

Muscle Specification in the *Xenopus laevis* Gastrula-Stage Embryo

Kathleen Wunderlich,^{1,2} Jean K. Gustin,^{1,3} and Carmen R. Domingo^{1*}

Recent fate maps of the *Xenopus laevis* gastrula show that mesodermal tissue surrounding the blastopore gives rise to muscle (Keller [1991] *Methods Cell Biol* 36:61–113; Lane and Smith [1999] *Development* 126:423–434). In a significant deviation from earlier data, the new maps demonstrate that cells in the ventral half of the gastrula are precursors to a significant portion of trunk somites. However, these posterior somites are not formed until tadpole stages (stages 38–44). We therefore set out to determine the timing of muscle specification within the ventral half of the gastrula. Our approach was to generate a series of tissue explants from gastrula-stage embryos and then culture them to either stage 28 (tailbud) or stage 44 (tadpole). At each endpoint, the presence of muscle in explants was assessed with a muscle-specific antibody. Interestingly, we found that muscle tissue is detected in ventral explants. However, these explants must be cultured to the tadpole stage. This is perhaps not unexpected, as this is the point at which this tissue normally gives rise to muscle. We further show that muscle specification of the involuting marginal zone does not change over the course of gastrulation. Together, these results suggest that dorsalizing signals emanating from the midline during gastrulation are not necessary for muscle specification of the ventral half of the involuting marginal zone. *Developmental Dynamics* 233: 1348–1358, 2005. © 2005 Wiley-Liss, Inc.

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INTRODUCTION

During embryonic development, cells are faced with progressively fewer choices as they advance from their initial pluripotent condition to a final, single fate. In this context, “fate” is defined as the repertoire of cell types that a particular cell can normally give rise to during its development. The next step, “specification,” occurs when a cell requires no further instruction from its environment in order to achieve its “fate.” Previous studies examining dorsal mesoderm

specification concluded that at the onset of gastrulation, mesodermal tissue adjacent to Spemann’s Organizer is specified to form muscle tissue, and that mesodermal tissues originating from the ventral-lateral and ventral-marginal zones are not specified to give rise to muscle tissue (Dale and Slack, 1987a). These results led to an early theory of mesoderm induction known as the “Three Signal Model” (Slack and Forman, 1980; Smith and Slack, 1983; Slack et al., 1984, 1987; Dale et al., 1985; Smith et al., 1985;

Dale and Slack, 1987a). The first two inductive signals occur during the cleavage and early blastula stages. These events lead to the establishment of the Nieuwkoop Center, which then stimulates the overlying mesoderm to form Spemann’s Organizer (Jones and Woodland, 1987; Godsave and Slack, 1991). The third inductive signal occurs during gastrulation, when graded concentrations of secreted molecules such as noggin (Smith and Harland, 1992) and gooseoid (Cho et al., 1991; Niehrs et

¹Department of Biology, San Francisco State University, San Francisco, California

²Biomolecular Resource Center, University of California, San Francisco, San Francisco, California

³Department of Molecular Microbiology & Immunology, Oregon Health Sciences University, Portland, Oregon

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*Correspondence to: Carmen Domingo, Department of Biology, 1600 Holloway Ave., San Francisco State University, San Francisco, CA 94132. E-mail: cdomingo@sfsu.edu

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al., 1994) emanate from the organizer region and dorsalize neighboring tissues. A fourth ventralizing component has been proposed that limits dorsalization by antagonizing the "third signal" emanating from Spemann's Organizer (Kimelman et al., 1992; Sive, 1993). This fourth signal is thought to be communicated via molecules such as bone morphogenetic protein-4 (BMP-4) (Fainsod et al., 1994; Dosch et al., 1997).

Fate-mapping experiments facilitate the determination of the location of specific cell lineages with respect to the entire embryo. Early fate maps of the *Xenopus laevis* gastrula showed that trunk muscle is derived primarily from the dorsal and dorsal-lateral lip regions of the gastrula-stage embryo, while the ventral-lateral lip contributes little to muscle formation (Keller, 1976). In these experiments, embryos were cultured to stage 22, at which time only 9 to 10 somite pairs have formed. Because the full complement of 45 somite pairs is not achieved until stage 40 (Nieuwkoop and Faber, 1967), tail muscle, blood, and other mesodermal derivatives are under-represented in fate maps that terminate within the tailbud stages (Lane and Smith, 1999). More recently, Keller (1991) revised the gastrula fate map to include the contributions of the ventral marginal zone to the somitic mesoderm. This revision resulted from the observation that cells positioned within the ventral blastopore region converge dorsally and contribute to the somitic mesoderm (Wilson et al., 1989). To provide further support for this revision of the gastrula fate map, Lane and Smith (1999) conducted additional fate-mapping experiments that were carried out to stage 41 to allow for the formation of the entire posterior axis. These experiments clearly showed that posterior somites are derived from the ventral-lateral and ventral marginal zones (Lane and Smith, 1999; Lane and Sheets, 2000).

In this report, we have attempted to more clearly define those regions of the involuting marginal zone that are specified to give rise to muscle. This was accomplished by first generating a set of marginal zone ex-

plants at regular angular intervals around the entire circumference of the blastopore. These explants were then cultured to either stage 28 (tailbud) or stages 38–44 (tadpole). Finally, the presence or absence of muscle in each explant was assessed by immunostaining for the muscle marker 12/101 (Kintner and Brockes; 1984). Our results suggest that muscle specification occurs throughout the marginal zone by the onset of gastrulation, but the anterior-to-posterior progression of muscle formation obscures this fact until later in development (i.e., tadpole stages). We observe no regional differences in muscle specification over the course of gastrulation. Moreover, we find that explants derived from the ventral half of the circumblastoporal mesoderm give rise to muscle in the absence of notochord tissue. These results suggest that dorsalizing signals emanating from the midline are not necessary for muscle differentiation of mesoderm tissue derived from the ventral half of the blastopore at the onset of gastrulation.

RESULTS

Based on the recent revisions to the *Xenopus* fate map (Keller, 1991; Lane and Smith, 1999), we will refer to the blastopore lip regions as "upper" and "lower" as illustrated in Figure 1. We will reserve the terms "dorsal" and "ventral" for our discussion of the tadpole stages, as these are times during which the nomenclature is not debated.

Notochord Is Derived From Only the Upper and Upper-Lateral Circumblastopore Region

All fate maps (Nakamura and Kishiyama, 1971; Keller, 1976, 1991; Dale and Slack, 1987b; Moody, 1987a,b; Lane and Smith, 1999; Lane and Sheets, 2002) agree that notochord is primarily derived from the upper lip region and not at all from the lower lip region. We began by confirming that our explant dissection technique was sufficiently precise to replicate these earlier results. For these

experiments, explants were isolated at three distinct stages of gastrulation. These included the onset of gastrulation, at which time the bottle cells are beginning to ingress from the upper lip region (stage 10), mid-gastrulation, a time when cells at the lower lip region are beginning to ingress into the blastocoel (stage 11), and the end of gastrulation, when the blastopore has nearly finished closing and the majority of the mesoderm has involuted into the blastocoel cavity (stage 12) (Fig. 1). Explants from each of these starting points were cultured to tailbud (stages 28–30) and then stained for the notochord marker Tor 70 (Bolce et al., 1992). Our results showed a high correlation with the fate map predictions and are summarized in Figure 6. Explants made from embryos at the onset of gastrulation (stage 10) stained positively for the notochord marker in upper lip (88%) and upper-lateral lip (57%) explants (Fig. 2A,B; Table 1). Notochord tissue was not detected in explants made from the lower-lateral or lower lip regions of the blastopore (Fig. 2C,D; Table 1). We obtained similar results with explants made from late gastrulae (stage 12). In this case, the majority (86%) of explants derived from the upper lip region expressed the notochord marker, while slightly fewer (59%) positively staining upper-lateral explants were observed. Notochord staining was never detected in explants derived from the lower-lateral or lower lip regions (Fig. 3; Table 1). While these results assured us of the validity of our approach, they also provided us with a measure of the "purity" of our explants. Because notochord can instruct somite development and differentiation (Slack and Forman, 1980; Dale and Slack, 1987b; Lettice and Slack, 1993), the detection of lower-lip derived muscle may be interpreted as a sign of explant contamination by notochord cells from the upper lip. Our control explants allowed us to rule out this possibility, as, in agreement with previously published results, we did not detect the presence of notochord in our lower-lateral and lower lip explants.

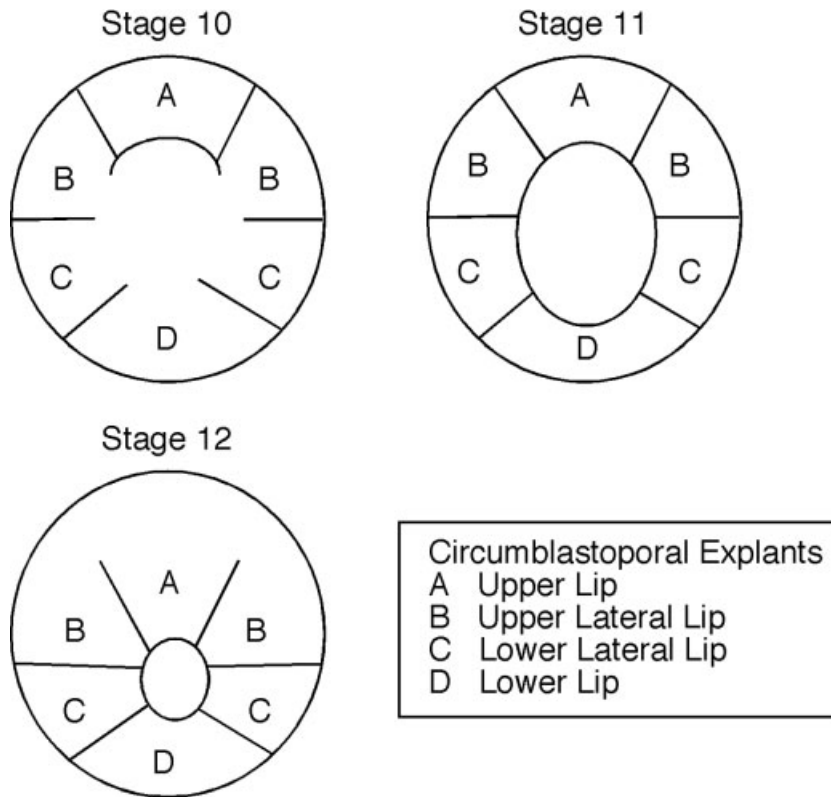


Fig. 1. Schematic diagram of explants generated from gastrulating embryos at stages 10, 11, and 12. Explant A comprises the upper lip region, which has in the past been referred to as the “dorsal marginal zone.” Explant B, which is situated adjacent to the upper lip region, is comprised of the upper-lateral lip, which has previously been referred to as the “dorsal-lateral marginal zone.” Explant C comprises tissue from the lower-lateral lip region, which was previously referred to as the “ventral-lateral marginal zone.” Explant D comprises the lower lip region of the blastopore that has traditionally been referred to as the “ventral marginal zone.” It should be noted that each explant covers an angular distance of approximately 60° around the blastopore.

Muscle Specification Is Most Frequently Observed in the Upper and Upper Lateral Circumblastopore Regions When Explants Are Cultured to Tailbud (Stages 28–30)

Earlier gastrula fate maps (Keller, 1976) established that muscle is largely derived from the upper and upper-lateral lip regions. Later experiments performed by Dale and Slack (1987a) suggested that by the onset of gastrulation, mesodermal tissues from the upper and upper-lateral lip regions of the blastopore are specified to give rise to muscle, whereas mesodermal tissues from the lower-lateral and lower lip regions are not. An important caveat is that this study used tissue that had been isolated from embryos between stages 6 (early blastula) and 10 (onset of gastrulation). Unfortunately, their experiments only

provided specification data up to, but not past, the onset of gastrulation. Therefore, the possibility still remains that the ventral half of the blastopore may become specified to give rise to muscle tissue over the course of gastrulation. For example, the closure of the blastopore may bring the lateral and lower lip regions into close proximity with dorsalizing signals emanating from the midline. In this way, the lateral and lower lip regions may gradually become specified to form muscle over the course of gastrulation. To test this possibility, we made explants from stages 10 (early gastrulation), 11 (mid-gastrulation), or 12 (late gastrulation) embryos. These explants were cultured to tailbud stages, fixed, and immunostained with the muscle-specific antibody 12/101. We found that explants made from embryos at the onset of gastrulation most frequently expressed the muscle marker

in our upper (85%) and upper-lateral lip (100%) explants (Fig. 4A,B; Table 2). Muscle was detected less often in lower-lateral lip explants (40%), and muscle was never observed in lower lip-derived explants (0%) (Fig. 4C,D; Table 2). Explants made from late-gastrula stage embryos gave very similar results; muscle was frequently found in upper (87%) and upper-lateral (96%) explants, and it was observed to a lesser degree in lower-lateral (33%) explants (Fig. 5; Table 2). In only a few cases (2%) did we observe muscle in explants made from the lower lip region (Table 2). In general, the morphology of the 12/101-positive tissues was similar between the explants and consisted of large blocks of cells in the parallel alignment typical of elongated myotome fibers. These results show that for gastrula-stage explants cultured to tadpole stage 28, the marginal zone displays a graded potential to give rise to muscle, with the highest potential in the upper marginal zone and the lowest potential in the lower marginal zone. Moreover, the behavior of each sub-region of the marginal zone remains unchanged over the course of gastrulation (Fig. 6). This suggests that although closure of the blastopore may bring mesoderm positioned in the lower blastopore region into closer proximity to dorsalizing signals, it does not increase the likelihood of muscle developing in this tissue.

Muscle Specification Is Observed Throughout the Circumblastopore Region When Explants Are Cultured to Swimming Tadpole (Stages 38–44)

One interpretation of the above results is that by the end of gastrulation, the lower and lower-lateral lip regions have not received sufficient instruction to form muscle. Instead, they may require further dorsalizing signals that are presumably emanating from the notochord region of the developing embryo (Smith and Slack, 1983). Alternatively, the lower and lower-lateral lip regions may have received muscle-determining instructions at gastrulation, but they may not express that potential until such time as this region contributes to pos-

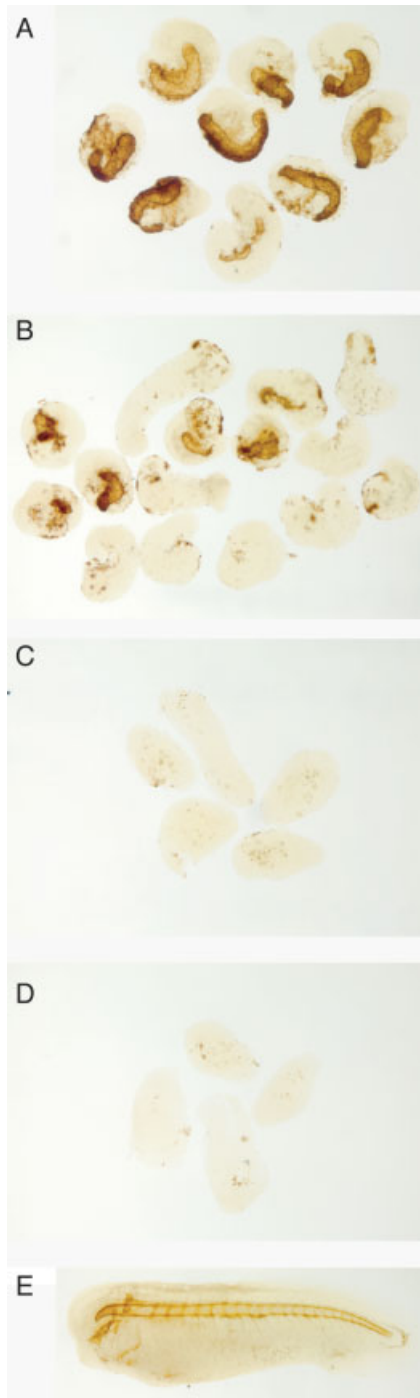


Fig. 2. Notochord-specific staining is restricted to the upper and upper-lateral lip regions in explants made at the onset of gastrulation (stage 10) and then cultured to tailbud stage 30. **A:** Explants made from the upper lip region of the blastopore contain notochord tissue as assayed with the notochord-specific antibody Tor 70. **B:** By comparison to the upper lip, a smaller percentage of explants from the upper-lateral lip stain positively for notochord tissue. None of the explants made from the lower-lateral lip (**C**) or lower lip region (**D**) of the blastopore display notochord staining. **E:** A control embryo at tailbud stage 30 that has been stained for the presence of notochord with Tor 70.

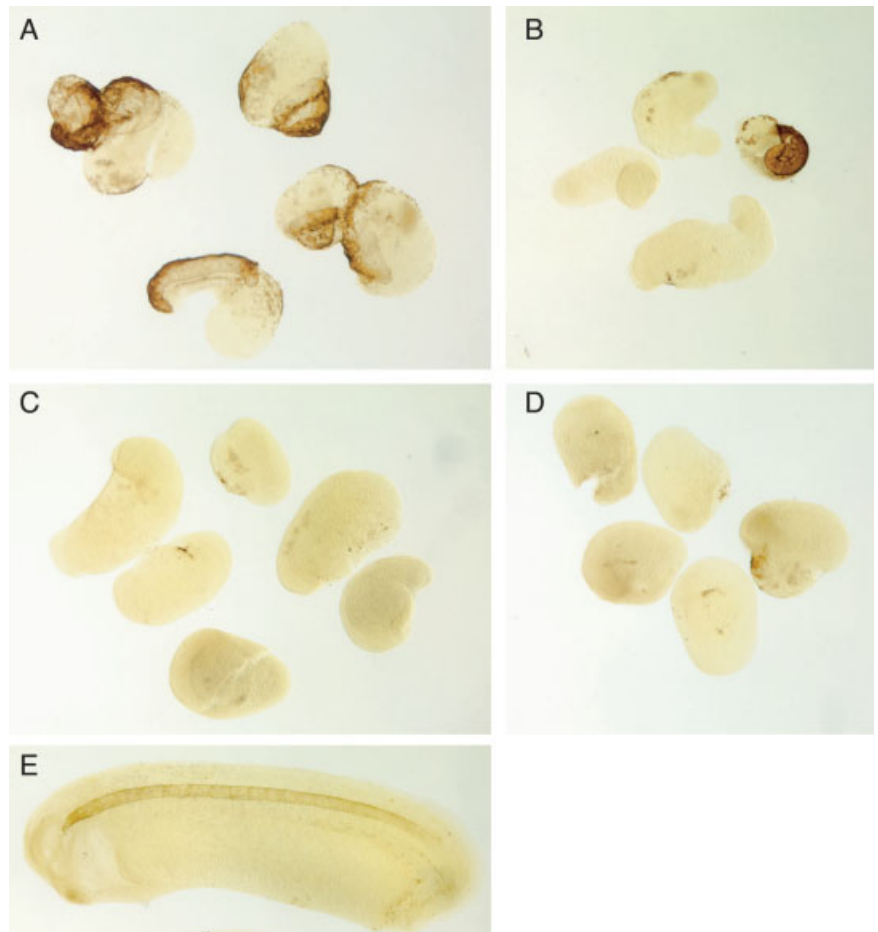


Fig. 3. Notochord-specific staining remains restricted to the upper and upper-lateral lip regions in explants made at the end of gastrulation (stage 12) and then cultured to tailbud stage 28. **A:** All explants made from the upper lip region of the blastopore contain notochord tissue as assayed with the notochord-specific antibody Tor 70. **B:** By comparison to the upper lip, a smaller percentage of explants from the upper-lateral lip stain positively for notochord tissue. None of the explants made from the lower-lateral lip (**C**) or lower lip (**D**) region of the blastopore contain notochord tissue. **E:** A control embryo at tailbud stage 28 that has been stained for notochord with Tor 70.

terior somites (i.e., tadpole stages 38–44). Lane and co-workers (Lane and Smith, 1999; Lane and Sheets, 2000, 2002) have carried fate-mapping experiments out to later stages than those previously characterized. Instead of terminating their experiments at stage 28, they extended their endpoint to the swimming tadpole stages, at which time the tail has completely developed. This key change in their experimental method allowed them to observe that posterior somites are actually derived from the lower and lower-lateral circumblastoporal regions of the gastrula-stage embryo. To test if tissue from the lower lip is specified during gastrulation but does not differentiate until later stages, we also cultured our explants to the swimming tadpole stages. As before,

after culturing the explants to this new endpoint, they were fixed and stained for the muscle marker 12/101. Because the Tor 70 antibody loses its specificity for notochord tissue beyond stage 28 (Bolce et al., 1992), notochord staining was not performed in these experiments. Interestingly, we found that our results matched the more current fate map predictions. Regardless of the stage at which the explants were made, we found muscle in the majority of explants from each region (Table 3). In explants made from embryos at the mid-gastrula stage, we detected the presence of muscle not only in the upper (100%) and upper-lateral (100%) explants, but also with increasing frequency in the lower-lateral (80%) and lower lip (52%) explants (Fig. 7, Table 3). This same trend was observed

TABLE 1. Notochord Detection in Explants Cultured to Tailbud Stage 28

Explant stage		Upper lip	Upper-lateral lip	Lower-lateral lip	Lower lip
		10	Mean \pm Std. error (%)	88 \pm 7	57 \pm 14
	No. of samples, clutches	17, 3	28, 3	25, 3	14, 3
11	Mean \pm Std. error (%)	90 \pm 7	58 \pm 11	6 \pm 6	0 \pm 0
	No. of samples, clutches	25, 5	41, 4	51, 5	27, 5
12	Mean \pm Std. error (%)	86 \pm 5	59 \pm 4	0 \pm 0	0 \pm 0
	No. of samples, clutches	39, 5	79, 5	69, 5	33, 5

among explants made at the end of gastrulation with muscle found in the upper (97%), upper-lateral (100%), lower-lateral (79%), and lower (58%) explants (Fig. 8, Table 3). We found that within explants derived from the lower lip, muscle cells were less organized and more scattered throughout the tissue (Fig. 8D') in comparison to muscle cells found in explants made from the other three regions (see, for example, Fig. 8C').

We clearly observe an increase in muscle formation among explants cultured to tadpole stages rather than tailbud stages. This result can be further highlighted by directly comparing the frequencies with which stage 11 explants gave rise to muscle when they were cultured to either stage 28 or to stage 44 (Fig. 9). By stage 28, only 25% of lower-lateral lip explants express the muscle marker, but by stage 44 this number increases to 80% of the explants. Perhaps more strikingly, at stage 28 none of our lower lip explants display muscle marker expression, but at stage 44 fully 50% of these explants harbor identifiable muscle morphology. We presume that this increase in muscle formation occurs in the absence of notochord-derived signals that are spatially restricted to the upper and upper-lateral blastopore lip (Fig. 6; Table 1). Taken together, these results demonstrate that by the onset of gastrulation, the majority of the mesoderm surrounding the blastopore is specified to give rise to muscle, and that in the case of the lower half of the blastopore, this potential is not revealed unless the tissue is permitted to develop to a stage at which the full complement of somites have formed.

DISCUSSION

In this report, we describe a re-examination of the role played by the involuting marginal zone in the specification of muscle. We show that muscle specification in the marginal zone does not change over the course of gastrulation, but rather appears to be established by the onset of gastrulation. However, explants must be cultured for a length of time that is coincident with posterior muscle development in the intact embryo. Moreover, we show that regions distal to the organizer (lower-lateral and lower lip) are competent to form muscle by the onset of gastrulation, and that this requires no further organizer-dependent signaling.

Muscle Specification Is Not Restricted to Areas Contacting Dorsalizing Signals

Previous studies examining muscle specification concluded that by the onset of gastrulation, only the upper half of the blastopore lip is specified to form muscle (Slack and Forman, 1980). If specification were regionally restricted at the onset of gastrulation, we would predict that regardless of the length of time for which we cultured our explants, we would not have observed the presence of muscle in a significant percentage of explants made from the lower half of the blastopore. Surprisingly, when explants were made from any region at any stage of gastrulation and then cultured to swimming tadpole stages, we observed that more than 50% of explants from each region, including the lower lip, were positive for muscle. Therefore, our data does not support the hypothesis that muscle specification is restricted to those regions that are in close contact with dorsalizing signals.

The Capacity to Form Muscle in the Involuting Marginal Zone Does Not Change Over the Course of Gastrulation

Given both the dramatic cell movements that occur during gastrulation and the presence of dorsalizing signals in the upper lip region, one can hypothesize that muscle specification is a gastrulation-restricted temporal event. During this stage, cells throughout the blastopore converge towards the midline, thus driving the closure of the blastopore (Keller, 1991). Therefore, as the distance across the blastopore is reduced during gastrulation, cells positioned in the lower blastopore region may be brought into contact with dorsalizing signals present in the upper blastopore region. According to this view, muscle specification would progress from the upper to the lower blastopore lip, and the lower lip would not be specified until either the end of gastrulation or perhaps even later. However, our data do not support this hypothesis. Regardless of the stage of gastrulation (i.e., stages 10, 11, or 12) at which they were made, explants from each region behaved nearly identically to one another. We, therefore, find no evidence for a regionally progressive muscle specification event that takes place over the course of gastrulation.

Mesoderm Derived From the Lower Half of the Blastopore Lip Is Specified to Form Muscle, But This Potential Is Not Observed Until Tadpole Stages

It is well established that somitogenesis progresses in an anterior-to-posterior fashion. This leads to the hy-

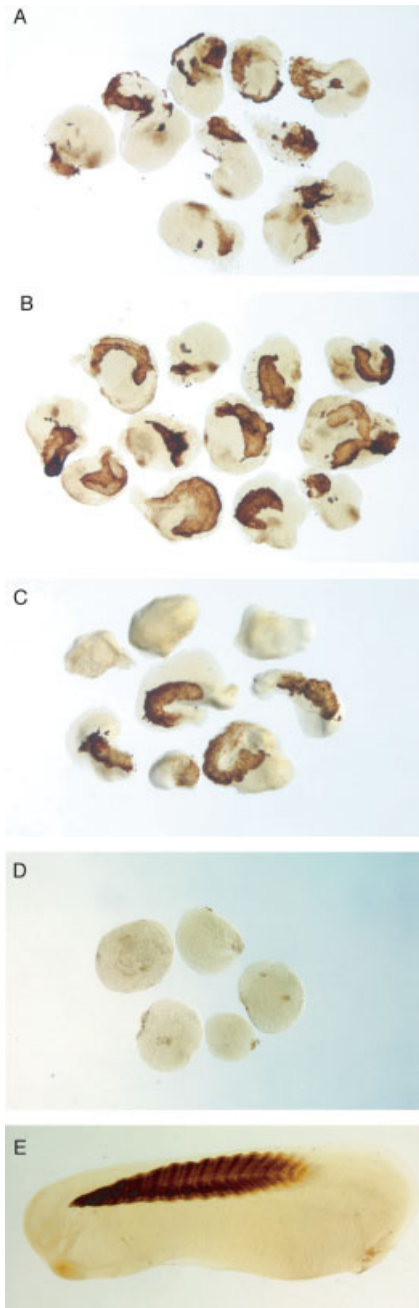


Fig. 4. Muscle-specific staining is absent from lower lip explants generated at the onset of gastrulation (stage 10) and then cultured to tailbud stage 28. **A:** The majority of explants made from the upper lip region of the blastopore contain muscle tissue as assayed with the muscle-specific antibody 12/101. **B:** All explants from the upper-lateral lip region of the blastopore contain muscle tissue. Relatively fewer explants made from the lower-lateral lip (**C**) contain muscle tissue, and no muscle tissue is detected in explants derived from the lower lip region (**D**). **E:** A control embryo at tailbud stage 28 that has been stained for the presence of the muscle marker 12/101.

pothesis that although muscle is specified at the onset of gastrulation, the detection of lower-lateral and

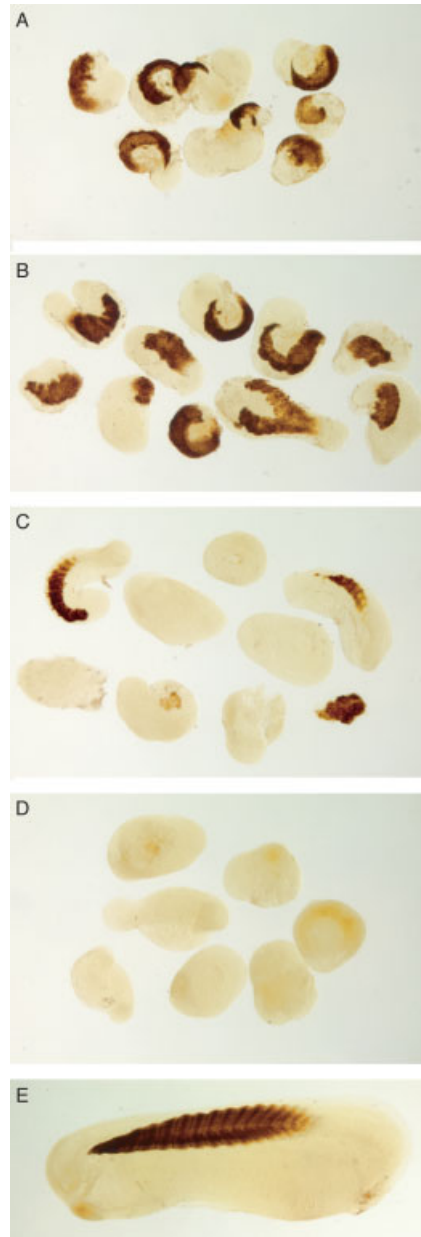


Fig. 5. Muscle staining remains absent from lower lip explants made at the end of gastrulation (stage 12) and cultured to tailbud stage 28. **A:** Most explants made from the upper lip region of the blastopore contain muscle tissue as assayed with the muscle-specific antibody 12/101. **B:** All explants from the upper-lateral lip region of the blastopore contain muscle tissue. A subset of explants made from the lower-lateral lip contains muscle tissue (**C**), whereas no muscle tissue is detected in explants made from the lower lip region of the blastopore (**D**). **E:** A control embryo at tailbud stage 28 that has been fixed and stained for muscle with the 12/101 antibody.

lower lip-derived posterior muscle is not possible until the time when the formation of posterior somites has completed. When we cultured ex-

plants to tailbud stage 28, only a small amount of muscle was detected in lower-lateral lip explants, and no muscle was detected in the lower lip explants. However, when explants generated from these same two locations were cultured to stages 38–44, we observed a significant increase in their muscle content (Fig. 9). These results support the hypothesis that by the time the embryo has reached the gastrula stage, muscle is specified throughout the circumblastoporal region. However, in order to distinguish whether or not specification has in fact occurred, explants must be cultured for a longer period of time to allow for the continued development of posterior muscle that apparently takes place between stages 28 and 44.

The Organizer May Recruit Cells to Participate in MIB for Normal Muscle Morphology

As expected, our lower-lateral and lower blastopore explants do not contain notochord tissue and are, therefore, not likely to be under the influence of dorsalizing signals associated with the organizer. Although these lower lip-derived explants eventually express the muscle marker, we observed that their 12/101-stained tissue is less organized than muscle derived from other regions of the blastopore lip. Recently, Lane and Sheets (2004) have proposed that the primary function of the organizer is to recruit cells into medial-lateral intercalation behavior (MIB). The recruitment of cells into MIB is essential for coordinating the convergence and extension movements of dorsal tissues along the anterior posterior axis (Keller et al., 1992; Keller, 2002). Given that the lower lateral and lower lip explants do not contain notochord tissue, it seems unlikely that they would receive MIB cues from the organizer. This may result in the formation of the scattered muscle cells that we observe in explants made from the lower half of the gastrula, as opposed to the typical chevron-shaped somites that one might expect.

Taking this model even further, one can envision that the organizer (upper lip) provides MIB signals that lead to the recruitment and organization of

TABLE 2. Muscle Detection in Explants Cultured to Tailbud Stage 28

Explant stage		Upper lip	Upper-lateral lip	Lower-lateral lip	Lower lip
10	Mean ± Std. error (%)	85 ± 15	100 ± 0	40 ± 7	0 ± 0
	No. of samples, clutches	21, 4	36, 4	32, 4	18, 4
11	Mean ± Std. error (%)	79 ± 9	99 ± 1	25 ± 6	0 ± 0
	No. of samples, clutches	39, 8	54, 7	75, 8	43, 8
12	Mean ± Std. error (%)	87 ± 13	96 ± 3	33 ± 10	2 ± 2
	No. of samples, clutches	30, 5	71, 5	64, 5	35, 5

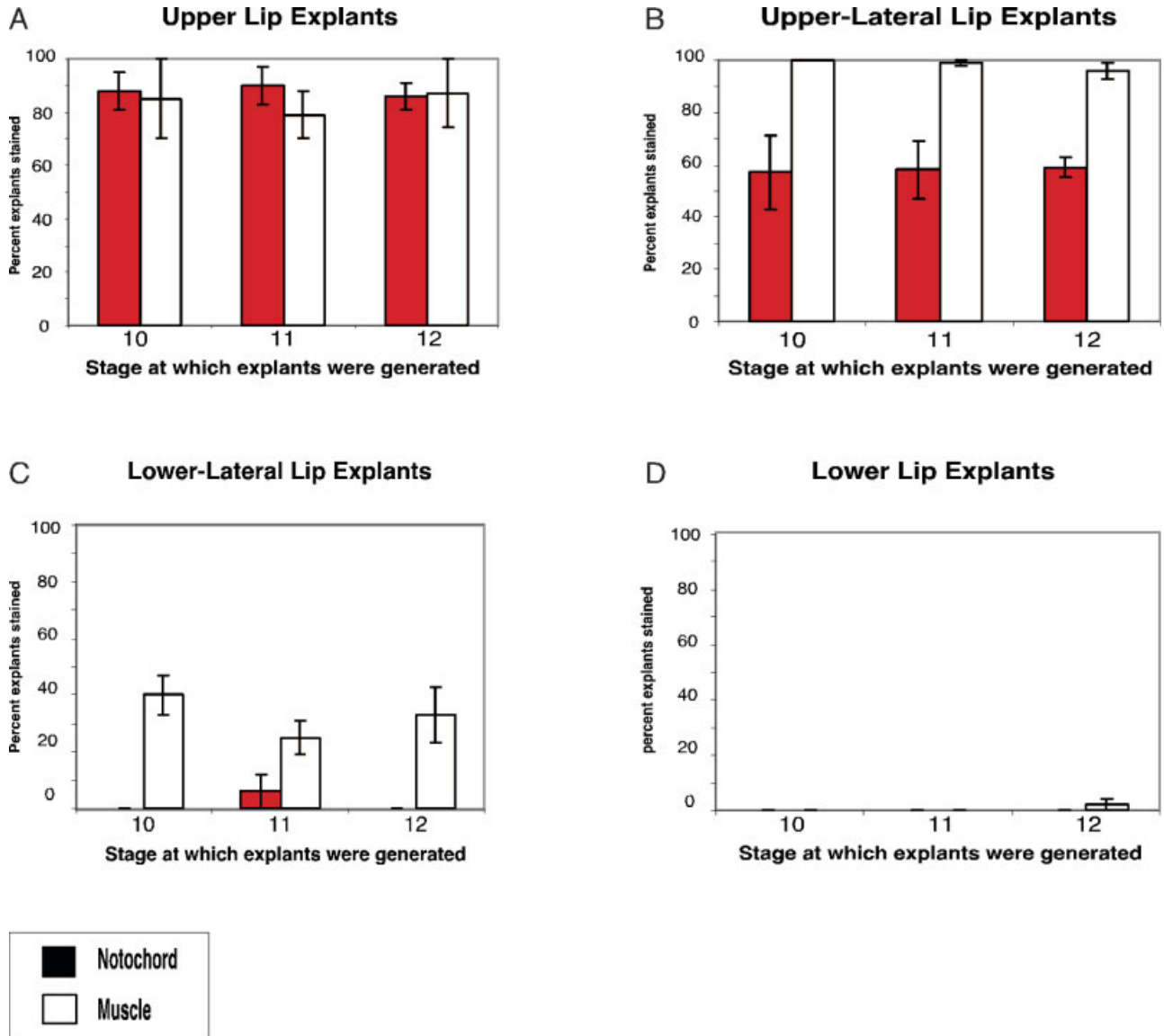


Fig. 6. Comparison of frequencies at which notochord and muscle markers are expressed in cultured explants. **A:** When cultured to tailbud stage 28, upper lip explants made from early gastrula (stage 10), mid gastrula (stage 11), and late gastrula (stage 12) embryos express both notochord and muscle markers at high frequency. **B:** Explants derived from the upper-lateral lip are more predisposed to express the muscle marker than notochord marker. As with the upper lip data, this profile does not change over the course of gastrulation. **C:** When cultured to tailbud stage 28, explants from the lower-lateral lip region give rise to muscle in less than 50% of cases and notochord tissue was rarely observed. Again, little variation is observed among explants generated at different time points during gastrulation. **D:** Notochord tissue is never observed in explants that are made from the lower lip region and then cultured to tailbud stage 28. In rare cases, muscle tissue is observed in these same explants. With few exceptions, no differences are observed among explants made at different stages of gastrulation.

TABLE 3. Muscle Detection in Explants Cultured to Tadpole Stage (38–44)

Explant stage		Upper-lateral	Lower-lateral	Lower lip	
		Upper lip	lip		lip
10	Mean \pm Std. error (%)	61 \pm 20	100 \pm 0	52 \pm 13	63 \pm 22
	No. of samples, clutches	11, 3	26, 4	69, 4	25, 4
11	Mean \pm Std. error (%)	100 \pm 0	100 \pm 0	80 \pm 7	52 \pm 13
	No. of samples, clutches	10, 3	34, 4	28, 4	23, 4
12	Mean \pm Std. error (%)	97 \pm 3	100 \pm 0	79 \pm 4	58 \pm 9
	No. of samples, clutches	31, 4	42, 3	51, 4	27, 4

cells from the lower-lateral and lower lip regions. After receiving these instructions, the cells can then go on to form the posterior trunk and tail myotome. Further support for this model comes from our previous work, in which we showed that signals capable of instructing cells to differentiate into muscle tissue are present in the circumblastoporal region through the early tailbud stages (Dali et al., 2002).

Temporal Regulation of the Anterior-to-Posterior Progression of Muscle Development

While BMP antagonists such as Noggin (Smith et al., 1993; Re'em-Kalma et al., 1995; Zimmerman et al., 1996), Chordin (Piccolo et al., 1996), and Goosecoid (Niehrs et al., 1994; Steinbeisser et al., 1995) can induce anterior somites at gastrula stages, they cannot induce the formation of posterior somites from the lower lip regions (Zetser et al., 2001). On the other hand, BMP-4, which is expressed in the lower lip region, has been shown to induce ectopic tails that include muscle (Beck et al., 2001). Thus, the signaling requirements may differ between anterior and posterior somite development. In the context of our current specification study, it is likely that the upper and upper-lateral blastopore lip explants give rise to anterior muscle and rely on signals emanating from the organizer region. Explants from the lower-lateral and lower blastopore lip regions may receive sufficient instruction from signals such as BMP-4 and XmyoD to initiate posterior muscle development. However, given that explants from the lower-lateral and lower blastopore lip regions show a temporal delay in muscle differentiation, the

question remains as to how the appropriate timing is achieved outside of the context of the intact embryo. One possibility is that BMP-4 acts in concert with another factor that does not reach adequate levels until tadpole stages (stages 38–44). At earlier tailbud stages (stage 28), the action of BMP-4 alone would be insufficient to instruct muscle development. Such a model would certainly be supported by our data. Recently, Lane and Sheets (2004) have proposed a different role for BMP-4, in which BMP-4 acts to repress rather than to instruct muscle development. In this model, the muscle program would be inactive until BMP antagonists such as Noggin de-repress the BMP signal. With respect to our current results, it seems unlikely that explants derived from the lower-lateral and lower blastopore lips would come into contact with Noggin. However, it may be that BMP-4 protein within the explants degrades upon extended culture times. In this scenario, the slow loss of the BMP-4 signal would lead to a loss of repression, and the explants would then be expected to eventually complete the process of muscle differentiation.

Re-Evaluation of Previous Studies

Blastomere recombination and tissue isolation experiments have provided further insights regarding the timing and steps involved in muscle specification. For example, Slack and Forman (1980) found that when cultured to stage 46, small stage-10 explants derived from the lower blastopore lip were not able to form muscle. However, a closer inspection of their raw data shows that when explants were isolated at stage 10, 15% of their lower-lip explants were able to form mus-

cle. Despite this data, the authors concluded that the lower lip region is not capable of self-differentiation into muscle.

Similarly, Dale and Slack (1987a) cultured ventral marginal zone explants from early gastrulae to stage 40 and found that 26% of these explants contained muscle tissue. Given their fate map (Dale and Slack, 1987b), it was expected that ventral tissue would give rise to muscle. However, they proposed that this occurred only after receiving dorsalizing signals from the organizer. In the same report, they did not clearly address why a subset of ventral marginal zone explants were capable of forming muscle in the absence of these dorsalizing signals. Looking at an even earlier time point in development, Godsave and Slack (1991) found that when cultured to stage 40, isolated ventral blastula cells (stage 8) gave rise to muscle tissue in 21% of cases. However, it was interpreted that isolated cells may be more labile than intact embryonic tissue, and for this reason cultured isolated cells displayed an increased likelihood of forming muscle in the absence of dorsalizing signals. Together these studies concluded that the lower blastopore lip region is not specified to give rise to muscle. However, these reports of muscle forming from the lower half of the blastopore left the possibility that specification might be occurring prior to or at the onset of gastrulation. Thus, we re-examined the role played by the involuting marginal zone in the specification of muscle. We show that muscle specification is indeed established by the onset of gastrulation throughout the involuting marginal zone. However, the ability to give rise to muscle from the ventral half of the gastrula remains undetected unless the explants

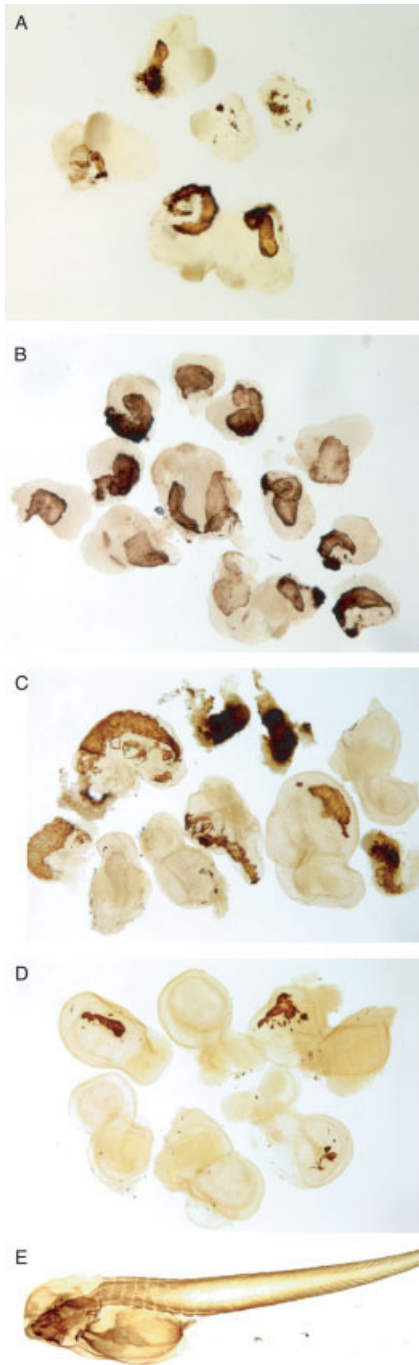


Fig. 7. Muscle is detected in all circumblastoporal explants made at the middle of gastrulation (stage 11) and then cultured to tadpole stage 42. **A:** Most explants made from the upper lip region of the blastopore contain muscle tissue as assayed with the muscle-specific antibody 12/101. **B:** All explants from the upper-lateral lip region of the blastopore contain muscle tissue. **C:** The number of explants made from the lower-lateral lip that contain clearly organized muscle tissue is substantially increased. **D:** In addition, a subset of explants made from the lower lip region of the blastopore contains muscle tissue. **E:** A control embryo at tadpole stage 42 that has been fixed and stained for muscle with the 12/101 antibody.

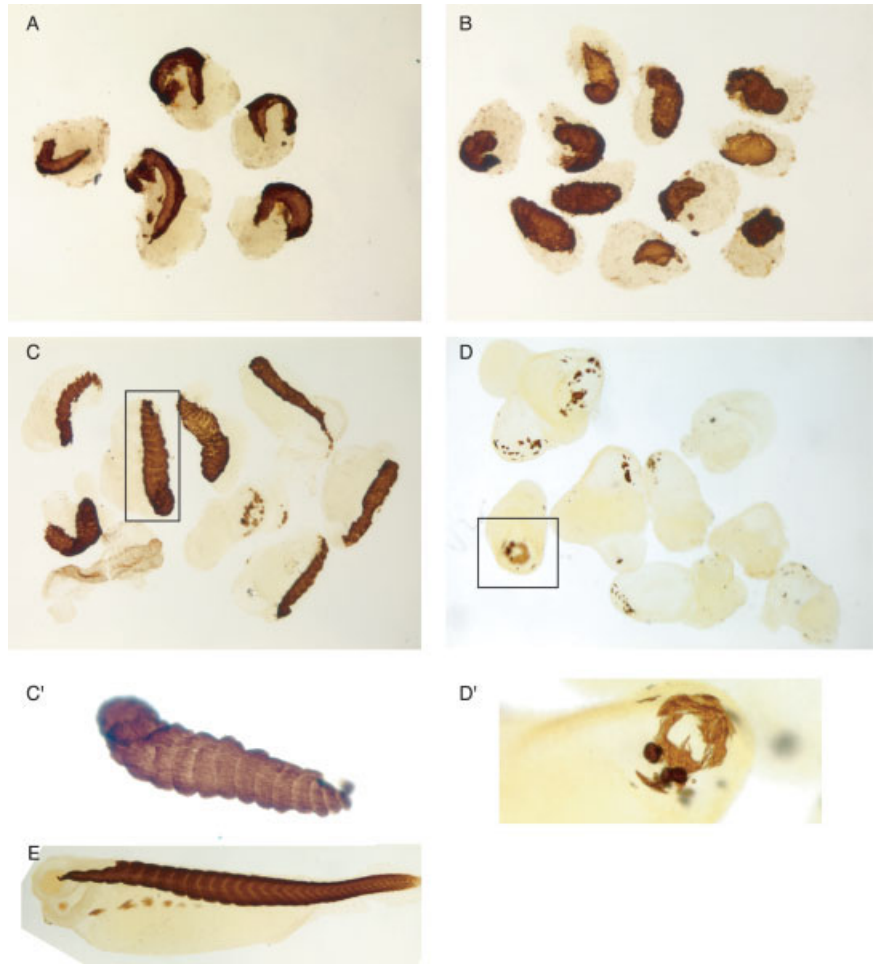


Fig. 8. All explants made at the end of gastrulation (stage 12) and then cultured to tadpole stage 38 harbor muscle tissue as assayed with the muscle-specific antibody 12/101. Explants made from the upper lip (**A**) and the upper-lateral lip (**B**) regions of the blastopore contain muscle tissue. **C:** All explants made from the lower-lateral lip contain muscle tissue that is organized and segmented. **C':** A closer view of an lower-lateral lip explant reveals normal parallel alignment of myotome fibers. **D:** In addition, a subset of explants made from the lower lip region of the blastopore contains muscle cells. **D':** A closer view reveals that these muscle cells have the characteristic myotome-like morphology. **E:** A control embryo at tadpole stage 38 that has been fixed and stained for muscle with the 12/101 antibody.

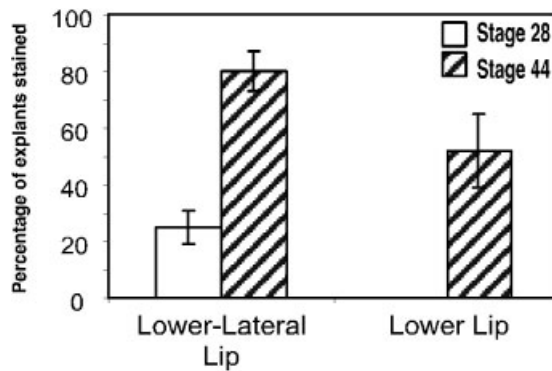


Fig. 9. A dramatic increase in muscle tissue is observed among explants made from the lower half of the blastopore of mid-gastrula embryos (stage 11) and that were then cultured to tadpole stage (stages 38 to 44). Under extended culture conditions, 50% of all explants made from the lower lip region of the blastopore express the muscle marker 12/101. When cultured to tadpole stage rather than tailbud stage, the frequency of muscle development in lower-lateral lip explants increased from 25 to 80%.

are cultured for a length of time that is coincident with posterior muscle development in the intact embryo. Interestingly, Holtfreter (1938) observed that a significant amount of muscle was derived from the ventral and lateral marginal zones in *Rana fusca* and *Rana esculenta*. Given our observations, it is likely that muscle formation in *Xenopus laevis* is similar to that observed in these previously studied anurans.

EXPERIMENTAL PROCEDURES

Culturing *Xenopus* Embryos

Eggs were obtained by standard methods (Kay and Peng, 1991) from adult *Xenopus* ovi-positive females. After *in vitro* fertilization, the eggs were dejellied in 2% cysteine and then cultured in 33% Modified Barth's Solution (MBS) at 15–23°C until they reached the desired stage.

Dissecting Circumblastoporal Explants

At stages 10, 11, and 12 (Nieuwkoop and Faber, 1967), embryos were transferred to 1X MBS in 60- x 15-mm Petri dishes coated with 1.5% agar. The vitelline envelopes were removed with fine forceps (Aesculap). Eyebrow-hair knives were used to dissect explants from the upper lip, upper-lateral lip, lower-lateral lip, and lower lip as shown in Figure 1. The site of initiation of involution within the upper lip (Keller, 1976) was used as a landmark to help establish the angular orientation of the embryos.

Explants (upper lip, upper-lateral lip, lower-lateral lip, and lower lip) were cultured in DFA media on 1.5% agar/1X MBS at 15–23°C until control embryos reached either the tailbud, stages 28–30, or the swimming tadpole, stages 38–44.

Immunohistochemical Assays

Whole embryos (with vitelline envelopes removed) and explants were fixed in 1X MEMFA for 1 hr on a nutator (Clay Adams). The endogenous pigment was removed from samples by bleaching for 2–3 days in a 2:1 mixture of methanol: 30% hydrogen peroxide with nutation under a fluores-

cent light. Bleached samples were transferred to 100% methanol and stored at 4°C for later analysis.

Immunostaining was performed according to the Cold Spring Harbor Manual (Hemmati Brivanlou and Harland, 1994). Primary monoclonal antibodies used in this study were the muscle-specific 12/101, diluted to 1:100 (Kintner and Brockes, 1984) and the notochord-specific Tor 70, diluted to 1:5 (Bolce et al., 1992).

Viewing and Scoring Explants

Explants were cleared in a benzyl benzoate/benzyl alcohol (2:1) solution and viewed on a Nikon Eclipse E600 fluorescent microscope with a 4x Nikon Plan Fluor objective. Images of the explants were captured using a SPOT-RT Slider digital camera and its companion SPOT software (Diagnostic Instruments) running on an IBM-compatible PC. Explants were scored positively if DAB precipitation was above background levels and the stained tissue displayed a morphology consistent with that of normal notochord or muscle. Samples that harbored cells with vacuoles and an elongated morphology were considered positive for the presence of notochord. Those samples containing cells that showed the typical myotome morphology were scored positively for muscle, as were samples harboring small groups of stained cells that are typical of the hypaxial musculature of the ventral body wall (Martin and Harland, 2001).

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