 (250μg) and E10.5 (250μg) fusion cDNA library DNA was transformed. Transformants were plated onto 25 X 25cm plates containing YD+Ade plates and small aliquot dilutions were plated onto YD+Ade+His 10cm plates to check the transformation efficiency.

From approximately $15X10^6$ colonies plated (from two large scale transformations), roughly$10X10^3$ colonies were His+. These colonies were tested for lacZ expression by the top-agarose assay. This method is much superior to the recommended filter assay in giving clean, strong positive signal and eliminating the background all together. Only 30 colonies scored as lacZ positive by this assay and they were picked for further characterization. Of these, 16 survived the picking and culturing during the next steps.

Since the number of positive clones was relatively small, the insert DNA from these clones were extracted directly. Crude yeast DNA prep was made and its plasmid DNA

was electroporated into DH5-alpha bacterial strain (note: it's important to use electroporation of yeast DNA rather than standard CaCl transformation. Contaminants from the yeast DNA prep inhibits CaCl transformation). Plasmids extracted from the bacteria were sorted by size (since both bait and fish plasmids are isolated together from the yeast prep, sorting of the plasmids had to be done at this step) and directly sequenced using the M13F primer and YTH1 primer. Due to the possibility of multiple transformation (the dose of DNA used errors on this side), some clones had to be re-purified several times before the right plasmid was extracted. False positive interactions were tested by re-transforming the isolated plasmids into L40/lexA-lamin. None of the identified clones showed false-positivity by this assay.

In situ hybridization

TIP 8, TIP12, and TIP18 inserts were moved into pBSII plasmid for further characterization. TIP8 and TIP12 were linearized either at BamHI (anti-sense) or EcoRI (sense) and transcribed with T3 or T7 RNA polymerases to make DIG-labeled riboprobes. Wholemount in situ using E9.5d and E10.5d embryos were performed as described in (Conlon and Rossant, 1992).

cDNA library screen

E10 mouse embryonic cDNA library was purchased from Novagen. Initial plating of phages were done at 3X10E4 plaques per plate. For tip12, total of 5.4X10E5 plaques were screened and one positive plaque was identified. For tip8, only 1.5X10E5 plaques were screened and no positive plaque was identified. After the initial round, the single tip12 positive plaque was purified two more round.

RESULTS

Data from chapters 1 and 2 suggest that there may be at least two functionally important domains in twist molecule. The bHLH domain retained in the truncated twist (twi.B) is capable of competiting for available E-proteins and hence inhibits MyoD in DNA binding assays as well as in transfected tissue culture cells. However, when the E-protein competition is eliminated by the use of tethered dimer MyoD~E47, twist bHLH region was not sufficient to inhibit MyoD~E47 when the full length twist was. The fact that the N-terminus of twist is required for full inhibition, as well as the fact that twist forms active DNA binding complexes with E-proteins, suggested that N-terminus of twist may facilitate interactions with other transcription factors or co-factors. Since we have not been able to show any transcriptional activation by twist, we separated the bHLH domain from the N-terminus in case that the N-terminus contains repressor activity.

Yeast two hybrid screen has been used successfully by many laboratories now to identify molecules that interact with each other as transcription factors, cell cycle machinery components, and transduction pathway complexes. The power of this screening approach lies in the fact that the only requirement for its successful use is that the two molecules interact with each other well enough that the complex formation can occur in yeast. The feasibility for such interaction using a mammalian bHLH family has been established by Olson's group (Cserjesi *et al.*, 1995; Li *et al.*, 1995) They have isolated several tissue specific bHLH factors using the E12 bHLH domain as the bait.

16 clones that interact with twist bHLH domain were isolated and named TIPs (Twist Interacting Proteins). Of these, five clones (TIP4,8,12,18,21) have been characterized and the rest either had no open reading frame (TIP3, 5, 6,11) or the original insert that

gave specific interaction was never recovered (TIP 2,14,15,17,19,20). One clone had no insert (TIP13), which is expected to give higher background lacZ expression level than a plasmid with random insert. As mentioned in the Materials and Methods section, all five of the characterized clones were sequence specific interactors of twist bHLH domain and the three clones with no ORF also showed sequence specific interaction. The complications due to multiple transformed plasmids per clone might have reduced the efficiency of plasmid recovery from some clones. Sequential plating of some clones from which I failed to isolate ORF containing plasmids gave rise to mixed population of lacZ-positive and lacZ-negative colonies as the plasmids segregated out later.

TIP8

TIP8 contains 255 nucleotide insert that shares identical bHLH domain with a known bHLH factor, E47. However, the insert also contains unique sequences both 5' and 3' of the shared region with E47. These unique sequences bracket potential splice donor and acceptor sites and hence it is possible that either TIP8 is a new splice variant of E2-5 gene, which gives rise to E12 and E47 by alternative splicing, or TIP8 is encoded by a novel E-protein gene. The possibility of TIP8 being a by-product of a cloning artifect cannot be ruled out at this time either. In order to distinguish these possibilities, cDNA library from E10 mouse embryo (Novagen) was screened using the 21 bp oligonucleotide specific to the unique sequence in the 5' end of TIP8 clone. 1.5X10E5 plaques were screened at high stringency but no positive clone was identified.

To test the abundance and authenticity of the TIP8 transcript, I used RT-PCR on E9.5 and E10.5 mouse total RNA using primers from unique 5' and 3' sequences. Both RNA samples gave strong signals of expected molecular weight, suggesting that the

transcript containing these sequences is expressed at a respectable level in the developing embryo. However, the small scale cDNA library screen failed to pull out corresponding cDNA from the E10 mouse embryo library. These results suggest that a larger screen is needed to conclude whether TIP8 is expressed at low levels but real or whether TIP8 is a cloning artifect.

A recent discovery of MITF-2B transcript makes the splice variant possiblity more probable (Skerjanc *et al.*, 1996). MITF is the mouse locus for ITF2 gene and it produces at least two transcripts, MITF-2A and MITF-2B. These transcripts give rise to two E-proteins with identical bHLH domains but opposing effects on MyoD activity. Both molecules dimerize with MyoD but MITF-2A acts as a positive co-factor whereas MITF-2B inhibits MyoD activity. MITF-2B differs from MITF-2A in its amino-terminus, and the negative regulatory activityof MITF-2B is localized to 83 amino acids in the N-terminus. This observation is particularly intriguing since twist acts as a negative regulator of MyoD function as well. Another group used the full length twist as bait in similar screens as this and found many E-proteins among the positive interacting clones. Therefore, TIP8 may be a genuine endogenous partner of twist. It will be interesting to test whether 1) twist:TIP8 complex contains different transcactivational activity than twist:E47 or other known positive acting E-proteins 2) TIP8 can interact with MyoD and other tissue specific factors (which is probable) and 3) TIP8 can act as a dominant negative co-factor. The challenge lies in isolating the full length cDNA corresponding to TIP8 since the unique sequence stretches are too short for efficient screening.

To test whether TIP8 shows tissue specific expression in the developing embryo, anti-sense TIP8 riboprobe and sense control probe were used in wholemount in situ hybridization experiments. Embryos from E9.5d and E10.5d were harvested and

processed as described. Similar to other ubiquitous E-proteins, TIP8 did not give strong tissue-specific expression pattern. This observation may be skewed by the fact that a large domain of the probe used is identical to E-47 and hence not specfic. Although riboprobes containing large domains of shared regions with other family members have been used successfully to show distinct expression patterns, TIP8 does not contain enough unique sequences. Hence, the expression pattern characterization also must wait for a longer cDNA clone.

TIP12

TIP12 transcript shows no homology to any known sequence in the databank. Its theoretical structural analysis revealed that TIP12 forms helix-loop-helix structure; however, it does not conform to the classical bHLH homology very well. Hence, either it is a member of a novel family of factors that can interact with the known bHLH proteins or it just sticks to bHLH domains because of the structural similarity. It may be a cytoplasmic protein which may not be a relevant interactor of bHLH nuclear factors, except perhaps in the process of nuclear transport. One of the major drawbacks in yeast two hybrid screen is the large number of background positive clones, although TIP12 does not seem to fall into this category.

cDNA library from E10 mouse embryo was screened for TIP12 cDNA using the whole insert as a probe (310 bp). Only one positive clone was isolated during the first round of screening which duplicated in later purification steps. The insert of this clone unfortunately was not bigger than TIP12 itself and no new sequence information was gathered. The fact that TIP12 sequence was isolated from a completely different library suggests that this transcript is real; however, the relative low abundance of this transcript, confirmed by RT-PCR, suggests that a larger number of cDNA clones should be screened for isolating the full length clone.

In situ hybridization using the anti-sense riboprobe of TIP12 revealed very low expression. It is hard to conclude whether the wholemount in situ is not sensitive enough to detect low level expression of TIP12, or whether there is wide spread low level expression of TIP12 since no tissue specificity could be assigned to these embryos. Again, a longer probe or a probe from a dfferent region might help bring out the signal to determine tissue specificity or general expression pattern.

TIP18

TIP18 clone contains a ~360 bp insert with an open reading frame. However, the insert does not match any known sequence in the data bank. It is rich in leucine residues and it interacts with twist bHLH region in a sequence specific manner in yeast. Primers from this sequence used in RT-PCR amplify an expected size band from E9.5 and E10.5 mouse embryonic RNA. However, nothing else is known about this molecule at the moment.

TIP21

TIP21 sequence is identical to murine twist. This insert is ~500 bps long and all of it matches the known murine twist sequence. Identification of twist in this screen demonstrates that at least in yeast, twist is capable of forming homodimers. This observation implies, but does not prove directly, that twist in mammalian tissues may also form homodimers. If this were true, twist is also likely to form heterodimers with dermo-1, which shares identical HLH domain and highly homologous basic and C-terminal regions adjacent to the HLH domain with twist. An implication is that twist may partner with many different bHLH proteins, including all known E-proteins (see chapters 1 and 2), twist itself, dermo-1 and related bHLH proteins, and hence bind to and regulate different kinds of promoters depending on its partner choice. The fact that

and regulate different kinds of promoters depending on its partner choice. The fact that dermo-1 and twist share overlapping domains of expression in dermomyotome and later in dermatome suggests possible interactions between these two factors in that lineage.

Discussion

To identify potential, endogenous partners of twist, twist bHLH region was used as a bait in a yeast two hybrid screen using the fusion cDNA library from E9.5 and E10.5 mouse embryos. The screen yielded twist itself, an E-protein, and several unknown sequences. This result suggests that in addition to the expected bHLH proteins, twist bHLH domain may interact with many different class of molecules. Recently, it has been shown that twist can interact with MEF2 family members when heterodimerized with E-proteins (Spicer *et al.*, 1996) No MEF2 sequence was identified from this screen but since only the bHLH domain was used in the screen, which is necessary for MEF2 interaction but may not be sufficient, it is hard to conclude the significance of this finding. MyoD, on the other hand, has been shown to interact with many non-bHLH factors such as Rb, E1A and c-jun. MyoD has also been shown to interact with MEF2 but since MyoD was not used as a bait in a similar screen, it is hard to tell how many of these physical interactions observed via biochemical means could be recapitulated in the yeast. What is clear though is that there are many non-bHLH factors that interact with bHLH proteins, often through the bHLH domain, and the yeast system is permissive enough to allow such interactions.

In a recent screen that resulted in the isolation of dermo-1, Li and colleagues identified 11 clones that fell into the "J9 class" (Li *et al.*, 1995). Members of this group share a 91-a.a. sequence homology that does not appear to encode a bHLH motif. Whether any of the TIPs that are "unknown" belong to this class of molecules is unclear. No sequence information for J9 class is available yet. It is probable that J9 class and some of the TIPs may encode for co-factors that interact with bHLH molecules to modulate their activity. A careful analysis of these molecules might yield interesting insights into the bHLH molecule regulation at the level of higher order complex formation.

It is possible that these unknown sequences are junk sequences that result from background interactions in the yeast. However, it is also possible that they may represent the missing co-factors that are necessary for some bHLH molecules to function. We and others have not been able to show transactivational activity of mammalian twist although the Drosophila twist has been shown to be an activator. For example, it would be interesting to see if twist can turn on the downstream reporter, an artificial promoter containing multimerized twist binding sites, in the presence of some TIP's. It has also been postulated that some of the cells lines that do not convert to muscle upon MyoD expression contain either dominant negative factors or that they are missing some key co-factors. Again, testing whether co-expression of J9 class or TIPs in these settings to see if they can convert these cells into myogenic pathway would be very interesting.

REFERENCES

Burgess, R., Cserjesi, P., Ligon, K. and Olson, E. (1995). Paraxis: a basic helix-loop-helix protein expressed in paraxial mesoderm and developing somites. *Developmental Biology* **168**, 296-306.

Conlon, R. A. and Rossant, J. (1992). Exogenous retinoic acid rapidly induces anterior ectopic expression of murine Hox-2 genes in vivo. *Development* **116**, 357-358.

Cserjesi, P., Brown, D., Ligon, K., Lyons, G., Copeland, N., Gilbert, D., Jenkins, N. and Olson, E. (1995). Scleraxis: a basic helix-loop-helix protein that prefigures skeletal formation during mouse embryogenesis. *Development* **121**, 1099-1110.

Li, L., Cserjesi, P. and Olson, E. N. (1995). Dermo-1: a novel twist-related bHLH protein expressed in the developing dermis. *Dev. Bio.* **172**, 280-292.

Skerjanc, I. S., Truong, J., Filion, P. and McBurney, M. W. (1996). A splice variant of the ITF-2 transcript encodes a transcription factor that inhibits MyoD activity. *J. Biol. Chem.* **271**, 3555-3561.

Spicer, D. B., Rhee, J., Cheung, W. L. and Lassar, A. B. (1996). Inhibition of myogenic bHLH and MEF2 transcription factors by the bHLH protein twist. *Science* **272**, 1476-1480.