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MAP Kinase Converts MyoD into an Instructive Muscle Differentiation Factor in *Xenopus*

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In amphibian development, muscle is specified in the dorsal lateral marginal zone (DLMZ) of the gastrula embryo. Two critical events specify the formation of skeletal muscle: the expression of the myogenic transcription factor, XMyoD, and the secretion of bone morphogenetic protein (BMP) antagonists by the adjacent Spemann organizer. Inhibition of BMP signaling during early gastrula stages converts XMyoD protein into an instructive differentiation factor in the DLMZ. Yet, the intracellular signaling factors connecting BMP antagonism and activation of XMyoD remain unknown. Our data show that BMP antagonism induces the activity of mitogen-activated protein kinase (MAPK), and that the activity of MAPK is necessary for muscle-specific differentiation. Treatment of gastrula-stage DLMZ explants with MAPK pathway inhibitors ventralized mesoderm and prevented muscle differentiation. Expression of XMyoD in ventral mesoderm weakly induced muscle formation; however, the coexpression of a constitutively active MEK1 with XMyoD efficiently induced muscle differentiation. Activation of the MAPK pathway did not induce the transcription of XMyoD, but increased its protein levels and transcriptional activity. Thus, MAPK activation is subsequent to BMP antagonism, and participates in the dorsalization of mesoderm by converting the XMyoD protein into a potent differentiation factor.

Key Words: **muscle; *Xenopus*; *MyoD*; MAP kinase.**

INTRODUCTION

Muscle is one of the first mesodermal tissues to be induced in the developing vertebrate embryo. In *Xenopus laevis*, muscle differentiates from cells located in the dorsal–lateral region of the marginal zone. Initially, dorsal–lateral marginal zone (DLMZ) tissue is ventrally fated. This region is not specified for muscle and it will not differentiate as muscle if removed from the embryo prior to gastrulation for explant culture (Dale and Slack, 1987). During gastrulation, "dorsalizing" signals are secreted from the adjacent dorsal marginal zone, the Spemann organizer region (reviewed in Harland and Gerhart, 1997). The organizer expresses a number of secreted bone morphogenetic protein (BMP) antagonist proteins such as noggin, chordin, and follistatin (Smith *et al.*, 1993; Lamb *et al.*, 1994; Hemmati-Brivanlou *et al.*, 1994; Sasai *et al.*, 1994). Dorsalization of the ventrally fated DLMZ to muscle requires inhibition of endogenous BMP signaling in the DLMZ region by these antagonist molecules (Sasai *et al.*, 1995; Re'em-Kalma *et al.*, 1995; Zimmerman *et al.*, 1996; Piccolo *et al.*, 1996). Thus, during early development, muscle arises through a multistep inductive process in which muscle cell fate is specified in the dorsal–lateral mesoderm region of the embryo.

The MyoD gene belongs to an extended family of muscle-specific basic helix–loop–helix DNA-binding proteins which commit cells to myogenic lineages (reviewed in Molkentin and Olson, 1996). In both vertebrates and invertebrates, MyoD protein has been shown to play a critical role in muscle cell differentiation (reviewed in Weintraub *et al.*, 1991; Arnold and Winter 1998; Abmayr and Keller, 1998). In *Xenopus*, ectopic expression of *Xenopus MyoD* (*XMyoD*) RNA in naive ectoderm induces transient expression of muscle markers without leading to a terminal commitment of these cells to muscle cell fates (Hopwood and Gurdon, 1990). *XMyoD* is initially expressed in all of the ventral mesoderm during blastula to gastrula stages (Frank and Harland, 1991; Hopwood *et al.*, 1992). However, by the end of gastrulation, *XMyoD* expression becomes fixed in the DLMZ mesoderm fated to differentiate as paraxial somite tissue (Frank and Harland, 1991). This observation suggests that some aspect of the dorsalizing process is required to maintain *XMyoD* expression in the

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muscle-fated DLMZ, while expression is extinguished in the ventral marginal zone (VMZ) region. In addition, it is not clear why ectopic ventral *XMyoD* expression does not commit cells to the myogenic lineage. It has been proposed that signals leading to *XMyoD* autocatalytic transcriptional activation are required for the maintenance of *XMyoD* expression in the presumptive somite region in the frog (Lun *et al.*, 1997; Steinbach *et al.*, 1998). Differential nuclear localization may also play a role in the ability of XMyoD protein to commit cells to myogenic cell fates (Rupp *et al.*, 1994).

In *Xenopus*, little is known about the signals which positively regulate *XMyoD* expression and protein activity in the DLMZ. BMP antagonists induce muscle differentiation and *XMyoD* expression in VMZ explants. However, the chain of signaling events leading to the activation of XMyoD protein as an instructive differentiation factor is poorly understood. In mammalian cell culture, attempts have been made to address the question of signaling pathways which interact with MyoD to direct myogenesis. In cultured mouse C2 myoblast cells, the activity of mitogen-activated protein kinase (MAPK) or extracellular regulated kinase (ERK) increased during myogenesis (Gredinger *et al.*, 1998). MAPK activity was required for terminal differentiation (Gredinger *et al.*, 1998). MAPK positively regulated the expression and activity of MyoD protein in mouse C2 myoblast cells, suggesting that MAPK signaling could play an early role in the conversion of MyoD to an instructive differentiation factor (Gredinger *et al.*, 1998).

In this study, we have examined the role of MAPK signaling in the muscle-specification process in *Xenopus*. We found that inhibition of BMP activity stimulated MAPK activity in dorsalized VMZ explants. During gastrula to neurula stages, MAPK activity in DLMZ explants is high relative to VMZ explants. Dorsalized VMZ and DLMZ explants treated with MAPK pathway inhibitors did not differentiate as muscle and maintained ventral mesoderm identity. Coectopic expression of activated *MAP kinase kinase* (*MEK*) and *XMyoD* RNAs induced *muscle actin* expression, cell-movement elongations, and terminal differentiation in VMZ explants. In VMZ explants, coectopic expression of activated *MEK* and *XMyoD* stimulated the ability of XMyoD to transcriptionally activate an E-box-dependent reporter gene. In these explants, the amount of XMyoD protein was increased and it appeared localized to the nucleus. These results suggest that BMP antagonists activate the MAPK cascade in the DLMZ, and that MAPK activation is required to convert XMyoD into a terminal differentiation factor.

MATERIALS AND METHODS

Xenopus Embryos, Explants, and RNA Microinjection

Ovulation, *in vitro* fertilization, embryo culture, and dissections were carried out as described in Re'em-Kalma *et al.* (1995). Embryos were staged according to Nieuwkoop and Faber (1967). Various synthetic RNA or DNA were injected into embryos at the one-cell stage. Capped synthetic *Xenopus* BMP dominant-negative (DN) receptor RNA (0.1 or 0.4 ng) was injected into the marginal zone (Graff *et al.*, 1994). Capped synthetic *Xenopus MyoD* RNA (pSP64T-XMyoD; Hopwood and Gurdon, 1990) was injected at a concentration of 2 ng/embryo. RNA encoding the constitutively active MEK (MEK1S217E/S221E; Umbhauer *et al.*, 1995) protein was injected at a concentration 2.5 or 5 ng/embryo. Plasmid containing the *4R-tk-Luciferase* reporter gene (gift of Dr. Stephen Tapscott) was injected at a concentration of 50 pg/embryo. Ventral marginal zone (VMZ) and dorsal–lateral marginal zone (DLMZ) explants were removed at stage 10–10.25, and cultured to different stages as described in Bonstein *et al.* (1998). In some experiments, explants were treated with the MEK inhibitors: PD098059 (50 μM) and UO126 (20 and 50 μM) (Calbiochem).

Western Blot Analysis

To prepare cell extracts, explants were washed in PBS and lysed (9 explants in 20 μl lysis buffer). Lysis buffer contains 25 mM Hepes (pH 7.7), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM dithiothreitol, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 1.7 μg/ml aprotinine, 100 μg/ml phenylmethylsulfonyl fluoride, 1 mM glycerolphosphate, and 20 mM p-nitrophenyl phosphate. The extract was cleared by three centrifugations at 12,000*g* for 5 min. Protein concentration was determined by the Bio-Rad protein assay. Equal amounts of extracted proteins were loaded (approximately 30 μg), separated by SDS–polyacrylamide gel electrophoresis, and transferred to nitrocellulose filters.

ERK MAPK Western blot analysis was performed by using the Phospho-Plus p44/p42 MAPK antibody kit (Cell Signaling–New England Biolabs). XMyoD Western blot analysis was performed by using the supernatant of D7F2 hybridoma (Hopwood *et al.*, 1992), diluted 1:2.5 in blocking solution. Proteins were visualized by using the enhanced chemiluminescence kit (Pierce).

Luciferase Assay

Extract preparation and luciferase assay were performed by using the Luciferase Assay System (Promega). Explants were lysed in the lysis buffer (1 explant in 3 μl of lysis buffer). The luciferase activity in the lysates was measured by integrating total light emission over 30 s with a Berthold luminometer. The luciferase activity was normalized to total protein concentration.

Immunohistochemistry

Embryos were derived from fertilization of albino eggs (except Fig. 4D, wild-type eggs) with wild-type sperm. Immunostaining was performed as described by Hemmati-Brivanlou and Harland (1989). Tissue culture supernatant of D7F2 hybridoma (anti-XMyoD; Hopwood *et al.*, 1992) was used undiluted. Tissue culture supernatant of 12/101 (mature muscle antigen; Kintner and Brockes, 1984) was diluted 1:1. Bound antibodies were detected with HRP-conjugated secondary antibodies (goat anti-mouse IgG; Pierce) diluted 1:100, and were visualized with the HRP color-developing reagent (DAB substrate; Amresco). After fixing the stain, some embryos were cleared in 1:2 benzyl alcohol/benzyl benzoate (Hemmati-Brivanlou and Harland, 1989).

Northern Blot Analysis and RT-PCR

RNA was extracted from embryos and prepared for Northern blot analysis as described in Re'em-Kalma *et al.* (1995). Electro-

phoresis, probe preparation, filter hybridization, and exposure were performed as described in Re'em-Kalma *et al.* (1995). Quantitation was performed by using a Fuji phosphorimaging system. *EF1α* was used as a positive standard for comparing amounts of RNA loaded per well at any given stage.

RT-PCR was performed as described in Wilson and Melton (1994), except that random hexamers (100 ng per reaction) were used for reverse transcription. Primers for *EF1α* were described in Hemmati-Brivanlou and Melton (1994). Primers for *XMyoD* were described in Nicolas *et al.* (1998). Primers for *Muscle Actin* were described in Wilson and Melton (1994).

RESULTS

Accumulation of High Levels of Phosphorylated ERK in the Dorsal–Lateral Marginal Zone Occurs during the Activation of XMyoD Expression

To study the possibility that the MAPK pathway was involved in the early development of skeletal muscle, we compared the levels of phosphorylated ERK in the ventral marginal zone (VMZ) and the dorsal–lateral marginal zone (DLMZ) during gastrula and neurula stages. Explants of VMZ and DLMZ were dissected at early gastrula stages (st. 10+–10.25), and cultured until sibling embryos reached later gastrula and neurula stages. Protein was extracted from explants and ERK was detected by Western blotting (Fig. 1A). Levels of phosphorylated ERK were high at early and late gastrula stages (sts. 10.5 and 12), and were hardly detected at late neurula stages in DLMZ explants. At gastrula stages, the absolute amount of phosphorylated ERK was significantly higher in DLMZ explants than in VMZ explants (Fig. 1, compare lanes 1 and 2 to lanes 5 and 6). Using an *in vitro* kinase assay to measure MBP protein phosphorylation (Gredinger *et al.,* 1998), we also determined that ERK activity was significantly higher in DLMZ versus VMZ explant extracts (not shown). The activation of XMyoD transcription occurs during gastrula stages (Hopwood *et al.,* 1989). Therefore, the initial expression of *XMyoD* occurred during gastrula stages when ERK activity was highest. Expression of later terminal differentiation markers such as *muscle actin* can be detected at late neurula stages; levels of phosphorylated ERK were greatly reduced at these stages (Fig. 1A, lane 7). In conclusion, the dorsal–lateral mesoderm is characterized by high levels of active ERK during the early stages of *XMyoD* expression.

Inhibition of BMP Signaling Induces ERK Phosphorylation and Skeletal Muscle Differentiation

It was previously demonstrated that inhibition of BMP signaling in ventral mesoderm changed its fate to a more dorsal mesoderm, that subsequently differentiated into skeletal muscle (Smith *et al.,* 1993; Graff *et al.,* 1994). We thought that if high MAPK activity plays an essential role in muscle development, then inhibition of BMP signaling in the VMZ might induce MAPK activity as part of the differentiation process. Embryos were injected with a BMP DN receptor that blocks BMP signaling in cells expressing this mutated receptor (Graff *et al.,* 1994). VMZ explants were isolated at early gastrula stages, and cultured until sibling embryos reached stage 12. Phosphorylated ERK was barely detectable in control VMZ explants of uninjected embryos (Fig. 1B, lane 1). VMZ explants dorsalized by injection with the BMP DN receptor expressed high levels of phosphorylated ERK in a dose-dependent manner (Fig. 1B, lanes 2 and 3). Similarly, injection of a constitutively active form of MAP kinase kinase/MEK1 (Umbhauer *et al.,* 1995; MEK1S217E/S221E) into embryos induced phosphorylation of ERK in a dose-dependent manner in VMZ explants (lanes 4 and 5). As expected, injection of the BMP DN receptor induced the expression of *muscle actin* in VMZ explants that were analyzed at neurula stage 15, whereas similar explants from uninjected embryos did not express *muscle actin* (Fig. 1C, compare lanes 1 and 3). Interestingly, if the injected explants were treated with a specific inhibitor of MEK1, PD098059, during their culture from st. 10+–10.25 to st. 15, the expression of *muscle actin* was highly inhibited (Fig. 1C, compare lanes 3 and 4). Overall, these results indicate that inhibition of BMP signaling induces the MAPK pathway as part of the program of skeletal muscle development.

Inhibition of the MAPK Pathway during Gastrulation Prevents the Formation of Skeletal Muscle

To explore whether the activity of the MAPK pathway was essential for myogenesis, DLMZ explants were isolated at early gastrula stages and cultured in the presence of PD098059 (Fig. 2A) or UO126 (Fig. 2B), specific pharmacological inhibitors of MEKs. Control DLMZ explants were dissected from the same embryos and were grown in the absence of these inhibitors. All explants were cultured until sibling embryos reached different gastrula and neurula stages. The expression of *muscle actin* was detected at late neurula stage by Northern analysis (Figs. 2A and 2B, left). At this stage, we observed high expression of *muscle actin* in control explants, which was markedly inhibited in explants that were treated with each of the MEK inhibitors (Figs. 2A and 2B, left). Both inhibitors prevented the phosphorylation of ERK in explants grown to stage 12 (data not shown).

MAPK is required for the proper establishment of mesoderm during early *Xenopus* development (Umbhauer *et al.,* 1995; LaBonne *et al.,* 1995; Gotoh *et al.,* 1995). Functional MAPK signaling is prerequisite for proper *XBra* expression, and in the absence of proper XBra activity, mesoderm formation is severely inhibited (Conlon *et al.,* 1996; Smith, 2001). Therefore, inhibition of the MAPK pathway during early gastrula stages could have caused mesoderm dedifferentiation. For that reason, we analyzed expression of the mesoderm-specific marker *XBra* during later gastrula stages, in explants treated with MAPK kinase (MEK) inhibitors (Figs. 2A and 2B, right). In comparison to controls, similar levels of *XBra* transcripts were observed in the

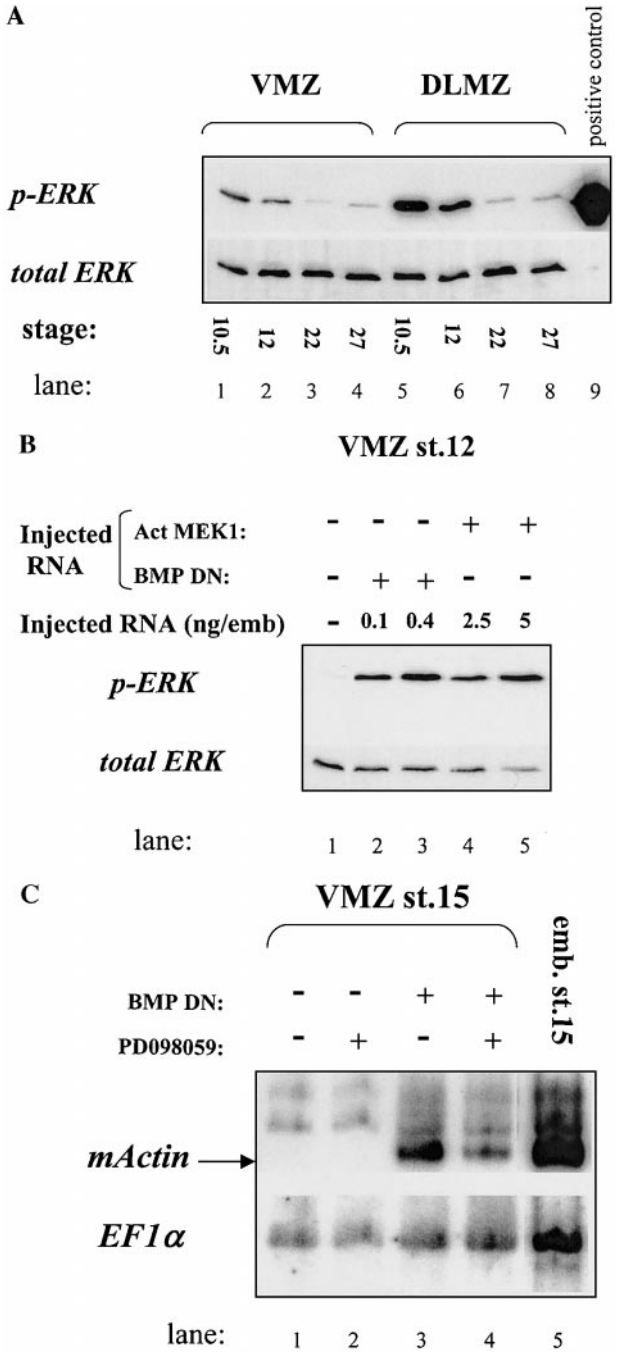


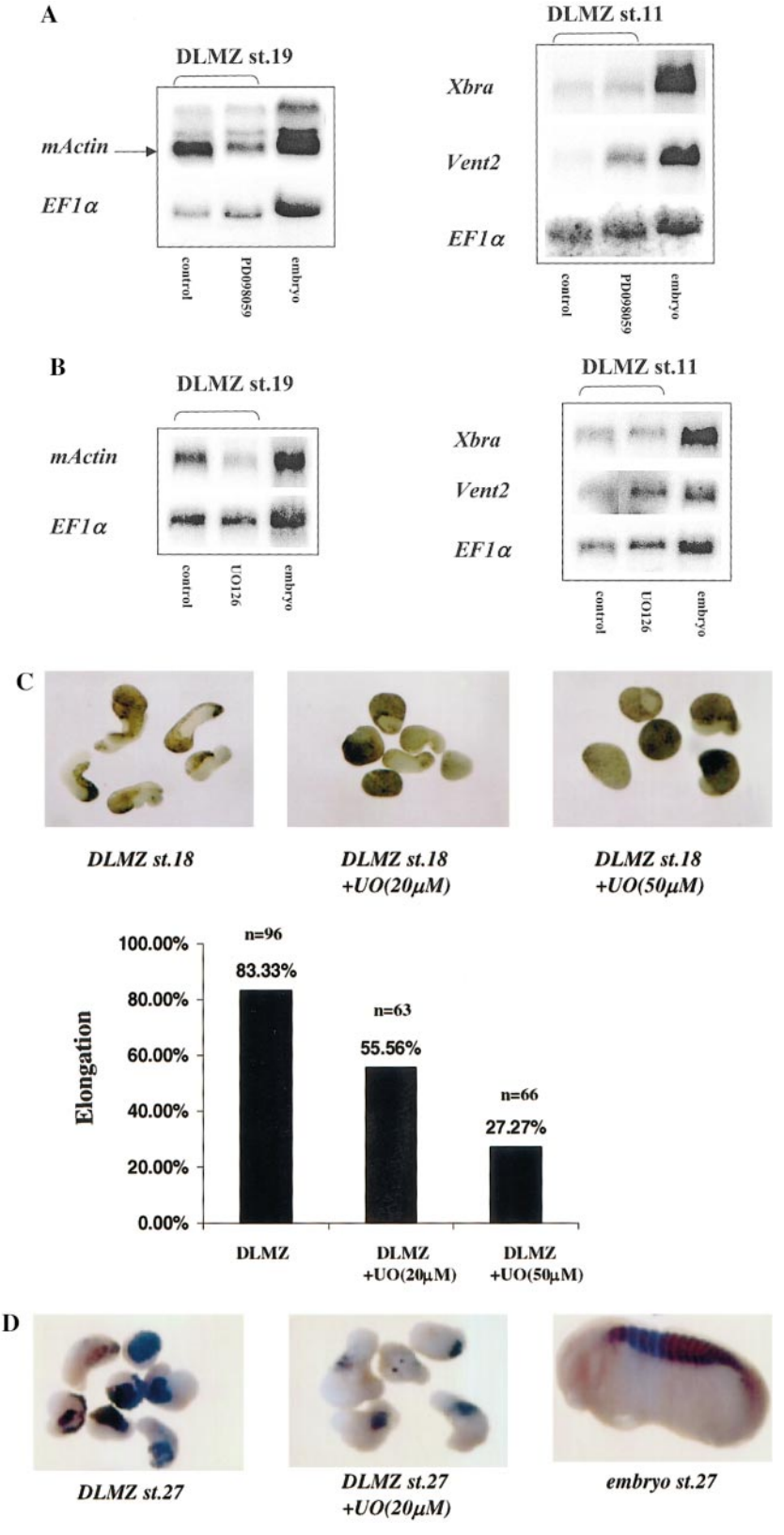
FIG. 1. BMP antagonism induces phosphorylation of ERK in the ventral marginal zone (VMZ). Dorsalization of the VMZ to muscle by BMP antagonism is dependent on MAPK activity. (A) Extracts from nine DLMZ or VMZ explants were separated by SDS–PAGE, Western blotted, and sequentially probed with a polyclonal antibody specific for phosphorylated ERK and with a polyclonal antibody specific for total ERK protein (New England Biolabs PhosphoPlus p44/p42 MAPK Antibody Kit). Phosphorylated p42 was used as a positive control (lane 9). Lanes are labeled according to the developmental stages (Nieuwkoop and Faber, 1967). (B) Embryos at the one-cell stage were injected with either 0.1 or 0.4 ng of *in vitro* transcribed *Xenopus* BMP DN receptor RNA, or with either 2.5 or 5 ng of *in vitro* transcribed constitutively active form of *MEK1* (*Act MEK1*) RNA. VMZ explants were removed at stage 10+–10.25 and cultured until sibling embryos reached stage 12. Protein was isolated from each treatment group (14 explants) for Western blot analysis using the New England Biolabs PhosphoPlus p44/p42 MAPK Antibody Kit. (C) Embryos at the one-cell stage were injected with 0.4 ng of *in vitro* transcribed *Xenopus* BMP DN receptor RNA. Thirty VMZ explants were removed from uninjected and injected embryos. Fifteen explants from each group were grown with (lane 4) or without (lane 3) 50 μM PD098059. Total RNA was isolated from pools of 15 neurula stage-15 VMZ explants from each treatment. All VMZ RNA was loaded per well for Northern analysis. One embryo equivalent of RNA was loaded (lane 5). The filter was sequentially hybridized with *Xenopus* cDNA probes for *muscle-specific actin* (*mActin*) and *EF1α*. *EF1α* served as a positive control for quantitating RNA levels in the different samples.

explants that were treated with MEK inhibitors, suggesting that inhibition of the MAPK pathway during gastrula stages did not affect mesoderm identity in explants. However, analysis of the expression of *XVent1* (not shown) and *XVent2* (Figs. 2A and 2B, right) indicated that their expression was increased in explants that were treated with MEK inhibitors relative to control explants. These markers are expressed during gastrula stages in the ventral mesoderm (Gawantka *et al.*, 1995; Papalopulu and Kintner, 1996; Schmidt *et al.*, 1996; Onichtchouk *et al.*, 1996). Hence, we can conclude that inhibition of the MAPK pathway during gastrula stages changes dorsal mesoderm to a more ventral fate, that during normal development is not a precursor of skeletal muscle differentiation.

To further validate that inhibition of the MAPK pathway repressed skeletal muscle differentiation, we analyzed the elongation of explants which is a characteristic of muscle development. DLMZ explants were elongated as expected, while the number of elongated explants was reduced with increased concentrations of the MEK inhibitor, UO126 (Fig. 2C). Explants were also stained for the 12/101 antigen, a marker of terminal muscle differentiation (Kintner and Brockes, 1984). Muscle was stained in well-defined blocks in control DLMZ explants (Fig. 2D). Treatment of explants with UO126 caused a significantly reduced staining of muscle. The specificity of the antibody was shown by the somitic staining of a whole embryo from the same developmental stage (Fig. 2D, right).

Activation of MAPK in Ventral Mesoderm Induces the Formation of Skeletal Muscle

To further explore the role of MAPK in myogenesis, we injected an RNA encoding a constitutively active form of MEK1 (MEK1S217E/S221E) and/or RNA encoding the XMyoD protein into fertilized eggs. Blastula (st. 8–9) animal cap and early gastrula (st. 10–10.25) VMZ explants were cultured until sibling embryos reached late neurula



stages, and *muscle actin* expression was determined by Northern analysis (Fig. 3A). The active form of MEK1 did not induce expression of *muscle actin* in animal cap and VMZ explants (Fig. 3A, lanes 2 and 6), whereas XMyoD induced some expression of *muscle actin* in the VMZ (Fig. 3A, lane 7). Injection of RNA molecules encoding XMyoD and active MEK1 induced strong expression of *muscle actin* in the VMZ (Fig. 3A, lane 8), and weaker expression in animal cap explants (Fig. 3A, lane 4). Therefore, it appears that XMyoD and activated MEK1 cooperate in the induction of *muscle actin* expression. We also analyzed the expression of *muscle actin* in animal caps by RT-PCR (Fig. 3B). Active MEK1 did not induce any *muscle actin* expression (Fig. 3B, lane 2), XMyoD induced its weak expression (Fig. 3B, lane 3), and both molecules induced a significantly higher expression of *muscle actin* (Fig. 3B, lane 4). From the levels of injected RNA encoding XMyoD, we can conclude that the increase in *muscle actin* expression was not due to different amounts of *XMyoD* transcripts (Fig. 3B, compare lanes 3 and 4). The synergism between XMyoD and active MEK1 was further assessed by analyzing explant elongation (Fig. 3C), and the expression of muscle antigen recognized by the 12/101 monoclonal antibody (Fig. 3D). By both criteria, XMyoD induced some skeletal muscle differentiation in VMZ explants; however, the combination of XMyoD and active MEK1 enhanced significantly the muscle phenotype (Figs. 3C and 3D).

Activation of MAPK Does Not Induce Expression of XMyoD in VMZ Explants

We observed that the inhibition of BMP signaling induced ERK activity (Fig. 1B), and the expression of *muscle actin* (Fig. 1C). Still, the direct activation of MAPK by ectopic MEK expression was not sufficient to activate the expression of *muscle actin* (Figs. 3A and 3B).

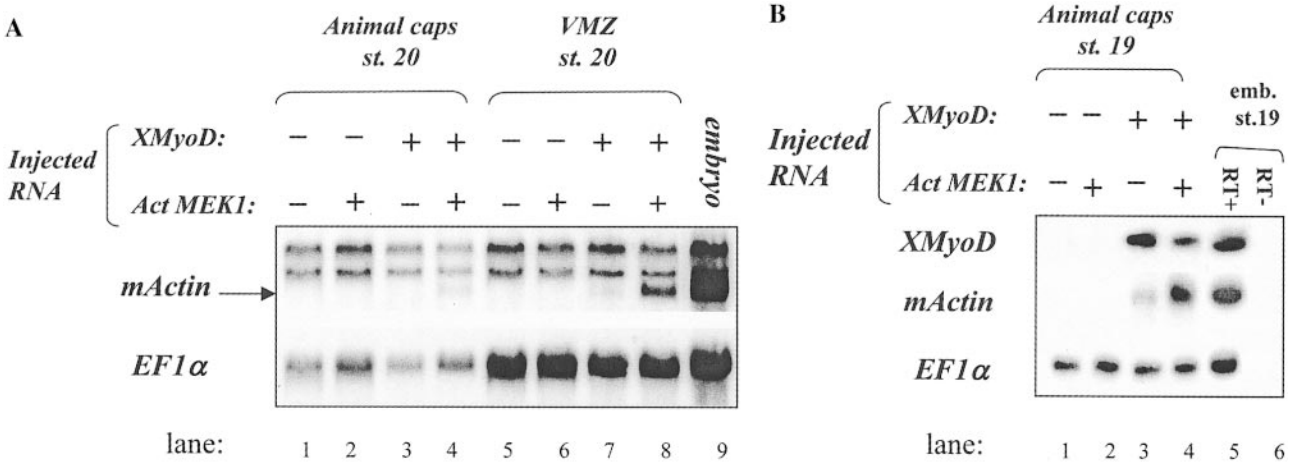
To further elucidate the different effects induced by BMP inhibition and MAPK activation, we injected embryos with RNA encoding an activated MEK1 protein or the BMP DN receptor. The expression of *XMyoD* was analyzed by RT-PCR in VMZ explants grown until at stage 14 (Fig. 4). The BMP DN receptor induced the expression of endogenous transcripts of *XMyoD*, while active MEK1 did not (Fig. 4). Therefore, in addition to its function in inducing MAPK activity, the BMP DN receptor could independently cause the accumulation of *XMyoD* transcripts. Activation of MAPK only, was not sufficient to induce expression of *XMyoD* transcripts.

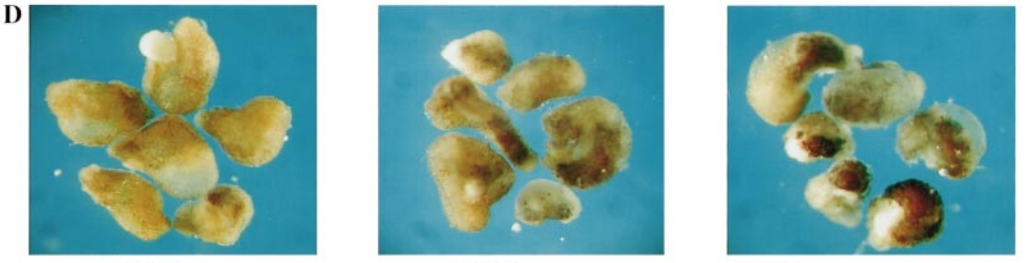
MAPK Induces Accumulation and Activity of XMyoD Protein

Although MAPK signaling cannot initiate the expression of *XMyoD*, it might affect the activity of preexisting XMyoD protein. To analyze this possibility, we studied how MAPK affected the transcriptional activity of XMyoD. For that, we injected a MyoD-responsive reporter gene (*4R-tk-Luc*), and RNA encoding MyoD and/or active MEK1 proteins into one-cell embryos. Luciferase activity was measured in VMZ explants grown to stage 12 (Fig. 5A). XMyoD significantly induced the activity of the reporter gene over the background levels of the VMZ explants (Fig. 5A, compare lanes 1 and 3). Active MEK1 did not effect the transcription of the reporter gene (Fig. 5A, lane 2), however, a combination of XMyoD and active MEK1 induced transcription in a cooperative manner (Fig. 5A, compare lanes 2–4). To analyze how the inhibition of the MAPK pathway affected the activity of XMyoD, we measured luciferase activity in DLMZ explants that were treated with UO126 (Fig. 5B). Injection of an RNA molecule encoding XMyoD significantly induced the activity of the reporter gene (Fig. 5B, compare lanes 1 and 3). Addition of UO126 to the injected explants inhibited reporter activity by about twofold (Fig. 5B, compare lanes 3 and 4). These results suggest that the MAPK pathway induces the transcriptional activity of XMyoD.

To continue and explore the possible reason for XMyoD-modified activity, we analyzed the levels of injected RNA encoding XMyoD by RT-PCR (Fig. 6A, right). The levels of *XMyoD* transcripts appeared equal in all the injected explants, suggesting that the amounts of ectopic *XMyoD* transcripts could not account for the differences in XMyoD

FIG. 2. Prevention of skeletal muscle formation by inhibition of MEK activity during gastrulation. (A) Thirty-six DLMZ explants were removed at stage 10+–10.25. Eighteen explants were cultured in the presence of 50 μM PD098059 until sibling embryos reached stage 19 (left) or stage 11 (right). Eighteen untreated explants served as controls. Total RNA was isolated from pools of nine explants from each group. All DLMZ explant RNA and one embryo equivalent of RNA were loaded per well for Northern analysis. The stage-19 filter was sequentially hybridized with *Xenopus* cDNA probes for *mActin* and *EF1α*. The stage-11 filter was sequentially hybridized with *Xenopus* cDNA probes for *Xbra*, *Vent2*, and *EF1α*. *EF1α* served as a positive control for quantitating RNA levels in the different samples. (B) Thirty-six DLMZ explants were removed at stage 10+–10.25 and cultured in the presence of 20 μM UO126 until sibling embryos reached stage 19 (left) or stage 11 (right). Eighteen untreated explants served as controls. Total RNA was isolated from pools of nine explants from each treatment. All the DLMZ RNA and one embryo equivalent of RNA were loaded per well for Northern analysis. The stage-19 filter was sequentially hybridized with *Xenopus* cDNA probes for *mActin* and *EF1α*. The stage-11 filter was sequentially hybridized with *Xenopus* cDNA probes for *Xbra*, *Vent2*, and *EF1α*. *EF1α* served as a positive control for quantitating RNA levels in the different samples. (C) DLMZ explants were removed at stage 10+–10.25 and cultured without or in the presence of 20 or 50 μM UO126 until sibling embryos reached stage 18. Explants were examined for elongation. (D) DLMZ explants from albino embryos were cultured until stage 27 without or in the presence of 20 μM UO126; embryos were stained with the muscle-specific 12/101 monoclonal antibody.





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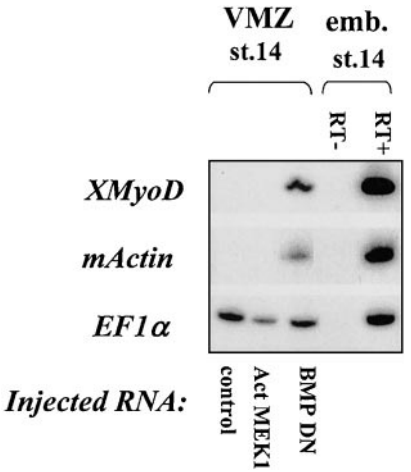

FIG. 4. Activation of MAPK does not induce *XMyoD* expression in VMZ explants. Embryos at the one-cell stage were injected with 0.4 ng of *in vitro* transcribed *Xenopus* BMP DN receptor RNA or with 2.5 ng of the constitutively active form of *MEK1* (*Act MEK1*) RNA. At stage 10.25, 18 VMZ explants were removed from each injected or uninjected embryo group. Explants were grown until sibling embryos reached stage 14, and total RNA was isolated. RT-PCR analysis was performed with the markers *XMyoD*, *mActin*, and *EF1α*. *EF1α* served as a positive control for quantitating RNA levels in the different samples. For controls, RT-PCR and −RT-PCR were performed on total RNA isolated from normal embryos.

activity, as presented in Fig. 5. Next, we analyzed the protein levels of MyoD using protein extracts from the same experiment (Fig. 6A, left). We found that XMyoD protein levels were reduced in VMZ and DLMZ explants treated with UO126, relative to control untreated explants (Fig. 6A, left). To further investigate the possibility that MAPK affected XMyoD protein levels, a complementary experiment that tested the effect of the activation of MAPK was performed (Fig. 6B). XMyoD protein levels were compared between VMZ explants that expressed injected XMyoD and those that expressed injected XMyoD and active MEK1. Levels of XMyoD protein were significantly higher in the second group of explants (Fig. 6B, left), although the levels of injected RNA encoding XMyoD were similar between the two groups (Fig. 6B, right). All the above experiments were analyzed at stage 12, the time when endogenous levels of XMyoD are induced, and the MAPK pathway is activated in the dorsal–lateral mesoderm.

Collectively, these results suggest that activity of the MAPK pathway affects a posttranscriptional event regulating the accumulation of XMyoD protein. A previous study suggested that, during the transient expression of *XMyoD* in the VMZ, XMyoD protein was localized to the cytoplasm (Rupp *et al.*, 1994). To analyze whether the cellular distribution of XMyoD protein was affected by the MAPK pathway, we immunostained *XMyoD*-injected DLMZ explants that were incubated in the presence or absence of UO126 with the D7F2 XMyoD monoclonal antibody (Hopwood *et al.*, 1992). Punctate staining of XMyoD was detected in DLMZ explants in contrast to UO126-treated explants (Fig. 7, upper/lower left). At higher magnification, strong nuclear localization was observed in control DLMZ explants versus the UO126-treated explants (Fig. 7, upper/lower right). This staining confirms results presented in Fig. 6, suggesting that the level of XMyoD protein was reduced in UO126-treated cells. However, we could not unequivocally show that this reduction in protein levels is the direct cause of differential cytoplasmic–nuclear localization.

DISCUSSION

In *Xenopus* embryos, dorsal–lateral mesoderm is specified to muscle during gastrulation by inductive signals emanating from the Spemann organizer (reviewed in Harland and Gerhart, 1997). Juxtaposed organizer cells secrete BMP antagonists such as noggin, follistatin, and chordin which recruit these ventrally fated cells to more dorsal fates

FIG. 3. Ectopic expression of a constitutively active form of MEK1 enhances muscle formation in the VMZ. (A) Embryos at the one-cell stage were injected with 2 ng of *in vitro* transcribed *XMyoD* RNA or/and with 2.5 ng of *in vitro* transcribed constitutively active form of *MEK1* (*Act MEK1*) RNA. VMZ explants removed at stage 10+–10.25, and animal cap explants removed at stage 8–9, were cultured until sibling embryos reached stage 20. Total RNA was isolated from each group of explants (15 explants per group) and from whole embryos. All explant RNA and one embryo equivalent of RNA were loaded per well for Northern analysis. The filter was sequentially hybridized with *Xenopus* cDNA probes for *mActin* and *EF1α*. *EF1α* served as a positive control for quantitating RNA levels in the different samples. (B) Embryos at the one-cell stage were injected with 2 ng of *in vitro* transcribed *XMyoD* RNA or/and with 2.5 ng of *in vitro* transcribed constitutively active form of *MEK1* (*Act MEK1*) RNA. Twenty animal caps were removed from uninjected and injected groups of blastula-stage embryos. Explants from each group were grown to stage 19 and total RNA was isolated. RT-PCR analysis was performed with the markers *XMyoD*, *mActin*, and *EF1α*. *EF1α* served as a positive control for quantitating RNA levels in the different samples. For controls, RT-PCR and −RT-PCR were performed on total RNA isolated from normal embryos. (C) Embryos at the one-cell stage were injected with 2 ng of *in vitro* transcribed *XMyoD* RNA or/and with 2.5 ng of *in vitro* transcribed constitutively active form of *MEK1* (*Act MEK1*) RNA. At stage 10+–10.25, 16–30 VMZ explants were removed from each injected or uninjected embryo group. Explants were grown until sibling embryos reached stage 18, when they were examined for elongation. (D) The same embryos and VMZ explants (see Fig. 3C) were grown to stage 35 and stained with the muscle-specific 12/101 monoclonal antibody, that recognizes mature skeletal muscle.

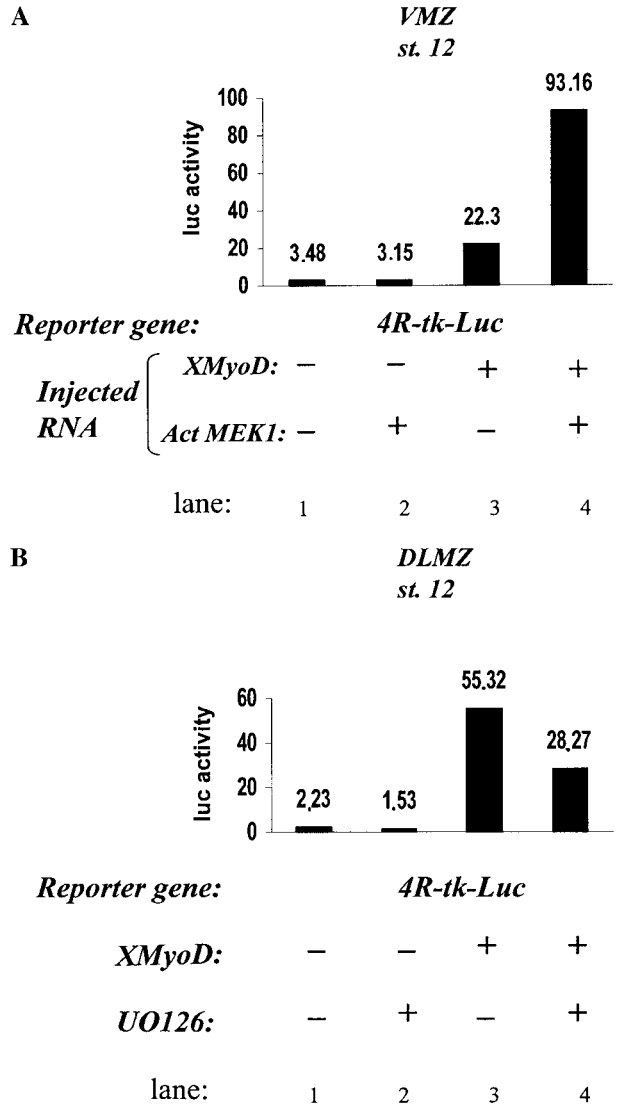

FIG. 5. Induction of XMyoD transcriptional activity by MAPK. (A) Embryos at the one-cell stage were injected with 2 ng of *in vitro* transcribed *XMyoD RNA* or/and with 2.5 ng of *in vitro* transcribed constitutively active form of *MEK1* (*Act MEK1*) RNA. All embryos were injected with 50 pg of the E-Box-dependent reporter gene plasmid, *4R-tk-Luc*. At stage 10+–10.25, 27 VMZ explants were removed from each injected embryo group. Explants were grown until sibling embryos reached stage 12. Protein extracts were examined for luciferase activity. One representative experiment is shown; experiments were performed four times. (B) Embryos at the one-cell stage were injected with 50 ng of the previously mentioned reporter plasmid (Fig. 5A). Embryos were injected with 2 ng of *in vitro* transcribed *XMyoD RNA*. At stage 10+–10.25, 27 DLMZ explants were removed from the injected and control embryo groups. Half of the explants were cultured in the presence of 50 μM UO126 until sibling embryos reached stage 12. Protein extracts were examined for luciferase activity. One representative experiment is shown; experiments were performed four times.

such as muscle (Harland and Gerhart, 1997). These extracellular signals act in concert with intracellular transcription regulators to establish cell fate in the embryo. The MyoD family of transcription factors is crucial in the muscle-specification process. In both cell culture and animal model systems, members of the MyoD family have been shown to be obligatory for the proper specification and differentiation of muscle tissue (reviewed in Weintraub *et al.*, 1991; Arnold and Winter, 1998; Abmayr and Keller, 1998).

In early *Xenopus* development, the *MyoD* gene undergoes a number of expression waves. Initially, the gene is expressed as a maternal transcript in the whole embryo (Harvey, 1990; Rupp and Weintraub, 1991). At the onset of zygotic transcription at the midblastula transition, MyoD is ubiquitously expressed at low levels throughout the embryo (Rupp and Weintraub, 1991; Harvey, 1991). Subsequently, high levels of induced zygotic transcripts and protein accumulate in all of the nonorganizer mesoderm during early–late gastrula stages (Frank and Harland, 1991; Hopwood *et al.*, 1992). Only as a result of the dorsalization process does *MyoD* transcription become fixed in the dorsal–lateral mesoderm fated for muscle (Frank and Harland, 1991). The question of how MyoD does not convert all the ventral nonorganizer mesoderm to muscle is still unanswered. In addition, it is also unclear as to how the dorsalization process fixes *MyoD* expression and converts MyoD protein into an instructive differentiation factor in the dorsal–lateral mesoderm.

In this study, we show that BMP antagonism in the VMZ activates high transient levels of MAP kinase (ERK) activity during gastrulation. MAPK activity is required for muscle to be specified in the DLMZ by BMP antagonism; in the presence of MAPK pathway inhibitors, BMP antagonism will not dorsalize DLMZ tissue to muscle. However, MAPK inhibition does not cause mesoderm dedifferentiation and a loss of mesoderm specification. A number of studies have shown that FGF signaling through the MAP kinase cascade activates *Xenopus* Brachyury (*XBra*) gene transcription (Umbhauer *et al.*, 1995; LaBonne *et al.*, 1995; Gotoh *et al.*, 1995). Expression of the XBra protein is a prerequisite for proper mesoderm induction (reviewed in Smith, 2001). Inhibition of MAPK activity in early gastrula-stage DLMZ explants did not perturb *XBra* expression in these explants. In addition, MAPK inhibition appeared to respecify the DLMZ to a more ventral character, since expression of the *XVent1* and *XVent2* markers was increased in DLMZ explants treated with MAPK pathway inhibitors. Increased levels of eFGF expression (Standley *et al.*, 2001) and MAPK activity (Curran and Grainger, 2000) have been detected in gastrula-stage dorsal mesoderm. eFGF has also been shown to mediate the community effect in *Xenopus* myogenesis (Standley *et al.*, 2001). Future experiments should determine whether BMP antagonism in the DLMZ indeed activates MAP kinase through eFGF signaling.

These results suggest that, in addition to a primary phase of MAPK activity regulating early mesoderm formation,

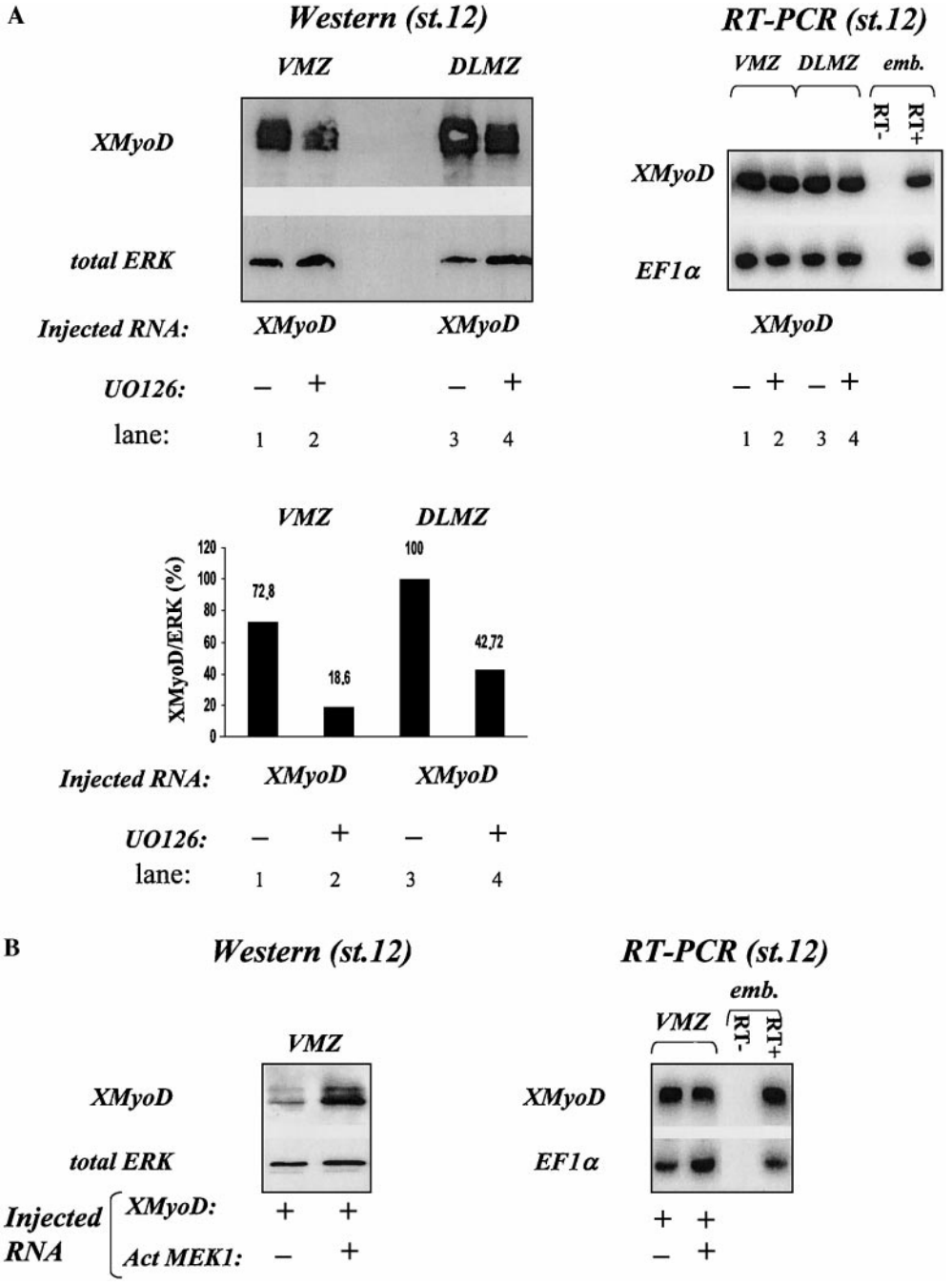

FIG. 6. Activation of MAPK increases XMyoD protein levels. (A) Protein extracts (9 explant equivalents), as described in Fig. 5B, were separated by SDS–PAGE, Western blotted, and sequentially probed with the XMyoD-specific D7F2 monoclonal antibody and with a polyclonal antibody specific for total ERK protein (left). Total ERK protein served as a positive control for quantitating protein levels in the different samples. A portion of the same extract was also used for total RNA preparation. RT-PCR analysis was performed with the markers *XMyoD* and *EF1α* (right). *EF1α* served as a positive control for quantitating RNA levels in the different samples. For controls, RT-PCR and −RT-PCR were performed on total RNA isolated from normal embryos. (B) Protein extracts (9 explant equivalents), as described in Fig. 5A, were separated by SDS–PAGE, Western blotted, and sequentially probed with the XMyoD-specific D7F2 monoclonal antibody and with a polyclonal antibody specific for total ERK protein (left). Total ERK protein served as a positive control for quantitating protein levels in the different samples. A portion of the same extract was used for total RNA preparation. RT-PCR analysis was performed with the markers *XMyoD* and *EF1α* (right). *EF1α* served as a positive control for quantitating RNA levels in the different samples. For controls, RT-PCR and −RT-PCR were performed on total RNA isolated from normal embryos.

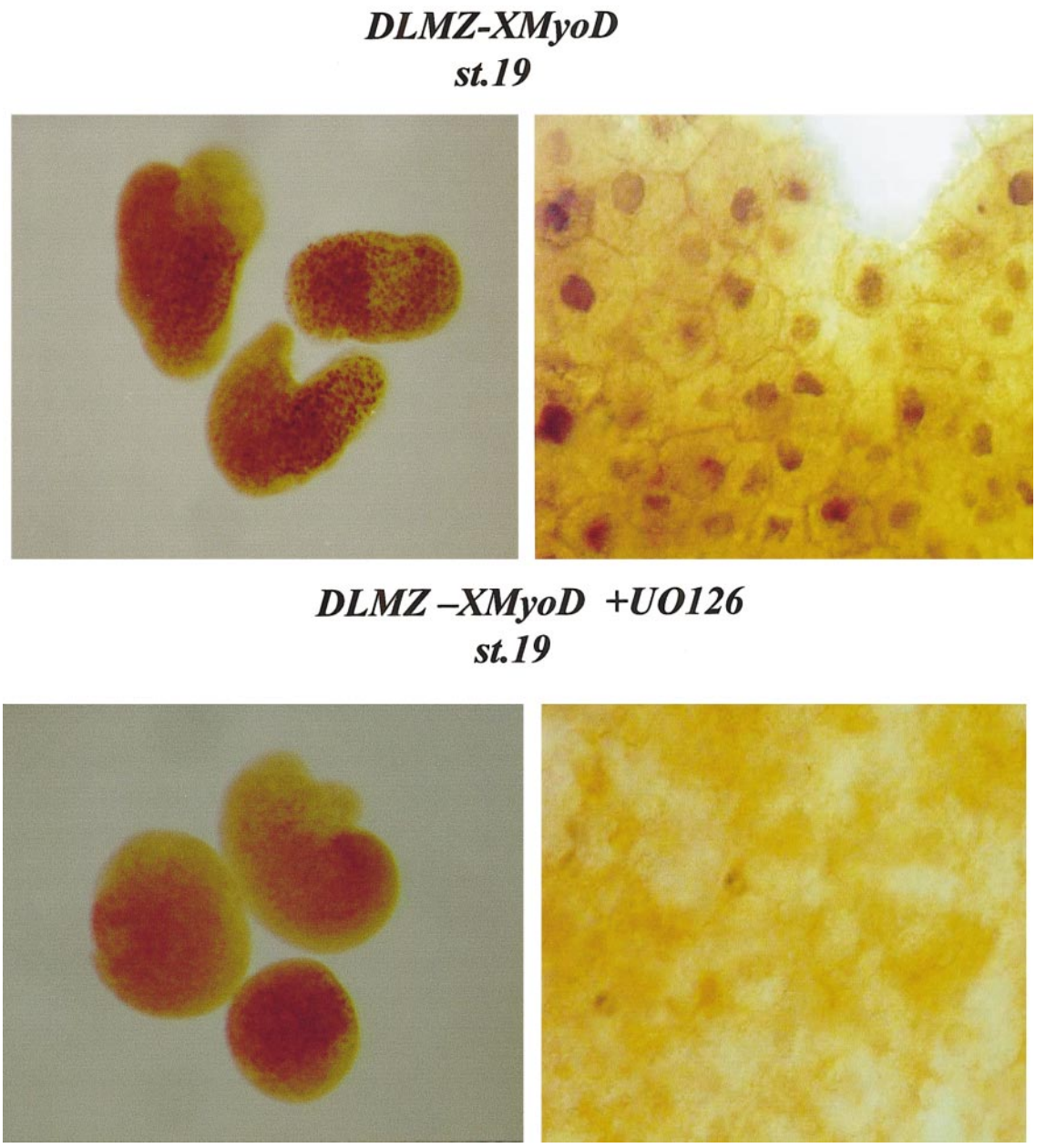

FIG. 7. Immunostaining of ectopically expressed MyoD protein following MAPK pathway inhibition. Embryos at the one-cell stage were injected with 2 ng of *in vitro* transcribed *XMyoD* RNA. Two DLMZ explants were removed from 36 injected embryos. One explant from each DLMZ pair was grown in the presence of 50 μM UO126, and the other was grown as a control, without treatment.

there is an additional stage in which MAPK activity is required for the specification of mesoderm to more dorsal fates by BMP antagonists. Experiments in *Xenopus* animal cap explants have also shown induction of MAPK activity by BMP antagonists (Uzgare *et al.,* 1998). In animal caps, it has been suggested that BMP antagonism may activate MAPK which leads to smad1 down-regulation, but the exact nature of this molecular chain of events is still unclear (Uzgare *et al.,* 1998). Recent studies in *Xenopus* have shown that FGF/MAPK activity is required for posterior neural patterning (Ribisi *et al.,* 2000); however, the role of MAPK in the initial induction of anterior-fated pan neural tissue by BMP antagonists is still an open question.

Previous studies using *Xenopus* animal cap explants have shown that active MEK1 can induce *muscle actin* expression (Umbhauer *et al.,* 1995; LaBonne *et al.,* 1995). In the former study (Umbhauer *et al.,* 1995; we used the same activated MEK1 construct), exceedingly low levels of

muscle actin RNA were induced relative to the panmesodermal *XBra* marker. These animal caps did not elongate like activin-treated or DLMZ explants which differentiate high muscle levels. The authors suggested that these animal caps differentiated to ventral mesoderm mimicking a classic FGF treatment. In the latter study (LaBonne *et al.*, 1995), activated MEK1 induced high levels of *muscle actin* expression in animal cap explants, even when compared to other MAP kinase-activating reagents. In their study, activated MEK1 also stimulated higher levels of RSK/MAP kinase activity in comparison to the other MAP kinase-activating reagents. The authors suggested that the high levels of MEK1 and resultant ERK activity might have stimulated a more dorsal induction; however, there is no report as to whether these explants typically elongated like activin-treated animal cap or DLMZ explants. The authors also suggested that high MEK1 levels may activate an alternative non-MAP kinase target to induce muscle (LaBonne *et al.*, 1995), although this has yet to be shown. In our system, we examined the role of MAP kinase in gastrula-stage VMZ and DLMZ explants which were already specified as mesoderm, thus circumventing the need for the pregastrula MAP kinase activity required for early mesoderm induction. Unlike blastula-stage animal cap explants, inhibition of MAP kinase signaling in VMZ or DLMZ explants allows the uncoupling of mesoderm patterning from the induction process.

We found that MAP kinase activity likely specifies muscle by modulating XMyoD protein activity in the DLMZ region. Ectopic expression of an activated MEK protein did not induce muscle formation in VMZ explants. However, the ectopic coexpression of XMyoD and activated MEK proteins shifted the VMZ to muscle fates in the absence of BMP antagonist treatment. Muscle differentiation occurred at a much higher frequency in MEK and XMyoD coinjected explants in comparison to explants solely expressing ectopic XMyoD protein. These coinjected VMZ explants elongated like DLMZ explants during neurula stages, and expressed high levels of terminal differentiation markers such as muscle-specific actin and the 12/101 antigen. XMyoD protein and the MAP kinase pathway synergize to specify muscle in the DLMZ region; thus, only in the presence of MAPK signaling can XMyoD act as an instructive differentiation factor.

To address the mode in which the MAPK pathway could modulate XMyoD activity, we coexpressed XMyoD and activated MEK protein along with a plasmid containing an E-box-specific luciferase reporter gene. In VMZ explants, MEK alone did not activate the reporter. However, in the presence of MEK and XMyoD proteins, luciferase activity was stimulated over fourfold versus XMyoD alone. In explants coexpressing MEK and MyoD, we detected higher XMyoD protein levels, although ectopic *XMyoD* RNA levels were identical.

These results support a model in which, the combination of BMP antagonism and the resultant induction of MAPK activity converts XMyoD protein into an active differentiation factor (Fig. 8). BMP antagonism fixes *XMyoD* transcription in the DLMZ, in addition to inducing MAPK activity. MAPK causes a further increase in XMyoD protein levels that accumulate in nuclei and can activate muscle-specific transcription (Fig. 8). Overall, it appears that BMP antagonism induces XMyoD by affecting transcriptional and post-transcriptional events. We do not know whether the resultant increase in XMyoD transcriptional factor activity on an E-box reporter gene is due to a direct change in the intrinsic activity of the MyoD protein or just a result of its accumulation in the nucleus. While these two possibilities are not mutually exclusive, further experiments are being performed to determine this point. However, it is clear that the MAPK pathway alone cannot suffice to activate *XMyoD* transcription in a VMZ explant, so BMP antagonism likely acts in concert with MAPK activity to initially stimulate *XMyoD* expression over a critical threshold. A similar observation was also observed in C2 mouse myoblast cells as they differentiated to myotubes in culture (Gredinger *et al.*, 1998). ERK activity increased as myoblasts underwent terminal differentiation; in these cells, ERK activity also induced increased MyoD transcriptional activity (Gredinger *et al.*, 1998).

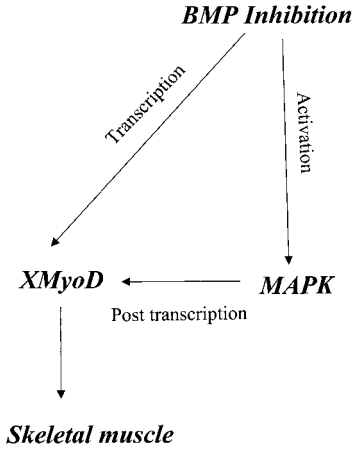

FIG. 8. Model of the role of MAPK signaling in muscle specification in the DLMZ. The inhibition of BMP activity induces *XMyoD* expression and increases MAPK activity in the DLMZ. MAPK activity causes posttranscriptional modification of the XMyoD protein, which converts MyoD into an instructive differentiation factor.

The accumulation of MyoD protein in *Xenopus* explants (this work) and in mammalian tissue culture cells (O. Ostrovsky and E.B., unpublished results) indicates that MAPK activity may decrease turnover of the MyoD protein. The mechanism is unknown at present, but we speculate that MAPK may stabilize MyoD by elevating levels of p21-WAF1 (Sewing *et al.*, 1997; Woods *et al.*, 1997). MyoD is phosphorylated at serine 200 by cdk/cyclin complexes, which target the protein for rapid degradation and inactivation (Kitzmann *et al.*, 1999). p21 inhibits these complexes

from phosphorylating MyoD, and the dephosphorylated protein is more stable (Kitzmann *et al.*, 1999; Reynaud *et al.*, 1999).

During gastrulation, XMyoD transcripts and protein are present throughout the whole VMZ, but they do not suffice to dorsalize the VMZ to muscle without antagonism of BMP signaling by the organizer. Our studies suggest that a major role of the organizer may be to induce MAPK activity in the DLMZ region. The lack of critical MAPK activity levels in nonmuscle fated tissues may explain why XMyoD exclusively triggers muscle differentiation in DLMZ mesoderm during early amphibian development. This MAPK-induced posttranscriptional modification of XMyoD protein could lead to an up-regulation of MyoD-dependent MyoD transcription, in addition to increased transcription of other MyoD target genes. Thus, perhaps as a result of dorsalization and autoactivation, MyoD could become a potent differentiation factor that specifies muscle formation in the DLMZ region of the gastrulating *Xenopus* embryo.

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