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Cadherin-mediated cell interactions are necessary for the activation of MyoD in *Xenopus* mesoderm

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ABSTRACT **Muscle progenitors in *Xenopus* interact in a community of 100 or more cells to activate their myogenic genes and the muscle differentiation pathway. We examine whether the cell adhesion molecule cadherin is involved in this process. Injections of dominant negative N-cadherin RNA into the region of 2- to 4-cell embryos that will give rise to muscle suppress MyoD expression in muscle progenitor cells. By contrast, Xbra expression is unaffected and levels of Xwnt-8 message rise with increasing doses of dominant negative cadherin RNA. MyoD inhibition in embryos injected with the dominant negative cadherin mRNA is rescued by coinjection of full-length cadherin RNA, showing that the inhibition of MyoD occurs through the cadherin pathway. These results show that cadherin-mediated cell interactions play a critical role in the signaling events required for muscle progenitor cells to differentiate, as judged by their stable activation of MyoD.**

The allocation of cells to functionally discrete masses is a critical step in the development of an organized body plan. Cell–cell interactions through the cadherins, a family of calcium-dependent cell adhesion molecules, are thought to play an important role in these processes (for reviews, see refs. 1 and 2). During development, different members of the cadherin family are expressed in spatially and temporally distinct patterns which often correlate with the demarcation of particular tissue territories; cells *in vitro* sort out from one another on the basis of cadherin-specific expression (3, 4). In amphibian muscle development, progenitor cells that have been induced to become mesodermal need to interact in groups of ≥100 in order to activate MyoD expression (5, 6). Thus, cells permitted to interact in a community of like precursors behave differently from those that are grown singly. This phenomenon, known as the "community effect," could be a common developmental mechanism that coordinates the activation of genes in groups of cells fated to form a particular type of tissue. The molecular basis of the community effect is not understood and the purpose of the present study is to determine whether cadherins are involved in mediating these cell interactions.

The cadherins are expressed on most, and possibly all, cells early in embryonic development. They have a single transmembrane domain linking a large extracellular domain, responsible for homophilic binding, to a highly conserved cytoplasmic tail which binds cytoplasmic proteins such as the catenins (2, 7). The cytoplasmic tail is required for functional adhesion among cells (7, 8). In *Xenopus*, U-cadherin (also known as C- or EP-cadherin) is expressed maternally in a ubiquitous pattern through early embryogenesis (9–12), E-cadherin is expressed in the ectoderm beginning at late blastula stages (9, 13, 14), and N-cadherin is expressed at the beginning of neurulation in the neural tube (15). A mutant N-cadherin which lacks the extracellular domain has been shown to act as a dominant negative inhibitor of cadherin function in *Xenopus* embryos (16). Injection of this mutant RNA into the animal pole of newly fertilized eggs severely disrupts cell adhesion, causing embryos to disaggregate, an effect that demonstrates the importance of cadherin-mediated adhesion in maintaining tissue integrity in embryos. Injection of this truncated N-cadherin RNA (N-cadΔE; ref. 16) or overexpression of full-length normal N-cadherin (15) leads to disruptions in tissue boundaries, supporting the idea that cadherins are involved in delimiting tissue territories. The truncated N-cadΔE protein is thought to act by binding competitively to cytoplasmic components normally bound by endogenous cadherins (16) and, since it acts to disrupt cell–cell adhesion in different tissue layers before N-cadherin is normally expressed, it can be regarded as a pan-cadherin inhibitor.

We have used dominant negative N-cadherin RNA to inhibit cell–cell adhesion in the presumptive dorsolateral mesoderm of *Xenopus* embryos. We show that cadherin-mediated interactions play a role in community signaling during muscle differentiation.

MATERIALS AND METHODS

Embryos, Explants, and Injections. Embryos were obtained by *in vitro* fertilization of hormonally induced eggs, raised in 0.1× MBS (17), and staged (18). Embryos that showed distinct dorsoventral polarity were injected with RNA on the lightly pigmented presumptive dorsal area on each side of the cleavage furrow in the equatorial region of the two- to four-cell stage (total of two injections; see Fig. 1*B*). Those embryos that did not show distinct dorsoventral polarity were injected at the equator on both sides of the embryo (total of four injections). Amounts of RNA ranging from 100 pg to 2 ng were delivered in 4–10 nl. Embryos were placed in 5% Ficoll/1× MBS for injection, transferred to 0.1× MBS 2–4 hr after injection, and incubated at 14°C. Dominant negative injected embryos were raised in 1× MBS from stage 11 onward to prevent death due to osmotic shock, since the epidermis failed to form a protective barrier around the embryo.

N-Cadherin Mutants and RNA Synthesis. N-cadherin constructs were kindly provided by C. Kintner (Salk Institute, La Jolla, CA) (16) (see Fig. 1*A*). Both mutants were Myc-tagged by inserting the 10-aa c-Myc sequence close to the signal sequence at the amino terminus (R. H. Riehl and C.E.H., unpublished work). Full-length *Xenopus* E-cadherin (19) and N-cadherin (16) constructs were a gift from A. Crawford, Wellcome/Cancer Research Campaign. Capped RNA was synthesized *in vitro* with SP6 RNA polymerase (MEGAscript kit; Ambion, Austin, TX).

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Abbreviation: NCAM, neural cell adhesion molecule.

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Histology and Immunochemistry. Embryos were fixed in MEMFA (20) for 2–4 hr at room temperature and kept in methanol at −20°C until further processing. Embryos were embedded in paraffin wax and serially sectioned at 10 μm. Antibody staining was carried out on sectioned material (21) and visualized by an alkaline phosphatase–anti-alkaline phosphatase coupled reaction (22). The following primary antibodies were used: anti-MyoD (21), anti-Myc and anti-neural cell adhesion molecule (NCAM) (23), and 12/101 (late muscle marker; ref. 24). *In situ* hybridization of *Xenopus* brachyury (Xbra) mRNA was done by using a digoxigenin-labeled RNA probe on paraffin sections (25). Sections were counterstained with Hoechst 33258.

[^{3}H]Uridine Injections and Autoradiography. At stage 14, each experimental embryo was injected with 40 nl of [5,6-^{3}H]uridine (Amersham) at 20 mCi/ml (1 Ci = 37 GBq) in trunk, tail, and head region. Injected embryos were incubated for 2 hr in a well containing [^{3}H]uridine at 2 mCi/ml in MBS, fixed for 4 hr in MEMFA, and processed for anti-Myc staining as whole embryos. Dewaxed sections were coated with K5 gel emulsion (Ilford) and exposed for 3 weeks at 4°C.

Northern Blot Analysis. RNA was extracted from two embryos at a time and was electrophoresed in a formaldehyde-containing agarose gel (10 μg of RNA per lane). The RNA was blotted onto GeneScreen nylon paper (NEN) and the loading was checked with ethidium bromide staining before and after the transfer. Blots were hybridized with radiolabeled cDNA probes for MyoD (26), Xwnt-8 (27), and elongation factor EF-1α (28) by published procedures (26, 29).

RESULTS

Inhibition of Cadherin Function Inhibits MyoD Expression in Explants of Dorsolateral Mesoderm. Synthetic message encoding the dominant negative mutant (N-cadΔE) or the nonfunctional mutant (N-cadΔE/C) was injected into embryos to target expression to the presumptive dorsolateral mesoderm (Fig. 1). At the beginning of gastrulation (stage 10.5), pieces of dorsolateral mesoderm were excised, cultured overnight as explants in MBS, and probed for MyoD protein expression when control embryos had reached stages 16–18 (22). At the time of excision, the tissue explanted from dominant negative RNA-injected embryos was less densely packed, indicating a loss of cell adhesion. By stages 16–18, sectioned explant tissue appeared normal with respect to cell packing (Fig. 2), suggesting that cells had regained adhesion.

Explants from uninjected embryos or from embryos injected with the nonfunctional mutant RNA all showed typical MyoD staining in 30–60% of the nuclei (Fig. 2 *A* and *B*; ref. 22). By contrast, the explants from embryos injected with dominant negative message were completely negative for MyoD or contained very few (1–10%) positive nuclei (Fig. 2 *C* and *D*). This result shows that inhibition of cadherin function can block the early events in muscle differentiation and suggests that cadherins may be involved in the cell–cell interactions that mediate the community effect.

Cadherin Function Is Necessary for MyoD Protein Expression in Whole Embryos. An alternative explanation for the above finding is that, as a consequence of their altered adhesion, the mutant-expressing cells reposition themselves in the embryo, leading to excision of inappropriate tissue (i.e., not presumptive somite) for explant culture. To investigate this possibility, intact embryos were probed for MyoD expression at stages 16–18 following injection of dominant negative message into two- to four-cell embryos (Fig. 1*A*). Analysis of sectioned embryos showed that more than half of the embryos did not express MyoD at all (Table 1; Fig. 3*B*). All but one of the remaining embryos expressed MyoD at a greatly or significantly reduced level (Table 1). The variability in MyoD expression probably arises from differences in the exact position of delivery and in the spread of the RNA.

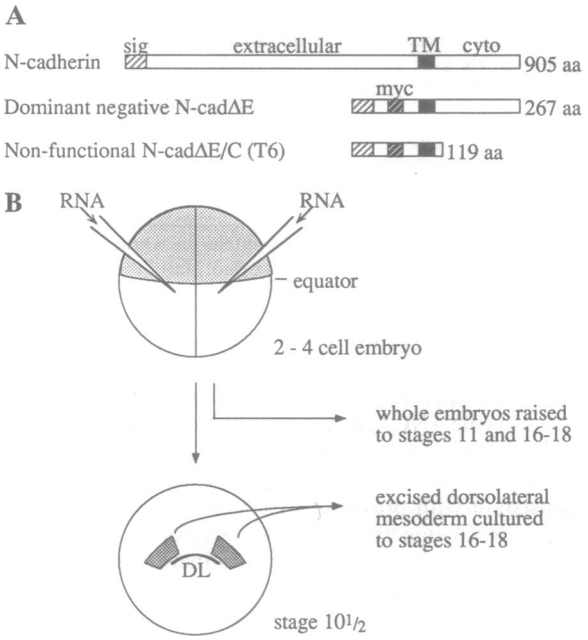

FIG. 1. Schematic diagram of the cadherin constructs (*A*) and summary of experimental design (*B*). (*A*) Normal full-length N-cadherin (top) has a large extracellular domain, a signal sequence (sig), a single transmembrane (TM) domain, and a short cytoplasmic (cyto) domain. The dominant negative form N-cadΔE (middle) has had most of the extracellular domain deleted. The nonfunctional mutant N-cadΔE/C(T6) (bottom) has had both the extracellular and cytoplasmic domains deleted (16). The two mutants contain a Myc tag (myc). (*B*) Cadherin RNA was injected into the dorsal region of embryos at the two sites shown or at four sites (mirror image to those shown). Whole embryos were processed for Xbra *in situ* hybridization at stage 11 and for MyoD immunostaining, Northern blot analysis, and [^{3}H]uridine autoradiography at stages 16–18. Excised pieces of dorsolateral (DL) mesoderm were probed for MyoD expression at stages 16–18.

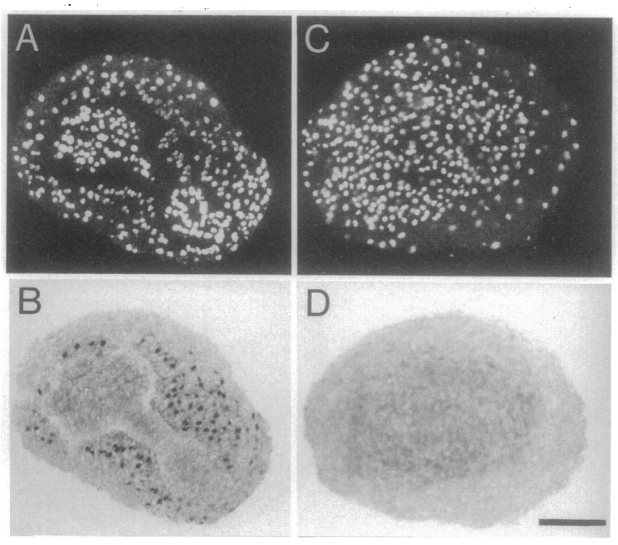

FIG. 2. MyoD expression in dorsolateral mesoderm explants from uninjected control embryos (*A* and *B*) and dominant negative N-cadherin RNA-injected embryos (*C* and *D*). *A* and *C* show staining with Hoechst 33258 to reveal the nuclei, and *B* and *D* show immunostaining for MyoD. In control explants (*B*) 30–60% of the nuclei are positive for MyoD, whereas MyoD staining is absent in dominant negative RNA-injected (600 pg of RNA) explants (*D*). Explants are equivalent to controls at stages 16–18. (Bars = 150 μm.)

Table 1. MyoD expression in whole embryos after injection of nonfunctional (N-cadΔE/C) or dominant negative (N-cadΔE) N-cadherin RNA

RNA injected	No. of embryos			
	–	+	++	+++
N-cadΔE/C	0	0	0	11
N-cadΔE	20	9	8	1

+, Greatly reduced MyoD (5–20 nuclei in whole embryo); ++, substantially reduced MyoD (<60% of normal); +++, normal level of MyoD.

To test for activation of later genes, dominant negative RNA-injected embryos were also stained with antibodies that recognize differentiated muscle (antibody 12/101) and central nervous tissue (NCAM antibody). No positive signal was detected with either antibody in samples from an experimental series of MyoD-negative embryos (data not shown).

Consistent with previous observations (16, 19), dominant negative RNA-injected embryos developed lesions in the ectoderm beginning at stage 11. Distinct dorsal lips formed and were followed by the formation of lateral and ventral lips as in normal embryos, but the blastopores failed to close and, indeed, seemed to "relax" around stage 12, leaving the yolk plug partly uninvaginated. The epidermis halted its epibolic movements around this time and usually relaxed to form a corrugated cap of cuboidal cells on top of a mass of mesoderm and endoderm (Fig. 3*B*).

These results show that inhibition of cadherin-mediated adhesion severely interferes with MyoD protein expression in whole embryos. This is consistent with the explant results and eliminates the possibility that the absence of MyoD expression in explants is due to the migration of dorsolateral mesoderm cells away from their normal position.

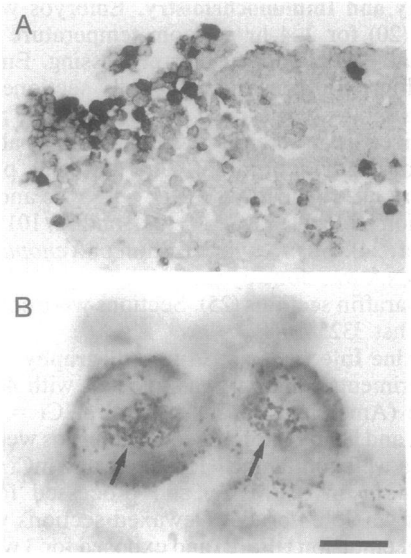

FIG. 4. Cells expressing the dominant negative N-cadherin protein are transcriptionally active. (*A*) Stage 16 embryo immunostained with c-Myc antibody to reveal cells expressing high levels of the Myc-tagged mutant protein (black and dark gray cells). (*B*) Two blue Myc-positive cells (dark gray) showing autoradiographic grains clustered above the nuclei (arrows) after [^{3}H]uridine labeling. Embryos received 600-pg injections of N-cadΔE RNA. (Bar = 80 μm in *A* and 10 μm in *B*).

The Effect Is Not Due to Toxicity in Cells Expressing Dominant Negative RNA. The effect of the dominant negative cadherin protein on MyoD expression might be a result of compromised cell viability due to a nonspecific toxic effect that temporally coincides with the normal developmental

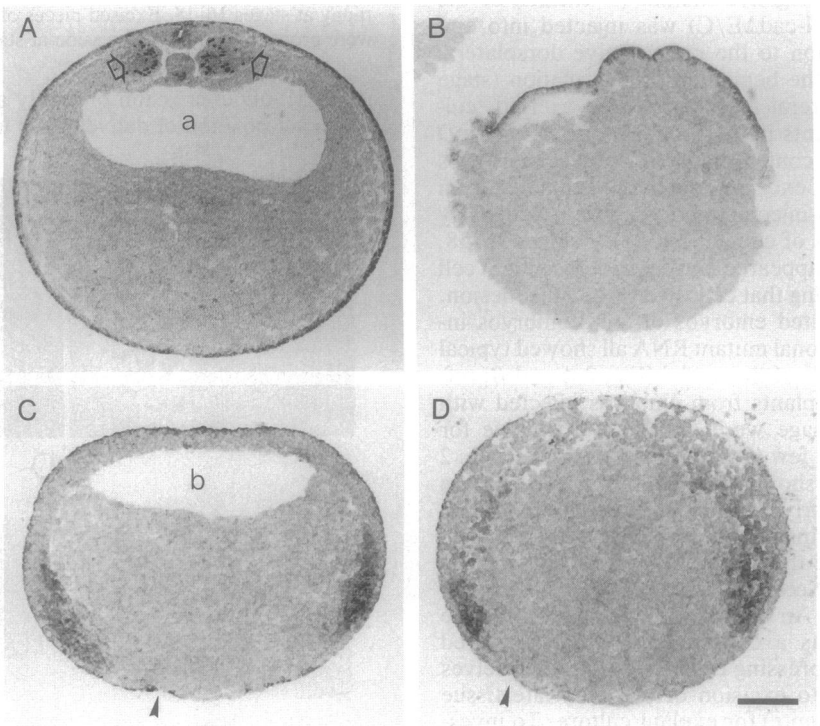

FIG. 3. MyoD and brachyury (Xbra) expression in embryos injected with dominant negative N-cadherin RNA. Transverse sections of stage 18 embryos injected with 900 pg of nonfunctional mutant N-cadΔE/C RNA (*A*) or 600 pg of dominant negative N-cadΔE RNA (*B*). Embryos in *A* have normal MyoD staining in the muscle (see area between open arrows), whereas MyoD staining is absent in *B*. In the latter, the epidermal tissue (pigmented) fails to extend around the embryo, leaving the internal tissue exposed. *In situ* Xbra staining on transverse sections of stage 11 embryos reveals that the pattern of Xbra staining is similar in control (*C*) and dominant negative-injected (600 pg of RNA) (*D*) embryos. Rounded, loosely packed cells indicate loss of cell adhesion in *D* and a recognizable blastocoel does not exist. Dorsal is up in *A*. The original animal pole is up in *C* and *D* and arrowheads indicate the position of blastopores. b, Blastocoel; a, archenteron cavity. (Bar = 200 μm.)

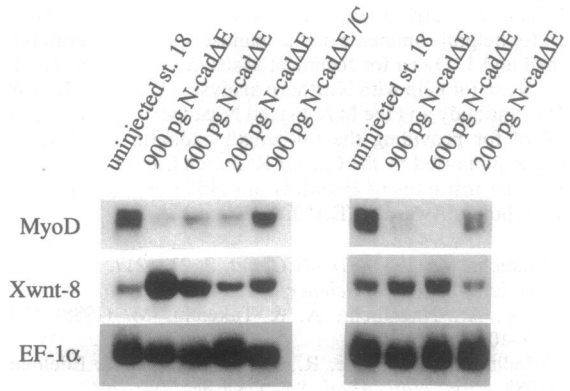

FIG. 5. Two Northern blots showing decreasing levels MyoD RNA in response to increasing amounts of injected dominant negative N-cadherin (N-cadΔE) RNA. Conversely, Xwnt-8 activity rises with increasing doses of injected dominant negative RNA. RNA from two embryos was loaded per lane. Levels of elongation factor EF-1α activity are similar across all the lanes.

time of MyoD expression. We therefore tested the transcriptional activity of dominant negative expressing cells by injecting [^{3}H]uridine into embryos at stage 14 with subsequent autoradiography and immunohistochemistry (c-Myc antibodies) 2 hr later at stage 16. Cells expressing mutant N-cadherin were easily identified with the Myc tag (Fig. 4*A*), and examination of their nuclei at high power frequently showed dense accumulations of autoradiographic grains (Fig. 4*B*). This agrees with our observation that most Hoechst-stained nuclei in dominant negative RNA-injected tissue are normal (Fig. 2 *A* and *C*) and do not show the highly condensed morphology typical of pycnotic cells. Our observation that levels of Xwnt-8 message rise with increasing doses of N-cadΔE RNA (see below) further supports the view that the cells expressing the mutant are viable.

MyoD Inhibition Is Dose Dependent at the Transcriptional Level. To investigate whether the dominant negative N-cadherin protein inhibits MyoD at the transcriptional level and whether it does so in a dose dependent manner, increasing doses of N-cadΔE RNA were injected into 2- to 4-cell embryos, and embryo RNA was isolated at stages 16–18 and subjected to Northern analysis. Fig. 5 shows two such Northerns probed for MyoD and Xwnt-8, a ventral mesodermal marker (27). MyoD transcripts decrease with increasing amounts of the dominant negative RNA. By contrast, Xwnt-8 RNA shows a reverse correlation, with the strongest expression at the highest doses of injected RNA, a result suggesting that Xwnt-8 may be synthesized in cells that would normally express MyoD.

Full-Length Cadherin Rescues the Dominant Negative RNA-Induced Block of MyoD. If competition with endogenous cadherins is responsible for the inhibition of MyoD expression by N-cadΔE, then it should be possible to rescue the defect with full-length cadherin RNA. Embryos were coinjected with 200 pg of dominant negative RNA (the minimal dose found to cause the dominant negative phenotype) and 1–2 ng of full-length N- or E-cadherin RNA. Injections of 1–2 ng of E-cadherin RNA alone gave rise to normal embryos, as did 1 ng of full-length N-cadherin RNA alone (data not shown), whereas 2 ng of N-cadherin RNA alone caused ectodermal lesions, in agreement with previous observations (16). Coinjections of 200 pg of N-cadΔE RNA with 1 ng of either N- or E-cadherin RNA gave phenotypic rescue, producing embryos which developed without ectodermal lesions and which completed blastopore closure (Table 2). At neurula stages (stages 16–18), 36% of the embryos coinjected with full-length N-cadherin RNA developed neural plates but the rest remained rounded, indicating that the rescue was only partial. Coinjection of 1 ng of E-cadherin RNA with 200 pg of N-cadΔE RNA gave complete phenotypic rescue, with 63% of the embryos developing normal neural plates. Rescue, as judged by normal neural plate formation, rose to even higher levels (92%) when 1.5 ng of E-cadherin RNA was coinjected with 200 pg of N-cadΔE RNA (Table 2).

Table 2. Rescue of a dominant negative phenotype by coinjection of full-length N- or E-cadherin RNA

RNA injected	No. of embryos	Stage 12 lesions,* %	Stages 16–18 with neural plate, %
None	14	0	100
N-cadΔE (200 pg)	18	100	0
+ N-cadherin (1 ng)	11	0	36
+ E-cadherin (1 ng)	16	0	63
+ E-cadherin (1.5 ng)	24	0	92

*Ectodermal lesions.

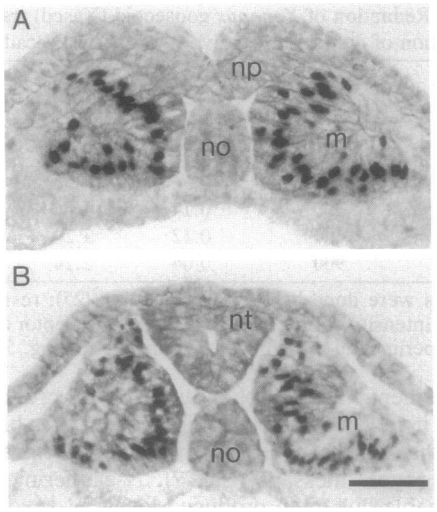

FIG. 6. Rescue of MyoD protein expression with full-length E-cadherin. Transverse sections of uninjected control at stage 16 (*A*) and E-cadherin-rescued embryo at stage 18 (*B*). Positively stained nuclei are present throughout the myotomes (m). The morphology and staining of the E-cadherin embryo are indistinguishable from controls. Two hundred picograms of N-cadΔE RNA was coinjected with 1.5 ng of E-cadherin RNA (*B*). np, Neural plate; nt, neural tube; no, notochord. (Bar = 100 μm.)

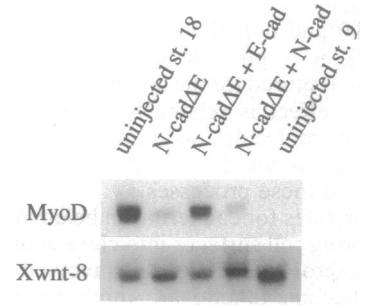

FIG. 7. Northern blot showing rescue of MyoD message by injection of full-length E-cadherin RNA. Coinjection of E-cadherin RNA (1.5 ng) with the dominant negative N-cadΔE RNA (200 pg) restores MyoD message to normal levels. Coinjection of the dominant negative RNA (200 pg) with N-cadherin RNA (1 ng) does not rescue as well as E-cadherin here, but other Northern blots have shown some increase in MyoD RNA. Xwnt-8 activity is similar in all lanes. The increases seen in Xwnt-8 activity in Fig. 5 do not occur here because the lowest dose of dominant negative RNA was used (200 pg). EF-1α activity was the same in lanes 1–4 (data not shown).

Table 3. Reduction of *Xenopus* goosecoid (Xgscd) transcription with injection of dominant negative N-cadherin (N-cadΔE) RNA

N-cadΔE RNA, pg	Normalized transcription	
	Xgscd	Xbra
0	0.56	1.96
200	0.14	1.84
600	0.12	1.26
900	0.04	2.24

Analyses were done by RNase protection (25); results are normalized to intensity of fibroblast growth factor receptor control RNA signal. Experimental design was the same as for Fig. 5.

In agreement with their phenotype, MyoD protein was also observed in rescued embryos (Fig. 6). Northern blot analysis showed that rescue by E-cadherin RNA permitted normal levels of MyoD message (Fig. 7). N-cadherin rescue was more variable but often produced levels of MyoD message that were significantly higher than those observed with dominant negative RNA alone. These results show that the dominant negative exerts its inhibitory effect on MyoD activation specifically through the cadherin pathway.

Cadherin-Mediated Interactions Are Not Needed to Activate Xbra Expression. The inhibition of MyoD expression might be due to a general suppression of mesoderm induction. *Xenopus* brachyury (Xbra), a pan-mesodermal marker, is expressed in the presumptive mesoderm during early gastrulation (30) and is a general indicator of mesoderm induction. When dominant negative RNA-injected embryos were probed for Xbra expression at stage 11 by *in situ* hybridization, they expressed normal levels of Xbra RNA in spite of showing evidence of cell disaggregation (Fig. 3 *C* and *D*). This shows that Xbra expression does not depend on cadherin-mediated cell adhesion. The *Xenopus* gene encoding MyoD is not the only early gene to be affected by the injection of dominant negative cadherin RNA, since Xgscd (31) expression is also much reduced (Table 3).

DISCUSSION

Defects in dorsal structures have been observed in *Xenopus* embryos in other studies in which cadherin function has been impaired (15, 16). Our results here show that the injection of a dominant negative N-cadherin RNA into the presumptive dorsolateral mesoderm suppresses the synthesis of *Xenopus* MyoD message and protein and affects the expression of later muscle-specific genes. It does not affect Xbra, but reduces Xgscd and enhances Xwnt8. Since early genes specific for muscle (MyoD) and notochord (MZ15) require community effects (6, 32), it is possible that cadherin-mediated cell interactions are required for the community effect. However, the community effect is related to dorsalization and may involve the same molecules operating in different ways (33). Our experiments do not show whether cadherin is involved in one or both of these processes.

Two possible roles for cadherin can be envisaged. It might be needed to bring cell surfaces into close enough apposition so that the receptor–ligand systems involved in community signaling can become functional. Another possibility is that cadherins directly mediate signals from cell to cell through the catenins, as suggested by previous work (12).

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