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MyoD stimulates Delta-1 transcription and triggers Notch signaling in the *Xenopus* gastrula

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The Notch signaling cascade is involved in many developmental decisions, a paradigm of which has been the selection between epidermal and neural cell fates in both invertebrates and vertebrates. Notch has also been implicated as a regulator of myogenesis, although its precise function there has remained controversial. Here we show that the muscle-determining factor MyoD is a direct, positive regulator of the Notch ligand Delta-1 in prospective myoblasts of the pre-involuted mesoderm in *Xenopus* gastrulae. Injection of a dominant MyoD repressor variant ablates mesodermal Delta-1 expression *in vivo*. Furthermore, MyoD-dependent Delta-1 induction is sufficient to activate transcription from promoters of E(spl)-related genes in a Notch-dependent manner. These results indicate that a hallmark of neural cell fate determination, i.e. the feedback loop between differentiation promoting basic helix–loop–helix proteins and the Notch regulatory circuitry, is conserved in myogenesis, supporting a direct involvement of Notch in muscle determination.

Keywords: Delta-1/muscle/MyoD/Notch signaling/*Xenopus*

Introduction

The process of neurogenesis is regulated by an interplay of proneural genes and the Notch signaling pathway in both flies (Artavanis *et al*., 1995) and vertebrates (Henrique *et al*., 1995; Lewis, 1996; Myat *et al*., 1996; de la Pompa *et al*., 1997; Haddon *et al*., 1998). Proteins of the Notch family are large, ligand-activated transmembrane receptors (Greenwald, 1994). Activating ligands include proteins of the DSL family (Delta, Serrate, Lag-2) (Artavanis *et al*., 1995; Weinmaster, 1997). Ligand binding induces proteolytic release of the Notch intracellular domain (NICD) (Kidd *et al*., 1998; Lecourtois and Schweisguth, 1998; Schroeter *et al*., 1998; Struhl and Adachi, 1998), which translocates to the nucleus where it associates with members of the CSL family of DNA-binding proteins [CBF1, Su(H), Lag-1] (Jarriault *et al*., 1995). In neurogenesis, transcriptional targets of the CSL–Notch complex are genes related to Enhancer of split [E(spl)], which antagonize the basic helix–loop–helix (bHLH) proteins related to Achaete-Scute. This results in preventing differentiation and maintaining cells as neuronal precursors as, for example, in the fly central nervous system (Doe and Skeath, 1996) and during primary vertebrate neurogenesis (Gridley, 1997; Weinmaster, 1997). Alternatively, Notch signaling permits cells to assume a non-neuronal fate by repressing the expression of neurogenic proteins in the fly parasympathetic nervous system (Artavanis *et al*., 1995). Transcription of DSL ligands is activated by Acheate-Scute-related bHLH proteins, forming a signaling loop (Ma *et al*., 1996). This loop provides the basis for lateral inhibition (Artavanis *et al*., 1995; Kopan and Turner, 1996; Lewis, 1996; Weinmaster, 1997).

In *Drosophila melanogaster*, selection of myoblast fate is also regulated by the Notch pathway (Corbin *et al*., 1991; Baylies *et al*., 1998). In addition, Notch antagonizes MyoD in cultured vertebrate cells (Kopan *et al*., 1994; Lindsell *et al*., 1995; Shawber *et al*., 1996; Kato *et al*., 1997) and *in vivo* (Kopan *et al*., 1994). These observations support the hypothesis that the regulation of myogenesis and neurogenesis is achieved analogously: positively acting bHLH transcription factors (e.g. MyoD or Achaete-Scute) are repressed by signals from the Notch pathway (Kopan and Turner, 1996). However, predicted precocious differentiation of myoblasts or expansion of myoblast pools at the expense of other mesodermal cells has so far not been detected in experiments where Notch signaling was inactivated (Conlon *et al*., 1995; Oka *et al*., 1995). Moreover, experiments in *Xenopus* (Jen *et al*., 1997) and mouse (McGrew and Pourquie, 1998) suggest that Notch signaling is involved primarily in setting the somite boundary, rather than affecting the pool of myogenic precursors. However, the possibility that Notch signaling contributes not to the selection of non-myogenic fates but rather to the maintenance of myogenic precursors in an uncommitted state has not been ruled out. Supporting the latter possibility is the observation that unlike neurogenesis, where cells subjected to Notch signaling for prolonged time choose an alternative fate (Nye *et al*., 1994), MyoD repression by Notch is reversible (Shawber *et al*., 1996).

An important component of the lateral inhibition loop during neurogenesis is the ability of proneural bHLH proteins, which are repressed by Notch signaling, to act as activators of Notch ligands (Artavanis *et al*., 1995; Kopan and Turner, 1996; Ma *et al*., 1996). Here we show that MyoD is a direct, positive regulator of Delta-1 expression in the muscle-forming mesoderm of the frog embryo. Furthermore, ectopic activation of Delta-1 by MyoD leads to the induction of the endogenous *ESR-1* gene (Wettstein *et al*., 1997) and of a *HES-1* reporter gene (Jarriault *et al*., 1995). *Xenopus* ESR-1 and mouse HES-1 are related to the *Drosophila* E(spl) proteins, which as transcriptional repressors, mediate the inhibitory functions

of the Notch signaling pathway (Wettstein *et al*., 1997), reminiscent of the relationship seen between Notch ligand and Notch targets in neurogenesis. These observations support the hypothesis that several aspects of myogenesis and neurogenesis are analogous, and suggest that Notch participates in regulation of myogenesis in addition to its role in somitogenesis.

Results

MyoD stimulates XDelta-1 transcription

The myogenic bHLH protein MyoD is induced in the prospective muscle-forming region of *Xenopus* embryos at the early gastrula stage (Hopwood *et al*., 1989; Steinbach *et al*., 1998). At about the same time, the Notch ligand XDelta-1 has been reported to be expressed in the ventrolateral mesoderm (Ma *et al*., 1996). Side-by-side RNA *in situ* hybridization for Delta-1 and MyoD (Figure 1A and B) revealed a striking overlap of the two expression domains. Double label analysis with slightly older embryos (Figure 1C, E and F) confirmed that the MyoD-positive cells are included in the somewhat broader horseshoe-like domain of Delta-positive cells in the marginal zone. Notably, Delta-1 expression in these cells is transient and disappears during involution, while MyoD expression persists (see asterisk in Figure 1E). These observations raised the intriguing possibility that MyoD may influence the expression levels of Delta-1 locally, and thus trigger Notch signaling transiently in an analogous fashion to proneural genes during neural fate determination (Chitnis *et al*., 1995; Kopan and Turner, 1996; Ma *et al*., 1996).

To test this hypothesis, we microinjected synthetic transcripts encoding XMyoD protein near the animal pole into each cell of the four-cell stage embryo, followed by RNA *in situ* hybridization for XDelta-1 at mid-gastrula. Compared with uninjected siblings (Figure 1G), MyoD-expressing embryos clearly showed ectopic Delta-1 expression in the injected region, at levels comparable with endogenous Delta-1 expression in the marginal zone (Figure 1H). Furthermore, isolated animal cap explants from injected embryos also contained elevated levels of Delta-1 mRNA (compare Figure 1I and J). By RT–PCR analysis, we found that Delta-1 mRNA levels were induced ~25-fold in XMyoD-injected explants at this developmental stage [12 h post fertilization (hpf); Figure 2, black bars, Figure 3, lane 4], while control injection of *lacZ* transcripts had no effect on Delta-1 mRNA levels (data not shown).

To ascertain whether or not during normal development the initiation of the mesodermal Delta-1 transcription can be attributed to transactivation by MyoD protein, we performed a time course analysis with developmentally staged RNA samples (Figure 2). Embryos (white bars) showed a significant overall increase in Delta-1 mRNA between 10.5 and 11.25 hpf (i.e. at mid-gastrula, or stage 11 of Nieuwkoop and Faber, 1967), mostly due to the normal increase of Delta-1 transcription in the pre-involuted mesoderm (see Figure 1). In the animal cap assay, Delta-1 mRNA levels were raised in the course of activin-mediated mesoderm formation (gray bars) and by microinjection of XMyoD mRNA (black bars). While the timing of the activin-mediated Delta-1 induction was the same as in the embryo, induction by MyoD was detected 45 min earlier. In normal development, MyoD induction occurs precisely between 9.75 and 10.5 hpf (see Steinbach *et al*., 1998). Therefore, these results are consistent with the hypothesis that Delta-1 activation in the mesoderm depends at least in part on MyoD protein accumulation.

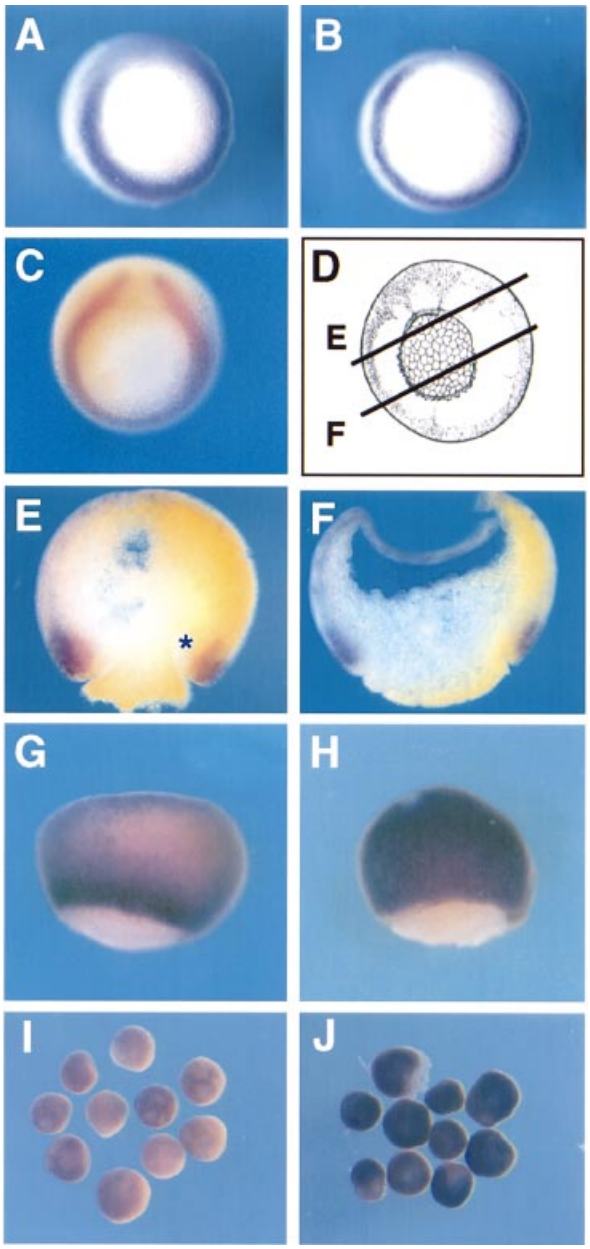

Fig. 1. Relative expression domains and ectopic Delta-1 mRNA induction by XMyoD at the mid-gastrula stage. Single- (**A** and **B**) or double-label (**C**, **E** and **F**) RNA *in situ* hybridizations show overlapping expression domains of endogenous XDelta-1 and XMyoD in the pre-involuted, ventrolateral mesoderm. Double-label pictures show XDelta-1 in purple and XMyoD in red. Whole mounts show vegetal views, dorsal side top; (**D**) indicates the plane of sections in (E) and (F). Note that XMyoD expression persists after involution [(C), dorsal-most part of the XMyoD domain], while XDelta-1 expression disappears from the involuted cells [marked by an asterisk in (E)]. Injection of XMyoD mRNA into each cell at the four-cell stage (400 pg total) causes ectopic XDelta-1 expression in the animal hemisphere and in animal cap explants. Lateral views of embryos (animal pole top), respectively of animal cap explants, from either uninjected (**G** and **I**) or XMyoD-injected (**H** and **J**) siblings are shown.

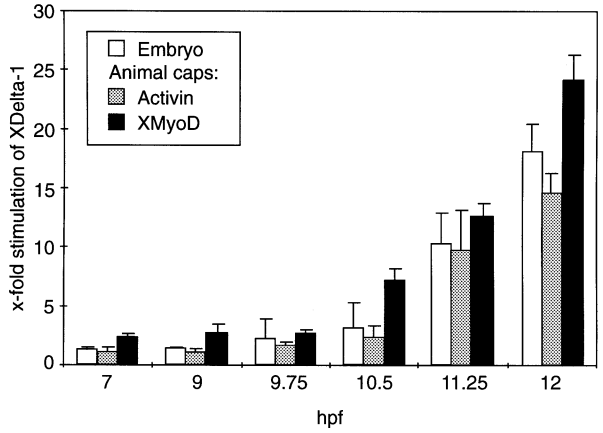

Fig. 2. Developmental time course analysis of XDelta-1 mRNA expression by RT–PCR. RNA samples were derived from control embryos (white bars) or from animal cap explants, which were either induced with activin protein at 7 hpf (gray bars), or have been injected with synthetic XMyoD mRNA at the two-cell stage (200 pg/embryo, black bars). Columns show the average increase of XDelta-1 steady-state mRNA levels as a function of time. The relative XDelta-1-induction is calculated as relative mRNA increase in embryos or stimulated animal caps over uninduced control caps of the same developmental age (see Materials and methods for details; $n = 3$ independent experiments, error bars = SD). We note that control caps show a gradual, 3-fold increase in Delta-1 mRNA levels over this time (data not shown). Delta-1 activation in embryos and activin-induced animal caps occurred between 10.5 and 11.25 hpf, whereas in MyoD-injected explants XDelta-1 was induced 45 min earlier.

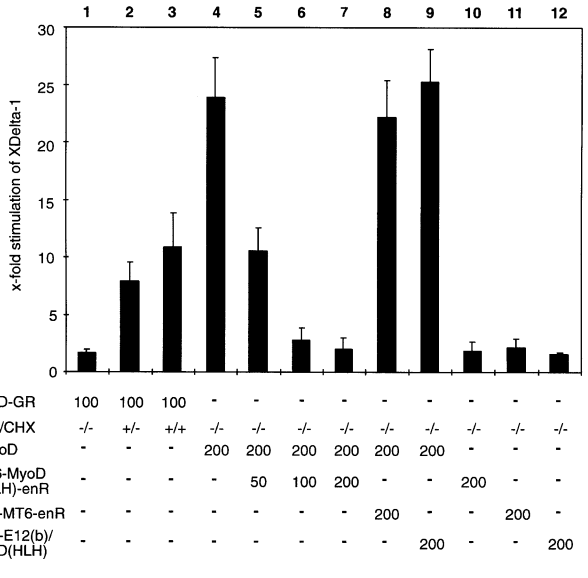

Fig. 3. Regulation of XDelta-1 expression by MyoD variants. Animal caps, pre-loaded with synthetic transcripts as indicated below the panel (numbers refer to the injected RNA dose in pg/embryo), were lysed at mid-gastrula (12 hpf), and relative XDelta-1 mRNA levels were quantitated by RT–PCR. The *y*-axis shows average stimulation of XDelta-1 expression over uninjected sibling explants, after normalization to histone H4 as an internal control ($n = 2$ independent experiments, error bars = SD). Where applicable, explants were treated with cycloheximide (CHX) from 9 to 9.5 hpf, while dexamethasone (DEX) was applied from 9.5 hpf onwards (see Materials and methods for details). Delta-1 mRNA levels are stimulated directly by the hormone-inducible MyoD–GR variant (see lanes 1–3). MyoD-dependent XDelta-1 expression in animal caps is inhibited specifically by the co-injected MyoD repressor variant MT6-MyoD(bHLH)–enR (lanes 4–7), but not by co-injection of the control constructs NLSMT6–enR (which lacks a DNA-binding domain; lane 8) and MT6-E12(b)/MyoD(HLH)–enR (containing a non-myogenic variant of MyoD's bHLH domain; lane 9). No effect was seen with the enR fusion proteins injected alone (lanes 10–12).

Delta-1 is a direct target gene of MyoD

These experiments do not tell us whether the increase of Delta-1 mRNA by MyoD is direct or indirect. To address this question, we injected transcripts coding for MyoD–GR, a hormone-inducible variant of mouse MyoD, into animal caps. This fusion protein contains the ligand-binding domain of the human glucocorticoid receptor inserted in-frame into the MyoD coding region (Hollenberg *et al*., 1993; Kolm and Sive, 1995). At the amount injected, MyoD–GR had no significant effect on XDelta-1 mRNA levels in the absence of dexamethasone (Figure 3, lane 1). In the presence of hormone, MyoD–GR caused a significant increase in Delta mRNA levels within <2 h (Figure 3, lane 2). Furthermore, this stimulation occurred in the presence of cycloheximide (CHX), an inhibitor of protein synthesis, which had been administered prior to hormone application (lane 3). Parallel control experiments demonstrated that hormone application alone had no effect on Delta-1 mRNA levels, and that protein synthesis was effectively blocked by CHX (data not shown; see Figure 5B). We conclude that MyoD can stimulate Delta-1 mRNA accumulation rapidly and directly, without synthesis of additional proteins.

Delta-1 repression by a dominant-negative MyoD variant

We recently have characterized a fusion protein of MyoD's bHLH domain and the transcriptional repressor domain of the *Drosophila* Engrailed protein [i.e. MT6-MyoD(bHLH)–enR] as a potent and specific dominant-negative MyoD variant (for details, see Steinbach *et al*., 1998). To gain additional evidence for the role of MyoD as a positive regulator of Delta-1, we tested whether or not MT6-MyoD(bHLH)–enR could ablate Delta-1 expression in the frog. In the animal cap assay, XMyoD-dependent induction of Delta-1 mRNA was abolished by MT6-MyoD(bHLH)–enR in a dose-dependent manner (Figure 3, lanes 4–7). Importantly, two related control constructs failed to show the same results (lanes 8 and 9), although comparable amounts of their protein products were found in the nuclei of injected cells (Steinbach *et al*., 1998). One of these constructs lacks a DNA-binding domain (i.e. NLSMT–enR). In the other construct, only the basic region of MyoD, which is pivotal for its myogenic activity, has been substituted with the corresponding region of its dimerization partner, the non-myogenic bHLH protein E12 [i.e. MT6-E12(b)/MyoD(HLH)–enR]. Since the remaining HLH domain of MyoD dictates its dimerization behavior, this variant is expected to form preferentially heterodimers with endogenous E-proteins; however, such complexes lack myogenic activity (discussed in Steinbach *et al*., 1998). In summary, we conclude that the enR peptide needs to be fused to a myogenic bHLH domain in order to compete efficiently with wild-type MyoD protein and to repress Delta-1 expression in this assay. These results are in agreement with our previous experiments, in which only MT6-MyoD(bHLH)–enR, but neither of the control constructs, antagonized MyoD protein activity (Steinbach *et al*., 1998).

Upon unilateral injection into the marginal zone at

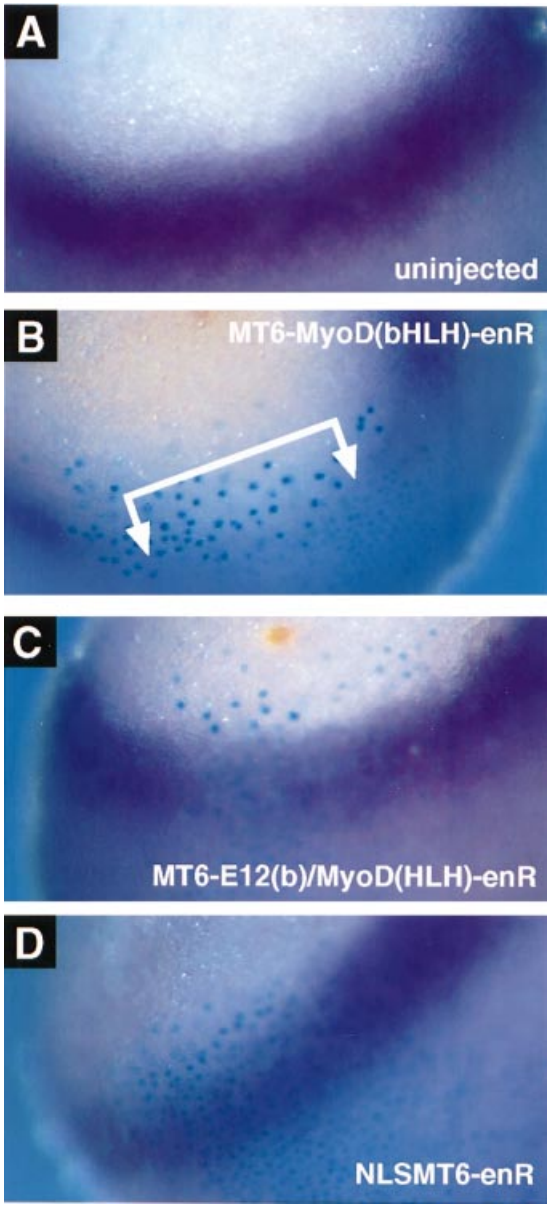

Fig. 4. MT6-MyoD(bHLH)–enR inhibits XDelta-1 induction *in vivo*. RNA *in situ* hybridization with an antisense XDelta-1 probe at mid-gastrula (NF 11). Panels show representative close-ups of XDelta-1 and β-galactosidase staining in the lateral marginal zone (yolk plug top). (**A**) Uninjected control embryos. (B–D) Embryos were injected equatorially into one cell at the two-cell stage with 50 pg of *lacZ* mRNA as lineage tracer, together with transcripts encoding the following enR fusion proteins: (**B**) MT6-MyoD(bHLH)–enR (50 pg); (**C**) MT6-E12(b)/MyoD(HLH)–enR (50 pg); and (**D**) NLSMT6–enR (200 pg). Arrows bridge the region in which MT6-MyoD(bHLH)–enR ablated XDelta-1 expression in (B).

the two-cell stage, MT6-MyoD(bHLH)–enR efficiently abolished Delta-1 expression at the early gastrula stage (compare Figure 4A and B), i.e. at the time when this gene is normally activated in the mesoderm, in the majority of the injected embryos (66%; see Table I). The ablation of Delta-1 expression was correlated spatially with the staining of the co-injected lineage tracer β-galactosidase, consistent with the assumption that MT6-MyoD(bHLH)–enR acts in a cell-autonomous manner (Figure 4B). Again, the NLSMT6–enR peptide had no effect on Delta-1 expression (Figure 4D; Table I). The non-myogenic

Table I. Inhibition of XDelta-1 expression *in vivo*

RNA	pg/embryo	*n*	wt (%)	pt (%)
Uninjected	na	56	56 (100)	0
MT6-MyoD(bHLH)–enR	50	62	21 (34)	41 (66)
MT6-E12(b)/MyoD(HLH)–enR	50	27	24 (89)	3 (11)
NLS-MT6–enR	200	30	30 (100)	0

Embryos were injected unilaterally into the marginal zone at the two-cell stage and scored for Delta-1 mRNA expression by RNA *in situ* hybridization at early gastrula (three independent experiments). Given are: *n*, number of embryos; wt, wild-type Delta-1 expression; pt, partial ablation of Delta-1 expression; na, not applicable.

MT6-E12(b)/MyoD(HLH)–enR variant weakly affected Delta-1 induction in the mesoderm (Figure 4C), although at much lower frequency than MT6-MyoD(bHLH)–enR (11 versus 66%; see Table I). Whether this was due to interference with the activity of other non-myogenic bHLH proteins involved in mesodermal Delta-1 expression or through forced homodimerization with the less abundant endogenous XMyoD protein is not known (see also Steinbach *et al*., 1998). Together, these results provide additional evidence that Delta-1 induction in the pre-involuted mesoderm involves MyoD protein activity.

MyoD triggers Notch signaling

In the frog neuroectoderm, Delta-1-mediated Notch activation induces the expression of the *Xenopus ESR-1* gene (Wettstein *et al*., 1997). XESR-1 is related to the *Drosophila* E(spl) proteins, which as transcriptional repressors mediate the inhibitory functions of the Notch signaling pathway (reviewed by Kopan and Turner, 1996). This conserved epistasis prompted us to test whether the level of MyoD-dependent Delta-1 induction could be sufficient to trigger Notch signaling as measured by induction of ESR-1 expression.

Notch is known to be expressed ubiquitously in the early embryo (Coffman *et al*., 1990). This allowed us to re-probe the same sets of animal cap RNA samples used previously to assess the Delta-1 induction by XMyoD and MyoD–GR (Figures 2 and 3). We found that XMyoD induced ESR-1 with a 45 min delay compared with Delta-1 (compare Figure 2 with 5A), suggesting dependence on protein synthesis. Indeed, CHX completely inhibited ESR-1 activation by dexamethasone-activated MyoD–GR (Figure 5B). Combined, these results suggest that MyoD induces ESR-1 transcription in the animal cap indirectly through upregulation of Delta-1 mRNA and protein levels, which subsequently leads to Notch signaling.

XESR-1 induction by Notch has been shown to involve the ubiquitously expressed CSL family member X-Su(H) (Wettstein *et al*., 1997). To address the possible role of Su(H) protein in the MyoD-dependent activation of ESR-1, we took advantage of *lacZ* reporter plasmids containing the promoter of the related *HES-1* gene from mouse. This promoter includes two copies of the Su(H) DNA-binding motif, through which Notch signaling stimulates *HES-1* transcription. Mutations in these binding sites render the *HES-1* promoter insensitive to Notch (see Jarriault *et al*., 1995). When injected alone, both *HES-1* promoter constructs were inactive (Table II), as determined

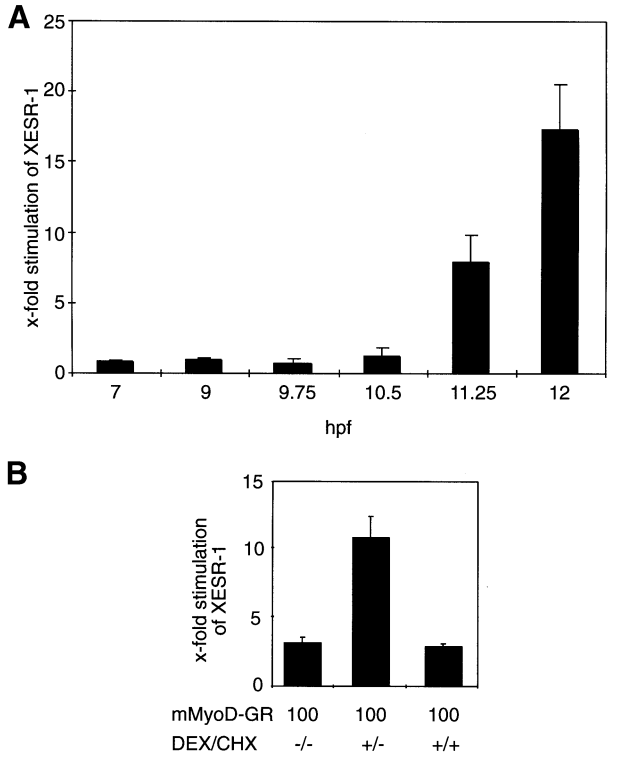

Fig. 5. MyoD indirectly induces the Notch signaling-dependent *XESR-1* gene. (**A**) Developmental time course analysis of XESR-1 expression by RT–PCR. RNA samples were derived from animal caps, which were injected with synthetic mRNA encoding XMyoD (200 pg/embryo). Activation occurs between 10.5 and 11.25 hpf ($n = 3$ independent experiments, error bars = SD). (**B**) The induction of ESR-1 mRNA by MyoD–GR requires protein synthesis. Explants were lysed at 12 hpf; for CHX and DEX treatment, see Figure 3.

by *in situ* β-galactosidase staining at the mid-gastrula stage (Figure 6A and D). Upon co-injection of synthetic transcripts encoding either the constitutively active intracellular domain of Notch (NICD, Figure 6C) or Delta-1 (data not shown), up to 90% of the embryos injected with the HES1–βgal construct contained one or two clusters of βgal-positive cells (see Table II). This reflects mosaic transcriptional activation of the reporter gene in some descendants of the injected blastomeres, which is the expected result for this type of experiment in *Xenopus*. Co-injection of XMyoD mRNA induced *lacZ* expression from the wild-type *HES-1* promoter almost as well as NICD (up to 76% of injected embryos), but failed to induce the mutHES1–βgal construct (compare Figure 6B and E; Table II). The mutHES1–βgal construct was not activated by NICD (Figure 6F, and Table II), indicating that Notch signaling activity was monitored faithfully with these constructs in the frog embryo. Thus, induction of the *HES-1* promoter by MyoD requires the same DNA motifs as its activation by Notch and Delta, probably involving the ubiquitously expressed X-Su(H) protein.

Discussion

The experiments presented here demonstrate that in *Xenopus*, MyoD is a direct, positive regulator of the Notch ligand Delta-1 in the pre-involuted mesoderm of the early gastrula stage, hours before somitogenesis commences. This is based on: (i) overlapping expression patterns of the two genes in the embryo; (ii) ectopic Delta-1 induction by exogenous MyoD in a rapid and CHX-insensitve manner; and (iii) ablation of Delta-1 mRNA after injection of a dominant MyoD repressor variant *in vivo*. In addition, we have found that exogenous MyoD induced ectopic expression of Notch target genes, such as the endogenous *ESR-1* gene or a co-injected *HES-1* promoter construct. While the Delta-1 activation by MyoD was CHX-insensitive, the induction of the E(spl)-like promoters required protein synthesis and the presence of CSL-binding sites. This suggests a regulatory cascade, in which MyoD upregulates Delta-1 protein levels, which in turn triggers Notch signaling, causing activation of target genes through Su(H). Additional evidence for Notch signaling at this location and stage of development comes from the observation that multiple *HES/ESR* genes are expressed in the pre-involuted mesoderm in a similar pattern to that of MyoD and Delta-1 (C.Niehrs and T.Pieler, personal communication). Taken together, these results show that a hallmark of neural cell fate determination, i.e. the feedback loop between differentiation promoting bHLH

Table II. MyoD activates a Notch reporter plasmid

Injected RNA[a]	pHES-lacZ reporter[b]				pmutHES-lacZ reporter[b]			
Dose (pg/embryo)	Dose (pg/embryo)	Experiment *n*	Embryos LacZ-positive *n*	Dose *n* (%)	Experiment (pg/embryo)	Embryos *n*	LacZ-positive *n*	*n* (%)
–	30	1	22	0	30	1	12	0
–	100	5	79	0	100	3	28	0
–	200	1	5	0	200	1	3	0
MyoD								
30	100	1	7	3 (43)	100	1	10	0
100	100	4	53	40 (76)	100	2	22	0
200–600	100	3	49	25 (51)	100	3	36	0
NICD								
30	100	1	17	6 (35)	100	1	18	0
100	100	4	50	45 (90)	100	3	34	0
300–600	100	2	17	13 (77)	100	2	15	0
XDelta-1								
100	100	2	35	18 (51)	nd	nd	nd	nd

[a]Embryos were injected into the animal hemisphere of two opposing cells at the four-cell stage.
[b]Reporter gene activity was assessed at mid-gastrula by β-galactosidase staining. nd, not done.

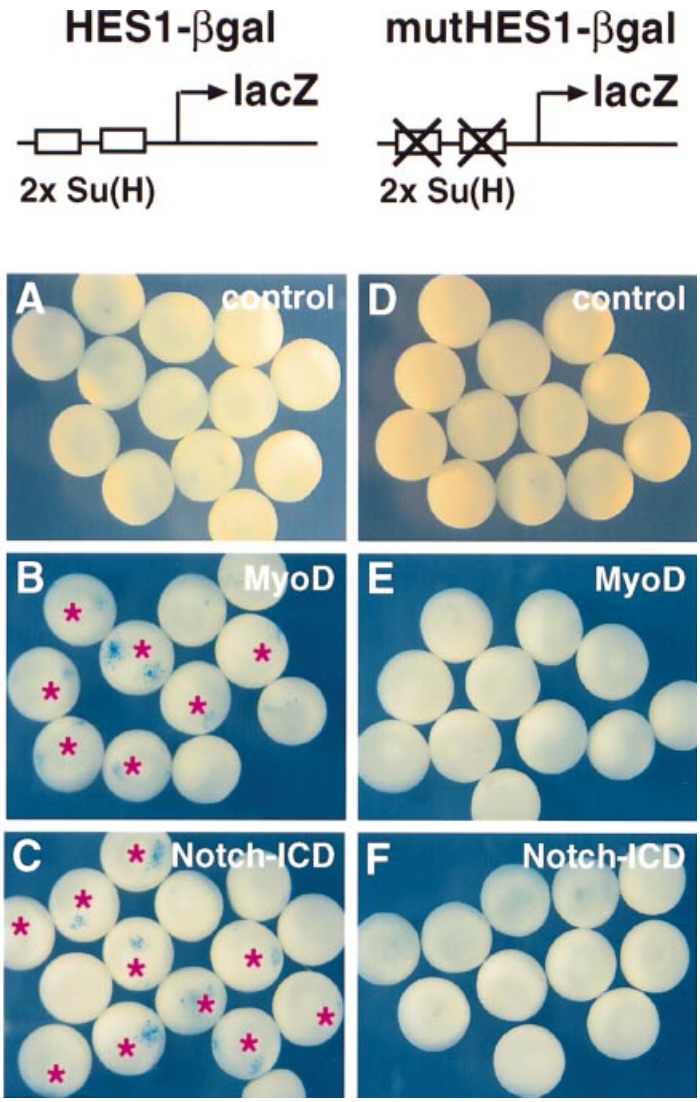

Fig. 6. XMyoD causes activation of a Notch signaling-dependent reporter gene. Embryos were injected near the animal pole into two opposite cells at the four-cell stage with one of the two reporter plasmids (50 pg/cell) shown on top: (**A**–**C**) HES1–lacZ, with the wild-type mouse *HES-1* promoter, or (**D**–**F**) mutHES1–lacZ, with point-mutated Su(H)-binding sites. Synthetic RNAs coding for XMyoD (B and E) or for NICD (C and F) were co-injected at 50 pg/cell. Red asterisks mark embryos with at least one spot of *lacZ*-positive cells, reflecting transactivation of the reporter plasmid. The embryos shown are from one experimental series.

proteins and the Notch regulatory circuitry, is conserved in myogenesis.

What might be the function of this feedback loop in myogenesis? Notch signaling in the somite is postulated to play a role in determination of somite boundary and rostrocaudal polarity (McGrew and Pourquie, 1998), and MyoD (and possibly Myf-5) may activate ligand expression. On the other hand, Notch signaling in vertebrate neurogenesis maintains neuroepithelial precursors by inhibition of the proneural bHLH proteins. In its absence, loss of progenitors coupled with their precocious differentiation is observed (de la Pompa *et al*., 1997; Henrique *et al*., 1997). While it is not known whether this feedback loop is conserved in other species, there is evidence for overlapping expression of myogenic genes and Notch ligands in the pre-somitic mesoderm and the myotome of mice (Kopan *et al*., 1994; Bettenhausen *et al*., 1995). Furthermore, MyoD mRNA is found in epiblast cells of chick embryos and when dissociated, these cells give rise to skeletal muscle *in vitro* (George-Weinstein *et al*., 1996). These experiments demonstrate that muscle differentiation, which occurs relatively autonomous in culture, can be prevented *in vivo* by cell and tissue interactions, possibly mediated by the Notch signaling pathway (George-Weinstein *et al*., 1996). A role for Notch proteins in regulation of myogenic bHLH proteins thus seemed likely, and is supported by our observation that ESR proteins are induced in response to MyoD activity. However, the role of CSL-dependent Notch signaling in muscle cells is controversial (Shawber *et al*., 1996; Kato *et al*., 1997), and none of the expected effects on skeletal muscle differentiation have been observed so far. It is possible that such effects will only be seen in compound, conditional mutant embryos due to the redundancy of Notch, Delta, Serrate and Fringe proteins in the mouse. In addition, determining the exact numbers of myogenic progenitors in the vertebrate is difficult, and this problem is compounded by the observation that both inductive and

repressive signals emanating from the axial and lateral structures continue to mold the myotome in the later somite. Thus, a subtle change in precursor numbers, caused for instance by the loss of a single Notch gene, may be obscured by somitogenesis defects and myoblast fusion. Further confusion stems from the observation that mice lacking the Su(H) homolog RBP-Jκ fail to express the myogenic bHLH protein myogenin, suggesting a block in muscle differentiation (Oka *et al*., 1995), while overexpression of a dominant-negative Su(H) variant in frogs has no effect on myogenesis (Jen *et al*., 1997). Clearly, more work is required to clarify these apparently conflicting results.

In contrast to the mouse, in which strong myogenic gene expression is coupled spatially and temporally to dermomyotome formation (Cossu *et al*., 1996), high level expression of MyoD (and Myf-5) in *Xenopus* is induced much earlier in presumptive mesodermal cells, around the onset of gastrulation. Nevertheless, these cells are not committed to myogenesis until early neurula (Kato and Gurdon, 1993). While we acknowledge the current controversy about Notch function(s) in myogenesis, it is tempting to speculate on the function of this transient Delta-1 expression, which is coupled directly to MyoD induction (Figures 1–3). The pulse of Notch signaling elicited by it may be required for progression of the specification process. The subsequent downregulation of Delta-1 mRNA and Notch signaling is then probably a prerequisite for differentiation, because forced, prolonged Notch signaling blocks synthesis of muscle structural proteins without inhibiting MyoD expression (Kopan *et al*., 1994; A.Authaler and R.A.W.Rupp, unpublished results). Alternatively Delta-1 expression in the pre-involuted mesoderm may be involved in keeping prospective myoblasts transiently uncommitted through Notch signaling, rather than just delaying muscle differentiation. In general, the ability to activate differentiation-promoting factors without immediate commitment may be a pivotal step in pattern formation, where cell populations commonly respond to induction by generating overlapping expression patterns of regulatory genes, which subsequently need to be refined into non-overlapping cell populations.

Materials and methods

Embryo manipulations

The *in vitro* fertilization of eggs, and the culture, microinjection and dissection of embryos have been described (Steinbach *et al*., 1998). To block protein synthesis, animal caps were incubated with 10 μg/ml CHX (Sigma) in 0.5× MBS/2% bovine serum albumin (BSA) for 30 min, then rinsed twice in 0.5× MBS/2% BSA. For hormone induction of the MyoD–GR variant, animal caps were treated with 10 μM dexamethasone (Sigma) in 0.5× MBS/2% BSA. Activin was supplied as a 1:4 dilution of conditioned medium of P388D1 cells. Stability and nuclear accumulation of the three enR fusion proteins in the embryo was comparable (for further information see Steinbach *et al*., 1998).

In vitro synthesis of capped RNA transcripts

The plasmids used as templates for *in vitro* transcription have been described before: for pBSKS$^+$-XMyoDb (*Xenopus* MyoDb), pCS2$^+$MT6-MyoD(bHLH)–enR, pCS2$^+$MT6-E12basic(bHLH)–enR and pCS2$^+$NLS–βgal (nuclear *Escherichia coli lacZ* variant), see Steinbach *et al*. (1998); pSP64T-MD–GR is the mouse MyoD–glucocorticoid receptor fusion gene (Kolm and Sive, 1995). Plasmids were linearized either with *Asp*718 [pCS2$^+$mMyoD, pCS2$^+$XMyoDb, pCS2$^+$NLSMT6–enR, pCS2$^+$MT6-mMyoD(bHLH)–enR and pCS2$^+$MT6-E12basic (bHLH)–enR], *Hin*dIII (pBSKS$^+$XmyoDb), *Bam*HI (pSP64T-MD–GR) or *Not*I (pCS2+NLS–βgal).

RNA analysis by quantitative RT–PCR

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. For details on multiplex PCR, cycle conditions and quantitation, see Steinbach *et al*. (1998). Data points of independent experiments were calculated as arithmetic means of duplicate or triplicate RNA samples. They represent average steady-state mRNA levels after normalization to histone H4 mRNA levels. The following PCR primers have been used (F = forward, R = reverse; standard annealing temperature 58°C): XMyoDb and histone H4 (Steinbach *et al*., 1998); XDelta-1 5′-AATGAATAAC-CTGGCCAACTG-3′(F), 5′-GTGTCTTTTGACGTTGAGTAG-3′(R) (position 1771–2099; DDBJ/EMBL/GenBank accession No. L42229; annealing temperature: 52°C); XESR-1 5′-ACAAGCAGGAACC-CAATGTCA-3′(F), 5′-GCCAGAGCTGATTGTT TGGAG-3′(R) (Wettstein *et al*., 1997).

In situ hybridization

Digoxigenin-labeled antisense RNA probes were *in vitro* transcribed from the plasmids 72-XDelta-1H/R (*Hin*dIII–*Eco*RV fragment of *Xenopus* Delta-1 cDNA; Chitnis *et al*., 1995) and pBSKS$^+$-XMyoDb, respectively. In general, whole-mount *in situ* hybridization was performed as described (Steinbach *et al*., 1998), with the proteinase K step reduced to 10 min for animal caps. Double-label analysis was carried out by simultanous hybridization of digoxigenin- (MyoD) and fluorescin-labeled (Delta-1) RNA probes, and successive color reactions (Fast Red, BM-Purple, Boehringer Mannheim), separated by heat inactivation of alkaline phosphatase (65°C, 30 min).

Reporter constructs and lineage tracing analysis

The reporter constructs HES–βgal and mutHES–βgal have been described (Jarriault *et al*., 1995). Promoter activity was detected *in situ* by β-galactosidase staining at the mid-gastrula stage. For lineage tracing analysis, embryos were fixed for 1 h, followed by β-galactosidase staining (color development <1 h).

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Note added in proof

A recent study in *Drosophila* provides independent evidence that Notch signaling through Su(H) is required for muscle determination. In addition, a Su(H)-independent requirement for Notch in muscle development was also revealed in holonull-mutants, which lack both maternal and zygotic expression of Notch [Rusconi,J.C. and Corbin,V. (1988) Evidence for a novel Notch pathway required for muscle precursor selection in *Drosophila*. *Mech. Dev.*, **79**, 39–50].