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Temporal Restriction of MyoD Induction and Autocatalysis during *Xenopus* Mesoderm Formation

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In *Xenopus*, the activation of the myogenic determination factors MyoD and Myf-5 in the muscle-forming region of the embryo occurs in response to mesoderm-inducing factors (MIFs). Different members of the FGF, TGF-*β*, and Wnt protein families have been implicated in this process, but how MIFs induce the myogenic regulators is not known. For MyoD, the induction process may serve to locally stabilize a transient burst of ubiquitous transcription at the midblastula transition, possibly by triggering MyoD's autocatalytic loop. Here we have sought to distinguish separate activating functions during MyoD induction by analyzing when MyoD responds to different MIF signaling or to MyoD autoactivation. We show that MyoD induction depends on the developmental age of the induced cells, rather than on the type or time point of inducer application. At the permissive time, *de novo* MyoD induction by Activin requires less than 90 min, arguing for an immediate response, rather than a series of inductive events. MyoD autoactivation is direct, but subject to the same temporal restriction as MyoD induction by MIF signaling. Further evidence implicating MyoD autocatalysis as an essential component of the induction process comes from the observation that both autocatalysis and induction of MyoD are selectively repressed by a dominant-negative MyoD mutant. In summary, our observations let us conclude that MyoD's expression domain in the embryo results from an interplay of timed changes in cellular competence, pleiotropic signaling pathways, and autocatalysis.

INTRODUCTION

MyoD, Myf-5, Myogenin, and MRF4 comprise a small family of myogenic transcriptional regulators in vertebrates. *In vitro*, each of these four bHLH transcription factors is capable of converting a large number of different cell types into striated muscle. This remarkable feature is in part explained by the fact that the myogenic proteins bind to specific DNA consensus sites called E-boxes, which are functionally important motifs of many muscle-specific promoters and enhancer elements. In addition, they owe much of their potency to auto- and crossregulatory interactions among themselves and with members of the MEF-2 protein family (see Buckingham, 1994; Molkentin and Olson, 1996; Weintraub *et al.*, 1991a). *In vivo*, this self-propagating genetic network is held in check by multiple control mechanisms, which regulate both expression and activity of the myogenic regulators (reviewed in Cossu *et al.*, 1996; Yun and Wold, 1996). Clearly, some of these mechanisms are dominant over the myogenic differentiation program, since widespread overexpression of MyoD or Myf-5 in embryos causes some ectopic muscle formation, but not a general transformation of embryonic cell lineages into muscle (Hopwood *et al.*, 1991; Ludolph *et al.*, 1994; Miner *et al.*, 1992; Rupp *et al.*, 1994; Santerre *et al.*, 1993).

Targeted mutations in the mouse have revealed both redundant and specific functions for the myogenic proteins (reviewed in Olson *et al.*, 1996; Olson and Klein, 1994; Rudnicki and Jaenisch, 1995). The emerging consensus is that MyoD and Myf-5 are involved in the formation or survival of two myoblast lineages and that Myogenin is required for efficient terminal myoblast differentiation, while MRF4 is likely to have postembryonic functions. The evidence for nonredundant functions, however, should be taken with caution, since myogenin, when knocked in into the myf-5 locus, can rescue the Myf-5 loss-of-function phenotype (Wang *et al.*, 1996). Functional diversity of myogenic proteins may therefore be encoded—at least in part—by different enhancer sequences, which control the spatiotemporal expression of individual members of this gene family (see Weintraub, 1993).

Skeletal muscle cells in vertebrates originate from the

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myotomal compartment of the somites. In amniota, spatial and temporal specific expression patterns of the myogenic genes are correlated with the formation of the myotome (Buckingham, 1992; Pownall and Emerson, 1992; Smith *et al.,* 1993). Experiments in the chick suggest a complex interplay of both stimulatory and inhibitory signals from axial structures and dorsal ectoderm to be involved in somite patterning and expression of the myogenic genes (reviewed by Lassar and Münsterberg, 1996). Sonic hedgehog, Noggin, Wnt, and BMP-4 proteins are likely candidates for some of these signals (see Pourquié *et al.,* 1996; Reshef *et al.,* 1998). Interestingly, the sequence of MyoD and Myf-5 activation is reversed between avians and rodents (Pownall and Emerson, 1992), and induction of MyoD and Myf-5 is uncoupled from somitogenesis in zebrafish (Weinberg *et al.,* 1996) and *Xenopus* (Hopwood *et al.,* 1989, 1991; Rupp and Weintraub, 1991). Whether these differences are due to different input signals, or whether the same signals become engaged in muscle induction at different times and location in different species, is currently not known.

A more profound, and still open, question is whether these signaling pathways induce myogenic gene expression or whether they are required to maintain or expand a population of already specified cells. Clearly, mouse and chick embryos contain low levels of MyoD and Myf-5 mRNAs at presomite stages (see Cossu *et al.,* 1996; Kopan *et al.,* 1994), and myoD and myogenin genes are transiently activated in chick somites, even in the absence of the neural tube (Bober *et al.,* 1994). Most strikingly, isolated chick epiblast cells express MyoD and differentiate autonomously into muscle at very high frequency *in vitro* (George-Weinstein *et al.,* 1996). This suggests that MyoD-driven muscle differentiation may be a kind of "default state" and that signaling of the embryonic environment controls this default state by general repression and local induction/relieve from repression.

We had proposed a similar model of permissive *Xenopus* MyoD induction (Rupp and Weintraub, 1991), based on the observation that in the frog, MyoD is ubiquitously expressed at low levels for a short period of time following the midblastula transition (MBT; Newport and Kirschner, 1982). This period precedes and overlaps with the induction-dependent high-level MyoD expression in the presumptive mesoderm, where MyoD mRNA (Frank and Harland, 1991; Hopwood *et al.,* 1989) and protein (Hopwood *et al.,* 1992) are detectable *in situ* from the midgastrula stage onward. At this time MyoD is expressed in a horseshoe-like pattern around the blastoporus in the preinvoluted mesoderm, whose dorsal gap corresponds to the Spemann organizer. This expression domain fits well to the muscle fate map, which predicts both lateral and ventral mesodermal cells to contribute to the trunk musculature (Dale and Slack, 1987). In contrast, Myf-5 expression is induced in two symmetric domains flanking the organizer, but not in ventral mesoderm (Dosch *et al.,* 1997). The early activation of MyoD and Myf-5 probably has a determinative function, because muscle-forming competence is lost in the embryo (Grainger and Gurdon, 1989; Steinbach *et al.,* 1997) before myogenic precursors are comitted (Kato and Gurdon, 1993). Interestingly, MyoD is also strongly expressed, although only transiently, in embryos, which have been ventralized by UV irradiation (Frank and Harland, 1991). Since such embryos develop no notochord, neural tissue, or muscle, axial structures are required for the maintenance, but *not* for the induction of MyoD expression in the frog.

The frog *Xenopus laevis* provides a particularly attractive system for studying inductive cell–cell interactions, due to the presence of pluripotential cells in the animal hemisphere of the blastula. As tissue explants ("animal caps"), these cells can be triggered to embark on multiple differentiation programs in a way, which closely resembles the normal induction and patterning of mesodermal and neuroectodermal tissues. Interestingly, these cells undergo with increasing developmental age a series of cell-autonomously programmed changes in competence (Servetnick and Grainger, 1991), which influence the tissue types formed by inductive stimuli. Mesodermal competence lasts from early cleavage to gastrula stages (Grainger and Gurdon, 1989; Jones and Woodland, 1987), when it is lost as a consequence of gene-selective transcriptional silencing by somatic H1 linker histones (Steinbach *et al.,* 1997). A large number of secreted proteins have been implicated as inducers in mesoderm induction (for reviews see Heasman, 1997; Smith, 1995), and some of these factors have been reported to promote myogenic gene expression and muscle differentiation. The latter include members of the FGF and TGF-β families of peptide growth factors (such as basic FGF, Activin, or Vg-1), Wnt family members (in particular XWnt-8), and bone morphogenetic proteins and their inhibitors (e.g., BMP-4 and Noggin) (see Dosch *et al.,* 1997; Hoppler *et al.,* 1996; Re'em-Kalma *et al.,* 1995; Smith, 1995).

It is not known how these signaling factors activate *Xenopus* MyoD. Because both MyoD mRNA and pre-mRNA levels rise during this process (Harvey, 1990), MyoD induction must be controlled—at least in part—on the transcriptional level. According to our model, induction may locally upregulate and stabilize basal MyoD transcription by establishing a positive autoregulatory loop (Rupp and Weintraub, 1991). In contrast, several other observations suggested that MyoD expression in the mesoderm may result from a cascade of inductive events. First, MyoD induction has been characterized as a delayed, cycloheximide-sensitive response to Activin (Harvey, 1991). Second, MyoD induction by Activin may be mediated by XWnt-8, because zygotic XWnt-8 signaling is required for MyoD expression in the ventrolateral mesoderm (Hoppler *et al.,* 1996) and because Activin is known to induce XWnt-8 transcription in animal caps (Christian *et al.,* 1992). Third, MyoD's autoactivation loop does not function at the MBT, although injected MyoD protein is transcriptionally active at this time (see Rupp *et al.,* 1994). This indicates that MyoD autocatalysis is developmentally regulated. In the present study, we have sought to investigate the epistatic relationships of mesoderm-inducing factors and

MyoD autocatalysis by time course analysis of MyoD induction in animal caps.

MATERIAL AND METHODS

Embryo Manipulations

The *in vitro* fertilization of eggs, culture of embryos, and dissections of animal cap explants has been carried out as described (see Rupp *et al.*, 1994; Rupp and Weintraub, 1991). At 23°C constant temperature in 0.1× modified Barth's Saline (MBS), we found embryos to develop slightly faster than described by Nieuwkoop and Faber (1967). Embryos were microinjected with capped synthetic transcripts, either into the animal pole at the two-cell stage (10 nl/embryo) for preloading of animal cap tissue or equatorially into one cell (5 nl) at the two-cell stage for targeting the transcripts to the somitogenic mesoderm. Animal cap explants were cut at late blastula (6.75–7.5 hours postfertilization (hpf)) and cultured singly in 0.5× MBS/2% BSA in 1% agarose/0.5× MBS-coated plastic dishes. Activin A was applied as conditioned medium of P388D1 cells (Sokol *et al.*, 1990) diluted in 0.5× MBS/2% BSA. Since animal caps round up rapidly after explantation, they were either transferred immediately to Activin A-containing medium or reopened mechanically with eyebrow knives to allow growth factor access at the desired time points. To block protein synthesis, animal caps were incubated with 10 μg/ml cycloheximid (CHX; Sigma) in 0.5× MBS/2% BSA for 30 min and then rinsed twice in 0.5× MBS/2% BSA. Under these conditions CHX inhibits ≥90% of protein synthesis for several hours (Cascio and Gurdon, 1987). For hormone induction of the MyoD-GR variant, animal caps were treated with 10 μM dexamethasone (DEX; Sigma) in 0.5× MBS/2% BSA (Kolm and Sive, 1995).

Plasmids and in Vitro *RNA Synthesis*

The pBSKS$^+$-XMyoDb (*Xenopus* MyoDb; Rupp and Weintraub, 1991), pCS2$^+$-mMyoD (mouse MyoD; Rupp *et al.*, 1994), p64T-MD-GR (mouse MyoD–glucocorticoid receptor fusion gene; Kolm and Sive, 1995), pSP64T-mβA (mouse Activin βA; Albano *et al.*, 1993), pCS2$^+$NLS-βgal (nuclear variant of *Escherichia coli* lacZ; Steinbach *et al.*, 1997), and pGEM5Zf (−)/RI-XWnt8 (*Xenopus* Wnt-8; Smith and Harland, 1991) plasmids have been described previously. pSP64T-HF37/bFGF encodes a bovine basic FGF variant, which by a single amino acid exchange has been converted into human bFGF; it also includes a signal peptide for efficient secretion. This variant is more active than wild-type *Xenopus* bFGF in mesoderm induction assays (D. Kimelman, personal communication).

The repressor domain of the *Drosophila*-engrailed protein (enR; amino acids 1–298; GenBank Accession No. M10017) was subcloned in frame into the pCS2$^+$MT6 and pCS2$^+$NLSMT6 (Rupp *et al.*, 1994) by PCR from a *Drosophila*-engrailed cDNA, using the forward primer 5′-GAAGGCCTACTAGTACAATGGCCCTGGAGGAT-3′ and reverse primer 5′-CCGCTCGAGAGGATCCCAGAGCAGATT-3′ (primer-based *Stu*I and *Xho*I restriction sites, which have been used for subcloning, are underlined). The mouse MyoD (bHLH) domain (amino acids 102–162) was amplified by PCR from the plasmid pVZ-11s (Davis *et al.*, 1987) using the forward primer 5′-CGGAATTCGGATATGGAGCTCAAGCGCAAGACCAC-3′ and the reverse primer 5′-GGACTAGTGTCGCGGCAG-CAGAGCCTGCA-3′ and subcloned via the underlined *Eco*RI and *Spe*I restriction sites into pCS2$^+$MT6-enR between the myc-epitope tag and the enR domain to yield pCS2$^+$MT6-MyoD(bHLH)-enR. pCS2$^+$MT6-E12basic(bHLH)-enR was generated by a similar strategy, starting however with the plasmid pEMSV-MyoDE12basic as PCR template and using a different forward primer, 5′-CGGAATTCGGATATGGAGCTTCAGAA GGCCGAGCGGGAG-3′, which matches to amino acid residues 552–557 of the human E12 protein (see Davis *et al.*, 1990).

For *in vitro* transcription, plasmids were linearized with Asp718 (pCS2$^+$mMyoD, pCS2$^+$XMyoDb, pCS2$^+$NLSMT6-enR, pCS2$^+$MT6-mMyoD(bHLH)-enR and pCS2$^+$MT6-E12basic(bHLH)-enR), *Hin*dIII (pBSKS$^+$XmyoDb), *Eco*RI (pSP64T-HF37/bFGF), *Bam*HI (p64T-MD-GR), *Sma*I (pSP64T-mβA), or *Not*I (pGEM5Zf(−)/RI-XWnt8, pCS2+NLS-βgal). The synthesis of capped SP6 transcripts and of the digoxygenin-labeled XmyoDb antisense probe for *in situ* hybridization was carried out as described (see Steinbach *et al.*, 1997).

RNA Analysis by Quantitative RT/PCR

Total cellular RNA from embryos and explants was prepared with the Tristar protocol (AGS, Heidelberg, Germany). Random-primed reverse transcription and PCR amplification of gene-specific fragments were performed under conditions that ensure a direct correlation between RNA template abundance and PCR product amounts and strict dependence of PCR products on cDNA synthesis (see Rupp and Weintraub, 1991; a detailed description is available upon request). After gel electrophoresis, the relative PCR product amounts were determined by quantitating incorporated [α-^{32}P]dCTP trace label with phosphor storage technology (Molecular Dynamics). Primer pairs for genes of interest were always used in multiplex PCR with H4 primers as internal standards. Due to the relative high abundance of H4 mRNA compared to other transcripts, 9 cycles of preamplification were performed with gene-specific primers, before the H4 primers were added, and amplification continued for 19 additional cycles. The standard temperature profile was denaturation (94°C for 30 s), primer annealing (58°C for 30 s), and extension (72°C for 1 min). Data points of independent experiments were calculated as arithmetic means of duplicate RNA samples, each consisting of three animal caps. They represent average increases in steady-state mRNA levels of induced over uninduced explants after normalization to histone H4 mRNA levels. Both XMyoDa and XmyoDb genes responded similarly to the various experimental conditions, although relative mRNA levels of XMyoDa were at least 10-fold lower than those of XMyoDb (not shown). For the sake of simplicity we only present data for XMyoDb. The following PCR primers have been used (F, forward; R, reverse): XMyoDb, histone H4 (see Rupp and Weintraub, 1991; Steinbach *et al.*, 1997); Xmyf-5, 5′-TCTAGCTGTTCAGATGGCA-3′ (F), 5′-TTAAGAGAGGCTTATAACAC-3′ (R) (positions 575–873; GenBank Accession No. X56738).

Whole Mount in Situ Hybridization

Whole mount *in situ* hybridization was performed as described (Steinbach *et al.*, 1997). XMyoDb sense RNA probes gave no hybridization signals (data not shown). For lineage-tracing analysis, embryos were fixed for 1 h, followed by β-galactosidase staining (color development <1 h).

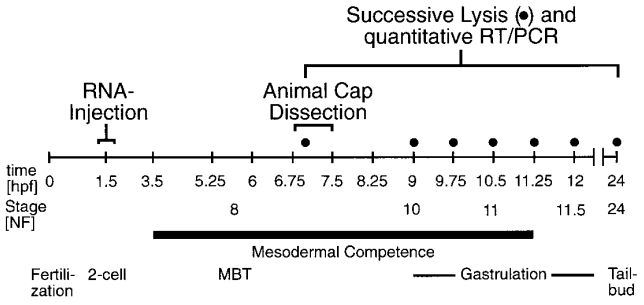

FIG. 1. Developmental time scale and flow diagram of experimental manipulations. Developmental time is given as hours post fertilization (hpf). NF, Nieuwkoop and Faber stages. The mesodermal competence period, represented by the black bar, lasts from about 3.5 to 11.25 hpf. Where applicable, embryos were injected into the animal hemisphere at the two-cell stage with capped, synthetic transcripts. The application regimens for soluble reagents are described in the respective figure legends. Animal caps were routinely cut shortly after the midblastula transition (MBT), i.e., between 6.75 and 7.5 hpf and cultured until successively later time points (●), at which time they were lysed and subjected to quantitative RT/PCR analysis.

RESULTS

MyoD Induction Is Temporally Restricted

Mesoderm induction is thought to occur from the 64-cell stage on to early gastrula (Jones and Woodland, 1987). Since zygotic transcription starts at the midblastula transition (Newport and Kirschner, 1982), there is 6 h time for the transcriptional activation of mesodermal genes before mesodermal competence disappears (Grainger and Gurdon, 1989; Steinbach *et al.*, 1997). In order to discriminate between the relative contributions of growth factors and MyoD autoactivation for MyoD expression we investigated in developmental time course experiments when different stimuli activate MyoD in animal caps (see Fig. 1). In preparatory experiments we had found that bFGF alone (≤50 pg RNA/embryo of HF37; see Materials and Methods) did not induce MyoD mRNA significantly (data not shown). As expected, however, it strongly synergized with Xwnt-8, which is not a mesoderm-inducing factor (Christian *et al.*, 1992), but is required for MyoD expression in the mesoderm (Hoppler *et al.*, 1996). To trigger mesoderm induction in animal caps, we therefore coinjected synthetic transcripts for bFGF and Xwnt-8 at the two cell stage or added Activin protein (Sokol *et al.*, 1990) to the explants immediately after dissection. To promote MyoD autoactivation we injected either mouse or high amounts of *Xenopus* MyoD transcripts (see Rupp *et al.*, 1994).

Unexpectedly, the kinetics of MyoD induction in animal caps as estimated by quantitative RT/PCR were initially indistinguishable between the various conditions (Fig. 2). In each case, a significant and quantitatively comparable increase in MyoD mRNA levels occurred between 9.75 and 10.5 hpf, i.e., around NF stage 10.25 (Nieuwkoop and Faber, 1967). The same result was found with animal caps, in which Activin had been provided early by RNA microinjection at the two-cell stage (data not shown). Subsequently, MyoD mRNA levels in general continued to accumulate over time (Fig. 2, 24 hpf), correlated with muscle differentiation (data not shown). An exception was XMyoD-injected animal caps, which showed only a marginal increase in endogenous XMyoD mRNA from 12 to 24 hpf and which was the only activator tested, which did not cause muscle differentiation in the explants (Hopwood and Gurdon, 1990; O. C. Steinbach and R. A. W. Rupp, unpublished results). The basis for the different activities of mouse and *Xenopus* MyoD proteins in this assay is unknown. Importantly, the timing of MyoD induction in animal caps was precisely correlated with the temporal upregulation of MyoD mRNA in the mesoderm of unmanipulated sibling embryos (Fig. 2, black bars), and therefore these results are relevant for normal development.

The 4-h time gap between the start of zygotic transcription at the MBT and MyoD induction seems to suggest that MyoD mRNA accumulation is a secondary consequence of mesoderm induction, which requires other immediate-early response genes. Consistent with this assumption, MyoD induction has been characterized as a delayed, cycloheximide-sensitive response to Activin requiring be-

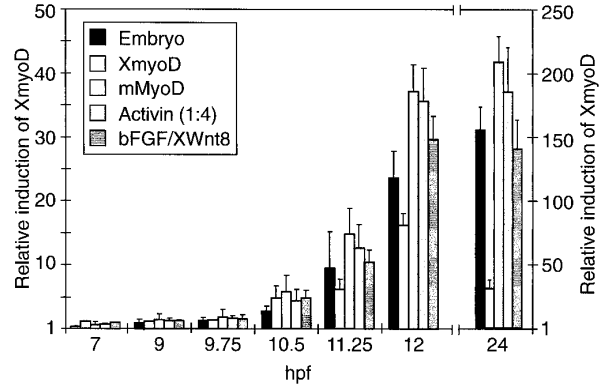

FIG. 2. The onset of MyoD induction. Columns show the average increase of XMyoD steady-state mRNA levels as a function of time. RNA samples were from control embryos or from animal cap explants injected either with synthetic transcripts encoding XmyoD (400 pg) or mouse MyoD (200 pg) or with a combination of bFGF (50 pg) and XWnt-8 (10 pg) transcripts. In this series of experiments, Activin induction was triggered by application of a 1:4 dilution of Activin-containing tissue culture supernatant at 7 hpf. For all five conditions tested, myoD induction occurs between 9.75 and 10.5 hpf. The relative XMyoD induction is calculated as mRNA increase of induced over control explants of the same developmental age, after normalization to H4 mRNA as internal standard (see Materials and Methods for details). (n = 3–5 independent experiments; error bars, SD). Note the broken x-axis and the different y-axis scale for the 24 hpf time point.

tween 6 and 10 h (Harvey, 1991). Puzzled by the synchronous activation of MyoD through several mechanistically unrelated stimuli, we decided to determine the minimal delay between Activin application and MyoD mRNA increase. To do this, we applied Activin at 9.75 hpf to animal cap explants, i.e., 45 min before MyoD activation occurs (Fig. 3a). Under these conditions, MyoD mRNA levels increased about 10-fold within 90 min, i.e., with a similar slope as in our previous experiments (Fig. 2) and comparable to induction kinetics of immediate-early response genes such as Xbra (Smith *et al.*, 1991) or Mix.1 (Rosa, 1989).

This rapid increase in MyoD mRNA in response to Activin induction seems incompatible with a hierarchic event, in which Activin triggers first transcription, translation, and secretion of Xwnt-8, which then induces MyoD. To test this argument, we coexpressed a dominant-negative XWnt-8 variant (dnXWnt-8; Hoppler *et al.*, 1996) with the various inducers in the animal cap. While we found that it had no effect on MyoD expression driven by activin or autocatalysis, dnXWnt-8 interfered—as expected—with MyoD induction by bFGF/XWnt-8 (Fig. 4). Interestingly, complete inhibition of MyoD activation by bFGF/XWnt-8 at 12 hpf required a time advance in the expression of the dnXWnt-8 variant over the wild-type protein (compare the second and third columns in Fig. 4a; see figure legend for details), probably reflecting a requirement for high protein levels of the interference mutant for efficient competition with the wild-type XWnt-8 protein. At 24 hpf (Fig. 4b), however, MyoD was silenced even when dnXWnt-8 was expressed simultaneously with bFGF/XWnt-8 (see second columns in Figs. 4a and 4b). Similar results were obtained in experiments, in which wild-type XWnt-8 function was supplied by plasmid-borne transcription (data not shown). Together, these experiments may indicate a dual function for post-MBT XWnt-8 signaling in both activation and maintenance of MyoD expression during (bFGF/XWnt-8)-mediated MyoD induction.

In summary, we conclude that MyoD activation depends primarily on the developmental age of the induced cells, rather than on the type or time point of inducer application. The same conclusion has been reported for the timing of muscle actin expression (Gurdon *et al.*, 1985). Indeed, the latter may be explained explained by the former, because *exogenous* MyoD protein is sufficient to trigger precocious muscle actin expression at the MBT (Rupp *et al.*, 1994).

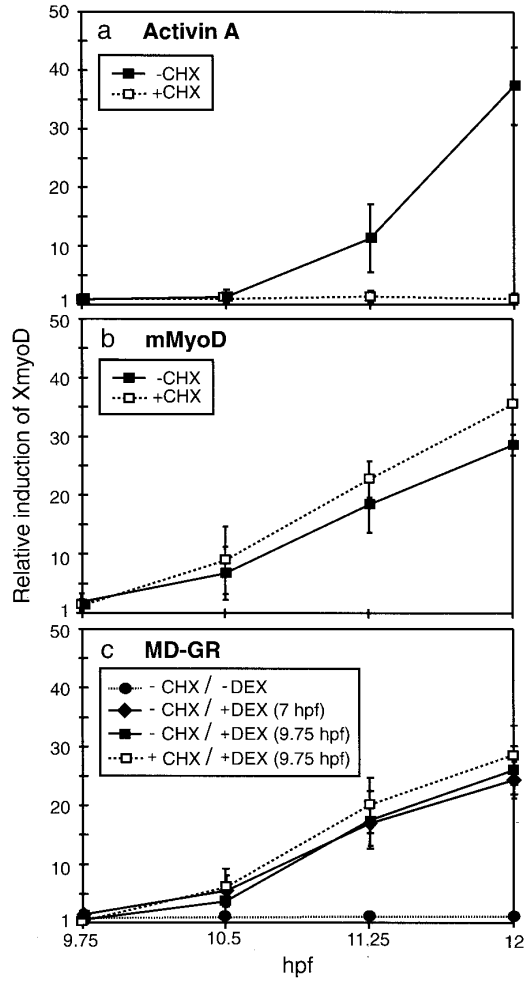

FIG. 3. MyoD activation is a fast response to Activin, but requires protein synthesis. (a) Addition of soluble Activin to animal caps at 9.75 hpf leads to an increase in XMyoD mRNA levels within 45 to 90 min. Explants, which were previously incubated with cycloheximide (CHX) from 9.25 to 9.75 hpf, show no increase in XMyoD mRNA. (b) mMyoD protein, translated from microinjected RNA, promotes autoactivation in the absence of protein synthesis (CHX treatment from 9.25 to 9.75 hpf). (c) MyoD autoactivation is direct. In this experiment, autocatalysis is triggered by the dexamethasone-inducible MD-GR protein, which has been preexpressed by mRNA injection at the two-cell stage (100 pg/embryo). After protein synthesis has been blocked by CHX treatment as in a, MD-GR is activated through dexamethasone application at 9.75 hpf. Note that MD-GR is completely inactive without dexamethasone ($n = 4$ for each panel).

MyoD Autoactivation Is Direct

Despite its fast response, MyoD induction by Activin is nevertheless completely inhibited by cycloheximide (Fig. 3a; see also Harvey, 1991), indicating the involvement of some unstable protein(s) in this process. Theoretically, this protein could be MyoD itself, since the gene is transcribed ubiquitously at low levels from MBT through gastrula stages (Rupp and Weintraub, 1991). Consistent with this idea, we show in Fig. 3b that cycloheximide did not interfere with myoD autoactivation, when either mouse MyoD protein or high levels of XMyoD protein (data not shown) were provided early through mRNA injection.

We also tested MD-GR, a hormone-inducible variant of

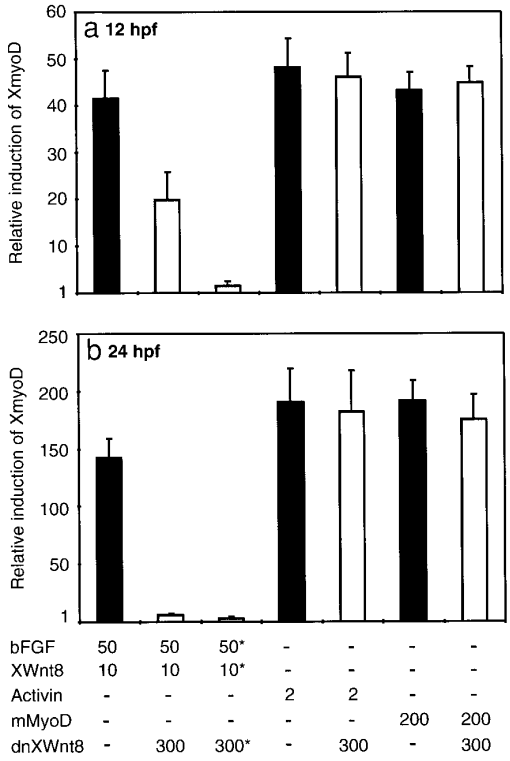

FIG. 4. Dominant-negative XWnt-8 blocks MyoD activation by bFGF/XWnt-8, but not by Activin or autocatalysis. RT/PCR analysis of relative XMyoD mRNA levels in animal caps, which were preloaded with synthetic transcripts as indicated below the panels (numbers refer to the injected RNA dose in pg/embryo). dnXWnt-8, dominant-negative XWnt-8 (Hoppler *et al.*, 1996). For the experiment in column 3 (*), dnXWnt-8 RNA was injected at the two-cell stage, followed by coinjection of bFGF/XWnt-8 RNAs at the eight-cell stage, to provide a head start for the intereference mutant. Panels show MyoD mRNA levels at: a, 12 hpf (i.e., shortly after activation); or b, 24 hpf (tail bud stage; during muscle differentiation) ($n = 3$).

mouse MyoD (Hollenberg *et al.*, 1993). This fusion protein contains the ligand-binding domain of the human glucocorticoid receptor inserted in frame into the MyoD-coding region, and its activity depends strictly on glucocorticoids (Hollenberg *et al.*, 1993; Kolm and Sive, 1995). In the presence of dexamethasone, MD-GR promoted MyoD transcription with the same efficiency as Activin or wild-type MyoD protein (compare Figs. 3a and 3b), even when it was activated after protein synthesis had been blocked by cycloheximide (Fig. 3c). Similar to wild-type MyoD proteins (see Fig. 2), early activation of MyoD-GR by dexamethasone at 7 hpf did not cause precocious MyoD activation (Fig. 3c). We conclude that the MyoD autoactivation loop is direct, but subject to the same temporal restriction as the signal input of mesoderm inducing factors in MyoD activation.

A Dominant-Negative MyoD Mutant Blocks MyoD Activation

While we have demonstrated that MyoD autoactivation can be triggered in animal caps by exogenous MyoD protein, we do not know whether it actually occurs *in vivo*. To address this issue, we fused the transcriptional repressor domain of the *Drosophila*-engrailed protein to MyoD's bHLH domain (together with a myc-epitope tag; see Materials and Methods). This follows an idea that has been first applied for the c-myb protein (Badiani *et al.*, 1994), as well as for the analysis of Xbra functions in *Xenopus* (Conlon *et al.*, 1996). This MT6-MyoD(bHLH)-enR variant is expected to compete with endogenous MyoD protein for cognate DNA-binding sites and to repress transcription of MyoD target genes.

Two control constructs were used to assess the specificity of the MT6-MyoD(bHLH)-enR variant. The first, called NLSMT6-enR, encodes a nuclear localized, myc-tagged version of the enR domain; in the absence of a DNA-binding domain, this variant should control for potential squelching effects of the engrailed repressor domain. The second construct, called MT6-E12basic(bHLH)-enR, is identical to MT6-MyoD(bHLH)-enR, except that MyoD's basic region has been replaced with the corresponding region from MyoD's nonmyogenic dimerization partner E12 (see Davis *et al.*, 1990). The basic region comprises the DNA-binding domain of HLH transcription factors, and it has been shown previously that a small number of specific residues within this region, which are not found at the corresponding positions in E12, encode the myogenic specificity of MyoD (discussed by Yun and Wold, 1996). In analogy to the related parental MyoD-E12basic protein (Davis *et al.*, 1990), MT6-E12basic(bHLH)-enR is expected to heterodimerize with E proteins and to bind to DNA, but to lack myogenic specificity. Since a myogenic basic region is required for MyoD autoactivation both *in vitro* (Weintraub *et al.*, 1991b) and *in vivo* (Rupp *et al.*, 1994), this variant might be predicted not to interfere with MyoD induction, unless overexpression of MyoD-enR fusion proteins would bypass the normal restrictions of MyoD functions. Using an antibody directed against the myc epitope tag, all three enR fusion proteins were shown by Western analysis to have similar stability in the embryo and by immunocytochemistry to accumulate preferentially in the nuclear compartment (data not shown).

In the animal cap system, MT6-MyoD(bHLH)-enR repressed MyoD autoactivation efficiently, when it was coexpressed with either mouse or *Xenopus* MyoD proteins (Fig. 5a and data not shown). It also inhibited Activin-dependent MyoD induction (Fig. 5b). In each case, repression was dose dependent and gene specific, because transcription of housekeeping genes like histone H4 was normal (data not shown), and—more importantly—Activin induction of the other early myogenic factor XMyf-5 was unaffected (Fig. 5d). In addition, repression required tethering of the enR moiety to DNA target sites via a myogenic

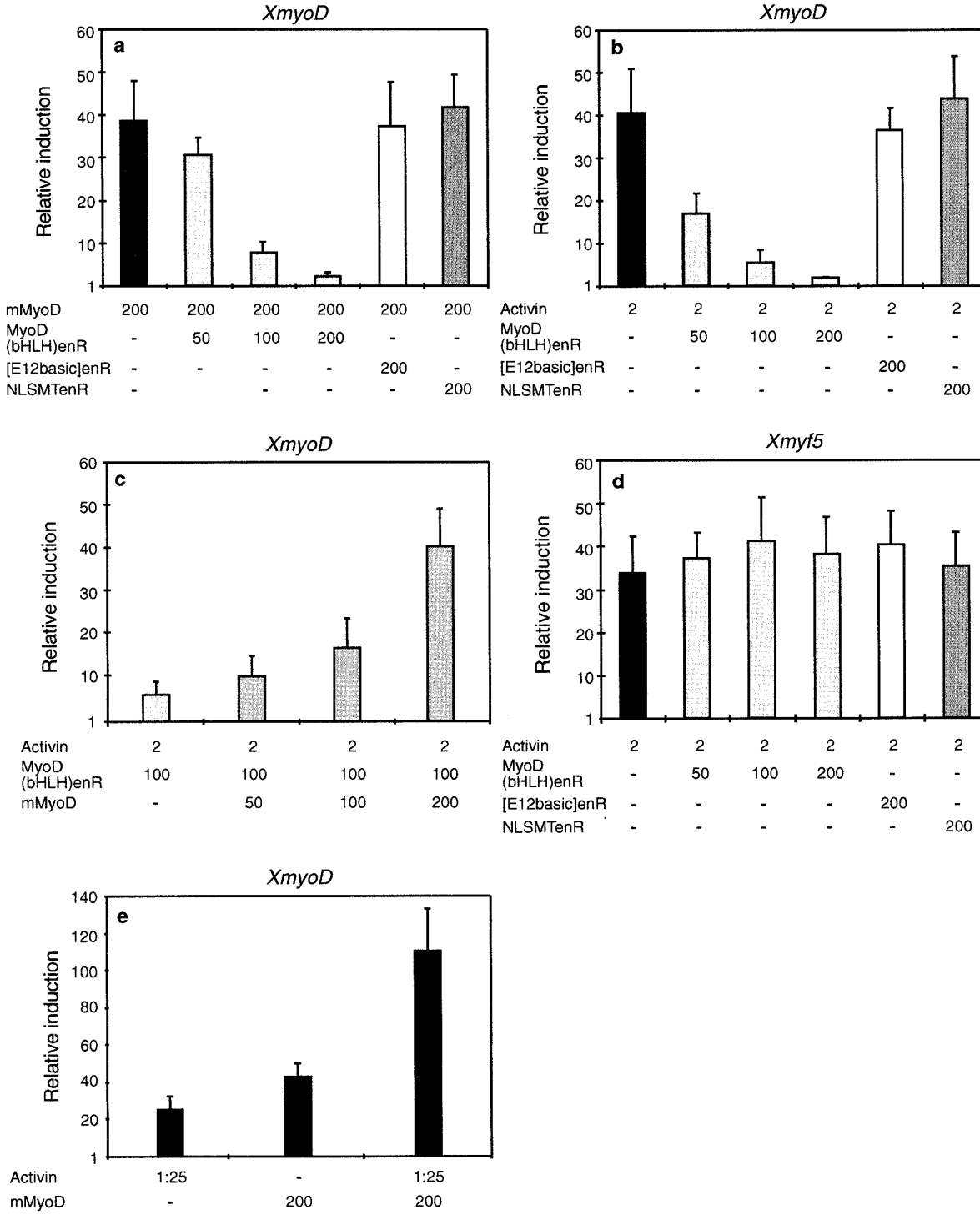

FIG. 5. MT6-MyoD(bHLH)-enR blocks MyoD induction in animal caps. RT/PCR analysis of XMyoD (a–c) or XMyf-5 (d) mRNA levels in animal caps, preloaded with synthetic transcripts as indicated below the panels (numbers refer to the injected RNA dose in pg/embryo). Panels show: a, MT6-MyoD(bHLH)-enR inhibits MyoD autoactivation; b, MT6-MyoD(bHLH)-enR interferes with Activin-induction of MyoD; c, Activin induction of MyoD is rescued by coinjection of MT6-MyoD(bHLH)-enR and wild-type MyoD protein; d, Activin induction of XMyf-5 is unaffected by MT6-MyoD(bHLH)-enR; e, Synergism of autocatalysis and Activin induction. Activin protein was applied as a 1:25 dilution of Activin containing tissue culture medium (Sokol *et al.*, 1990); mMyoD protein was provided by RNA injection (200 pg/embryo) ($n = 4$).

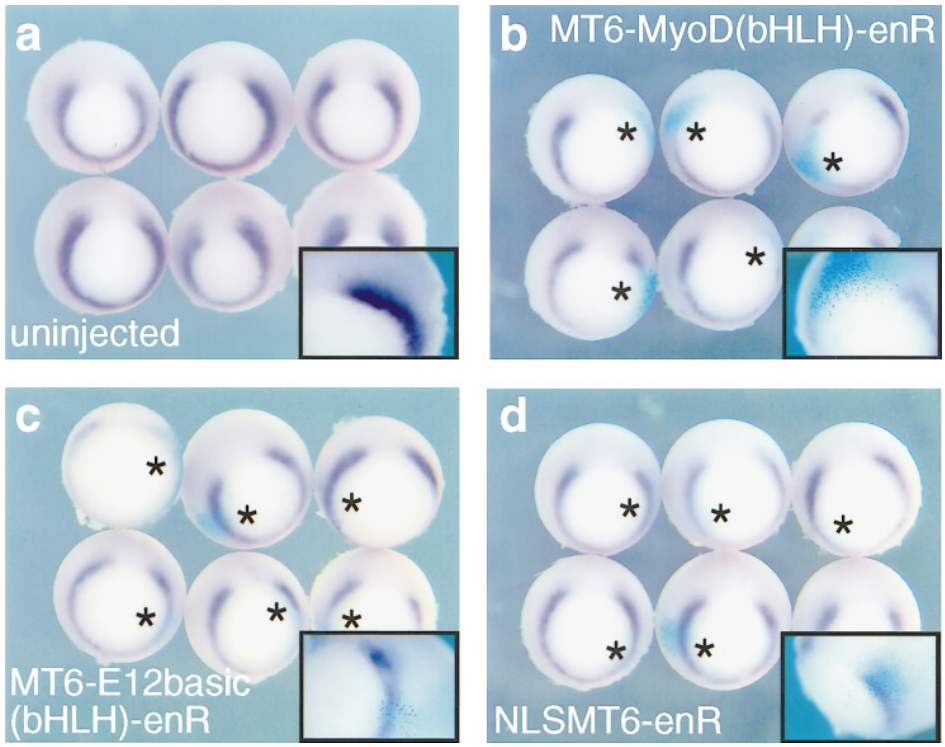

FIG. 6. MT6-MyoD(bHLH)-enR inhibits MyoD induction *in vivo*. RNA *in situ* hybridization with an antisense XMyoDb probe at midgastrula (NF 11), shortly after MyoD induction. (a) Uninjected control embryos. Embryos were injected equatorially into one cell at the two-cell stage with 100 pg lacZ mRNA as lineage tracer, together with transcripts encoding the following enR-fusion proteins: (b) MT6-MyoD(bHLH)-enR (50 pg/embryo); (c) MT6-E12basic(bHLH)-enR (50 pg/embryo); (d) NLSMT6-enR (200 pg/embryo). Asterisks mark the location of β-galactosidase-positive cells, indicating the injected region. Inserts show representative close-ups of MyoD and β-galactosidase staining.

DNA-binding domain, since neither NLSMT6-enR nor MT6-E12basic(bHLH)-enR proteins had any significant influence on the levels of the mRNAs tested (Figs. 5a–5d). Finally, coexpression of wild-type MyoD protein rescued the repression of Activin-driven MyoD induction by MT6-MyoD(bHLH)-enR (Fig. 5c). Together, these results exclude an unspecific poisoning of MyoD's feedback loop or the Activin signaling pathway by MT6-MyoD(bHLH)-enR. This fusion protein is therefore a potent repressor variant of MyoD, but not a deregulated allele.

Interestingly, Activin caused a four-fold superstimulation when coinjected together with wild-type MyoD and MT6-MyoD(bHLH)-enR transcripts, compared to the stimulation caused by the same ratio of these two RNAs alone (Fig. 5, compare the third bar in Fig. 5a with the last bar in Fig. 5c). This observation suggested a synergism between the autoactivation and induction modes, which has been verified in separate experiments in the absence of MT6-MyoD(bHLH)-enR (Fig. 5e).

Upon unilateral injection into the marginal zone at the two-cell stage, MT6-MyoD(bHLH)-enR repressed the endogenous MyoD expression in the mesoderm of embryos at the early gastrula stage, presumably from the time point of activation onward (Figs. 6a and 6b, Table 1). At the tadpole stage it was found to dominantly repress muscle differentiation (data not shown). The ablation of MyoD expression was spatially correlated with the staining of the coinjected lineage-tracer β-galactosidase, consistent with the assumption that MT6-MyoD(bHLH)-enR acts in a cell-autonomous manner.

The NLSMT6-enR control protein had no effect on MyoD expression (Fig. 6d, Table 1). However, the MT6-E12 basic(bHLH)-enR variant had a weak but notable influence on MyoD induction in the mesoderm (Fig. 6c, Table 1). The basis for the more potent activity of this variant in mesodermal cells of the marginal zone compared to Activin-induced animal cap cells is not known. Perhaps the induced state of marginal zone cells is less robust at this stage, and therefore they may be more vulnerable to interference than animal cap cells. Certainly, ablation of MyoD induction in the mesoderm required lower doses of MT6-MyoD(bHLH)-enR RNA than in animal caps (Table 1 and Fig. 5). It may also be of relevance that MyoD homodimers are known to exist *in vivo* (Maleki *et al.*, 1997). Thus, it is possible that in these circumstances the engrailed repressor domain is recruited to the MyoD locus by low amounts of pseudo-

TABLE 1
Inhibition of XMyoD Expression *in Vivo*

RNA	pg/embryo	*n*	wt (%)	pt (%)
MT6-MyoD(bHLH)-enR	50	27	3 (11)	24 (89)
	15	39	32 (82)	7 (18)
	5	37	36 (97)	1 (3)
MT6-MyoD-E12(bHLH)-enR	50	22	19 (86)	3 (14)
	15	38	38 (100)	–
	5	42	42 (100)	–
NLS-MT6-enR	200	32	32 (100)	–
	100	42	42 (100)	–
Uninjected	N/A	54	53 (98)	1 (2)

Note. Embryos were unilaterally injected into the marginal zone at the two-cell stage (three independent experiments). *n,* number of embryos; wt, wild-type MyoD expression; pt, partial ablation of MyoD expression.

homodimers between endogenous MyoD and exogenous MT6-E12basic(bHLH)-enR proteins. Most importantly, however, these results indicate that MyoD autoactivation occurs *in vivo* at the time of MyoD induction and therefore is likely to be an essential component of the induction process.

DISCUSSION

In an attempt to distinguish individual functions for inducing factors involved in MyoD expression during *Xenopus* mesoderm formation, we have discovered a dominant regulatory mechanism, which restricts the onset of MyoD induction to the early gastrula stage. The existence of such a mechanism is inferred from the following observations. First, zygotic transcription has already been going on for about 4 h (i.e., six intervals of our time course analysis), when MyoD mRNA levels rise significantly within 45 min (i.e., within one interval; Fig. 2). Second, *de novo* MyoD induction requires not more than 45–90 min (Fig. 3). Finally, all stimuli tested (Activin, bFGF/XWnt-8, and MyoD) are able to transactivate other genes at earlier developmental stages than MyoD (see Rupp *et al.,* 1994, and data not shown). Based on the synchrony between MyoD induction in animal caps and its activation in the mesoderm, this mechanism appears to operate in the whole animal hemisphere and to be independent from germ layer specification.

During the course of these experiments, we have found that dominant-negative XWnt-8 has no effect on autocatalysis or Activin-mediated MyoD expression, while it blocks MyoD activation by bFGF/XWnt-8 in animal caps (Fig. 4), as well as in the marginal zone of the embryo (Hoppler *et al.,* 1996). These results indicate that there is more than one mechanism by which MyoD can be activated in competent embryonic cells. Which, or how many of these, are physiologically relevant cannot be decided at the moment. For example, ventral marginal zone cells express Sizzled, a secreted XWnt-8 antagonist, from the MBT onward (Salic *et al.,* 1997). Overexpressed Sizzled protein blocks MyoD activation, just like dnXWnt-8. In the wild-type situation, however, Sizzled and MyoD are coexpressed in the ventral marginal zone, at least for some time (Fig. 6 and Salic *et al.,* 1997). This suggests that the ventral part of MyoD's expression domain may not depend critically on zygotic XWnt-8 signaling. Examples for regional and/or combinatorial signaling inputs into contigous expression domains have been reported for Xcad-3 and Xbra (Northrop *et al.,* 1995). The main finding of our study—a temporal restriction of MyoD activation—gains its strength from the fact that this phenomenon is observed under very different stimulation conditions *in vitro* and its precise temporal correlation with the normal MyoD activation in the embryo. Therefore, its validity does not primarily depend on the issue, whether each of the signaling factors we used reflects the normal route of MyoD induction.

Involvement of an Autoregulatory Loop in MyoD Induction

Based on MyoD's ubiquitous low-level transcription at the MBT we had previously postulated that the induction process may establish MyoD's autocatalytic loop (Rupp and Weintraub, 1991). The observations presented here lend further weight to this notion, although they do not prove it yet. First, autoactivation and induction of MyoD cannot be uncoupled temporally. Second, during the permissive period, MyoD induction is rapid, but nevertheless requires protein synthesis. Third, we have shown that autocatalysis is direct, synergizes with Activin induction, and occurs *in vivo.* Finally, we note that MyoD mRNA accumulates faster in mMyoD-injected animal caps compared with activin-induced explants (compare Figs. 3a and 3b). This advance is expected, given that the MyoD-RNA-injected animal caps should be ahead by one round of transcription and translation. Interestingly, this lead is not observed when Activin protein is applied hours before the onset of MyoD induction (Fig. 2, 10.5 hpf), perhaps because the induced cells are already poised for myoD transcription under these conditions, before the gene becomes responsive. This may involve endogenous MyoD protein synthesized from newly transcribed MyoD mRNA at the MBT (Rupp and Weintraub, 1991) or other proteins involved in myoD transcription.

In summary, the results presented here describe a perfect correlation between autoactivation and induction-dependent modes of MyoD expression under several independent experimental conditions. In *Drosophila,* expression of the Labial homeobox protein in the midgut (Tremml and Bienz, 1992), or of the proneural HLH protein Atonal in the developing eye (Baker and Yu, 1997), provides a further example for a tight coupling of feedback loops and inductive events. It is an open question whether such coupling is

merely advantageous, for instance, in fast developing systems, or whether it provides a basic function for establishing stable gene expression domains, as has been suggested on theoretical grounds (see Meinhardt, 1978).

Mechanism of the Temporal Control of MyoD Induction

Previously, we had reported that *Xenopus* embryos regulate the nuclear transport of MyoD, based on the observation that exogenous XMyoD protein was found to accumulate in the cytoplasm of cells in the animal pole, but to be nuclear in the mesoderm (Rupp *et al.*, 1994). We know now that plasmid-borne XMyoD protein is constitutively nuclear, and so only XMyoD protein, which is synthesized at pre-MBT stages, may be subject to differential localization (Rupp, unpublished results). Given that MyoD autoactivation by mouse MyoD protein, which is constitutively nuclear (Rupp *et al.*, 1994), occurs with the normal timing, it is very unlikely that the mechanism, which controls the onset of MyoD induction, involves regulation of MyoD's nuclear transport.

Furthermore, we have microinjected several mRNA-encoding factors known to provide a positive stimulus on myogenic gene expression in different vertebrate organisms. These include XE12, MyoD's dimerization partner (Lassar *et al.*, 1991; Rashbass *et al.*, 1992); SL-1, a member of the MEF-2 protein family of MyoD cofactors (Chambers *et al.*, 1992; reviewed by Olson *et al.*, 1995); Lef-1 and β-catenin, which are transcriptional mediators of Wnt signaling (reviewed by Heasman, 1997); and a dominant-negative variant of protein kinase A, which mimicks hedgehog signaling (see Hammerschmidt *et al.*, 1996; Münsterberg *et al.*, 1995). Neither of these, however, had any influence on the timing of MyoD induction (data not shown).

Furthermore, studies in mice have implicated Myf-5 and Pax-3 as upstream regulators of MyoD (Maroto *et al.*, 1997; Tajbakhsh *et al.*, 1997). In the frog, however, MyoD expression is independent from these factors for several reasons. First, XMyoD is induced prior to and without spatial overlap to Pax-3 (see Bang *et al.*, 1997). Second, there is only partial overlap between XmyoD and XMyf-5 expression (Dosch *et al.*, 1997). Finally, UV-ventralized embryos express XMyoD (Frank and Harland, 1991) but not XMyf-5 (C. Niehrs, personal communication). Just recently, BMPs and Nogging have been reported to determine the timing of myogenic gene expression in the chick myotome (Reshef *et al.*, 1998). These findings do not explain our observations, because MyoD induction in the frog occurs in the absence of Noggin expression and even when BMP-4 is overexpressed (Frank and Harland, 1991; Re'em-Kalma *et al.*, 1995; Smith and Harland, 1992). Based on these results, we expect the temporal control of MyoD induction in *Xenopus* to be exerted through a novel mechanism.

Changes in Cellular Competence and MyoD Induction

As we have shown here, MyoD cannot be induced before 10.5 hpf in development. Already at 12 hpf, MyoD induction has ended as a consequence of the general loss of mesodermal competence (see Steinbach *et al.*, 1997). We believe that this tight control of MyoD induction reflects an intrinsic function of early pattern formation, in a sense that the temporal control may provide specificity to pleiotropic signal transduction pathways. Concerning the onset of MyoD induction, we note that the Wnt/β-catenin pathway is activated in two bursts during dorsoventral axial patterning of *Xenopus* embryos—a transient one on the dorsal side of the embryo, which ends shortly after the MBT, followed by a ventrolateral one, which lasts from late blastula through gastrula stages (reviewed by Heasman, 1997). By gain- and loss-of-function analysis, Hoppler and colleagues have shown that zygotic Wnt signaling is required and sufficient for MyoD induction in all mesodermal cells, *including* the Spemann organizer (Hoppler *et al.*, 1996). This raises the question of why MyoD is never activated in the dorsal mesoderm. Our data suggest a simple answer—the first burst of dorsal Wnt signaling does not temporally overlap with the period, during which MyoD is transcriptionally responsive. This hypothesis is in agreement with data on the timing and duration of β-catenin activity on the dorsal side of the embryo (Schneider *et al.*, 1996) and the timing of myoD induction (Wylie *et al.*, 1996; this paper). Direct testing of this hypothesis would require means to shift MyoD's responsiveness to earlier developmental times, which are currently not available (see above). With respect to LMC, we have shown already that an extension of the mesodermal competence period by ablation of somatic linker histones leads to an increase of the number of MyoD-expressing cells in the mesoderm, as well as to ectopic MyoD expression in the neural plate (Steinbach *et al.*, 1997). The latter is not surprising, if one considers the evidence implicating FGF and Wnt signaling in both mesoderm and neural patterning (see McGrew *et al.*, 1997).

It is likely then that MyoD's expression pattern in the embryo is equally determined by local signaling as well as by temporal aspects of cellular competence. Given that similar conclusions have been formulated in a number of unrelated experimental studies for both *Xenopus* (Gamill and Sive, 1997; Tada *et al.*, 1997) and *Drosophila* (Parkhurst and Ish-Horowicz, 1991; Rodriguez *et al.*, 1990; Royet and Finkelstein, 1995), timing requirements may be a rather common feature of embryonic patterning processes. A detailed analysis of the mechanism(s) controlling cellular competence in various species will help address these questions in the future, and undoubtedly be a key issue for our understanding of signal transduction specificity during development.

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