Korean J Biol Sci 1: 329-335, 1997

Spatio-temporal Pattern Formation of Abdominal Muscle in *Xenopus laevis*

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Key Words:
Pattern formation
Abdominal muscle
Somite
Myotome
Heat shock
Xenopus laevis

The final pattern of the skeletal muscle of a vertebrate depends on the position-specific behavior of the muscle precursor cells during early developmental process and the abdominal muscle is made of cells which migrate a relatively long distance from their original tissue, myotome of dorsal mesoderm. We report the spatio-temporal migration pattern of abdominal muscle in *Xenopus laevis* by *in situ* hybridization and immunohistological studies. Shortly after hatching tadpole stage (stage 31/32), a group of myotomal cells detaches from the lower tip of the second somite and migrates ventrally to the lower position of abdomen. At stage 34/35, a second cell group migrates away from the third somite. Total 7 myotomal cell groups migrate ventrally one by one from the second to eighth myotome along their own pathways through the cell free space located between epidermis and subepidermal layer of the abdomen. During migration, the sizes of the cell groups (abdominal muscle anlagens) are increased to several tens fold. Around stage 40 all the abdominal muscle anlagens reaches their final positions and are interconnected side by side rostrocaudally. They are also connected to other types of muscles, forming a large multisegmented abdominal muscle. Heat shock study suggests that the disruption of segmentation of somites does not block the detachment of abdominal muscle anlagen, though the treatment gave stage- and dosage-dependent effects on the migration speed.

The mechanism of mesoderm induction and muscle differentiation in vertebrate has been one of the intensively studied topics of developmental biology. The mesoderm of the neural-stage embryo can be divided into five regions (choda, dorsal [somitic], intermediate, lateral plate mesoderm, and head mesenchyme). Of these regions, dorsal mesoderm gives rise to many kinds of muscles, including myotomal, lymph, heart, and trunk skeletal muscles. As a first step of muscle differentiation, dorsal mesoderm is segmented rostrocaudally blocks of the dorsal mesodermal cells are continuously separated out of the paraxial mesoderm in a cranial-caudal direction at the neural plate stage (Cooke and Zeeman, 1976; Cooke, 1978; Pearson and Elsdale, 1979). After segmentation, somite gives rise to sclerotome, dermatome and myotome and each tissue develops into ventral column, dermis, and muscle, respectively (Chevallier et al., 1977). Groups of myotomal cells are detached from several myotomes, migrate downward, and form abdominal muscle.

The process of abdominal muscle development in amphibian has been studied as a model system for the study of vertebrate skeletal muscle formation (Ryke, 1953; Seno, 1961). After experimental verification that the ventral muscles have a somitic origin in amphibian and avian (Pinot, 1969; Chevallier, 1979), the process of somitic cell migration during limb muscle development has been extensively studied (Jacob and Christ, 1980). However, the mechanism of abdominal muscle migration to form ventral trunk musculature has been unrevealed until electron microscopic observations showing the general migration pattern of abdominal muscle in *Xenopus laevis* (Lynch, 1990).

Genetic and molecular approaches to understand muscle development revealed a number of myoD family genes, the regulation mechanisms of gene expression, and morphological changes of muscle cells during differentiation. In *Xenopus laevis,* most of the findings of the myogenic genes and their functions are obtained from *in vitro* studies. To verify the functions of the genes *in vivo* and to interpret the data obtained from the *in vitro* studies, it is necessary to compare morphological changes of the manipulated embryos with normal ones. So far, however, insufficient information has been accumulated due to the difficulties of *in vivo* study and the lack of appropriate molecular markers.

The present study was done to establish the detailed chronology of abdominal muscle development and to reveal its relations with other muscle tissues in *Xenopus laevis.* Recently, it was revealed that the sarcomeric actin and α-cardiac actin genes are specifically expres-

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sed in the dorsal mesodermal cells and their descendants during the early development of *Xenopus laevis* (Gurdon et al., 1985; Mohun et al., 1990). For this reason, Digoxigenin-labeled α-cardiac actin cDNA probe and anti-sarcomeric actin monoclonal antibody were adopted in this study to trace the developmental process of the abdominal muscle differentiation *in vivo*.

Materials and Methods

Sources of embryos

Embryos were obtained from spawnings of laboratory reared stocks by artificial fertilization. Embryos were staged according to Nieuwkoop and Faber series (Nieuwkoop and Faber, 1967). A total of approximately thirty dozen embryos were examined during the courses of the present study.

Probe preparation

Two types of probes were used. 1) anti-actin monoclonal antibodies (MoAbs): The MoAbs were purchased from Sigma and used for immunohistological studies. 2) Digoxigenin labeled cDNA probes: cDNA probes were obtained by RT-PCR using α-cardiac actin specific primers. RNA for the RT-PCR was harvested as described (Condie et al., 1990) from neurular stage embryos. RT-PCR was carried out as described (Wilson and Melton, 1994). The α-cardiac actin cDNA fragment (252 bps) was primed by 5′-tccctgtacgcttctggtcgta-3′ (upstream) and 5′-tctcaaagtccaaagccacaata-3′ (down stream) (Rubb and Weintraub, 1991).

Whole mount immunohistology and in situ hybridization

Embryos for immunohistological work were fixed in absolute methanol (-20℃) and were passed through the graded alcohol series at room temperature for 30 min each. Epidermis of the embryos were removed while they were immersed in the 25% ethanol. Then they were washed with two changes of PBS buffer (pH 7.4) for 1 h each and the next steps were carried out by a standard procedure (Neff et al., 1989). After being mounted on a slide glass and stored at 4℃ for a week, the specimens were observed with a Nikon Fluophoto Microscope set up for epifluorescence or confocal microscope.

Embryos for *in situ* hybridization were fixed in MEMFA [10 mM MOPS (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde] for 2 h at room temperature with gentle agitation and then transferred to cold absolute methanol at -20℃. Embryos older than stage 35 were post fixed in acidic alcohol (95% ethanol: glacial acetic acid=7:1) for 1 h just after fixation in MEMFA. *In situ* hybridization was performed by a standard procedure (Hemmati-Brivanlou et al, 1990). Specimens were observed with Olympus Inverted microscope.

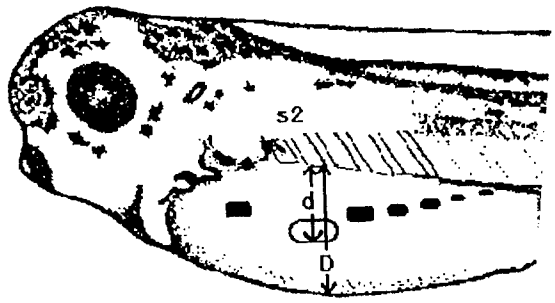

Fig. 1. Principle of the RMP calculation. RMP=d/D×100 / k×100. d, migration distance of the 2nd abdominal muscle anlagenj. D, distance between ventral margin of the 2nd myotome and that of the 2nd abdominal muscle analgen. k, d/D×100 in normal stage 40 embryo. s2, second somite.

Bleaching and clearing

After *in situ* hybridization, wild type *Xenopus* embryos were bleached with H_2O_2 (15% H_2O_2 in 25% PBS) in an open petri dish which was set on bright-background shaker at room temperature for 4 to 5 h. For better observation, embryos were dehydrated in absolute methanol and transferred to 2 benzyl benzoate: 1 benzyl alcohol (v/v).

Relative migration percent (RMP) analysis

To compare the migration distances of the abdominal muscle anlagens, the concept of RMP was applied (Fig. 1). RMP rather than real migration distance was measured because the sizes of abdomens and anlagens were different among the same offsprings. Only the RMPs of number II anlagens were measured and compared in present study because of its relatively long migrating distance compared to those of the other anlagens.

Results

Abdominal muscles migrate in a coordinated manner

To study the lineage of abdominal muscle, the expression pattern of α-cardiac actin gene was studied in early stage embryos. The earliest expression of α-cardiac actin gene was detected in the dorsal lip of gastrular (stage 10) embryo. Thereafter, this gene was expressed only in the somites of dorsal mesoderm and heart anlagen until stage 30, suggesting that this gene has a very high specificity to muscle tissues (Fig. 2A-C). After stage 30 the α-cardiac actin gene was evenly and continuously expressed in somites in a typical longitudinal array (Fig. 2).

Around stage 31 or 32 the first abdominal muscle anlagen (precursor tissue of abdominal muscle) starts to migrate ventrally as a small cell group from somite II (second somite from anterior-most somite) (Fig. 2A). At stage 36 or 37, more anlagens are clearly seen – they migrate sequentially from the somite III, IV, V and VI (Hereafter, each somite and anlagen will be num-

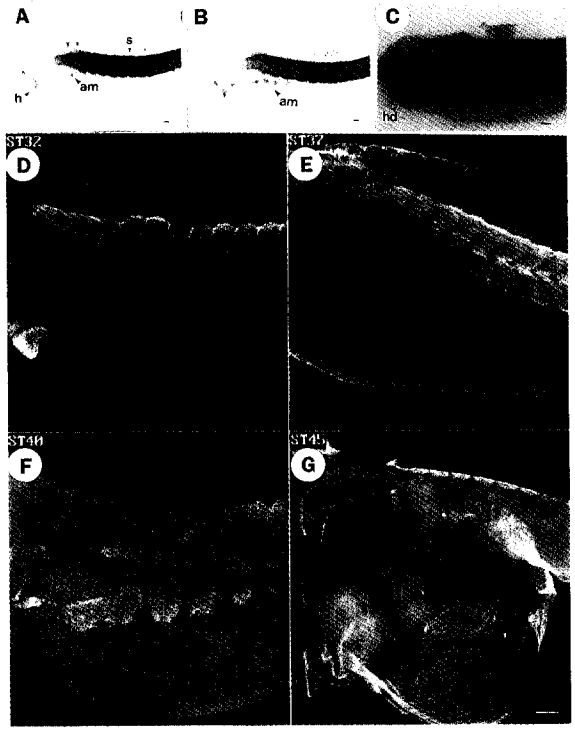

Fig. 2. Abdominal muscle migration in *Xenopus laevis*. A and D, Stage 32. B, Stage 36. E, Stage 37. C and F, Stage 40. G, Stage 45. Embryos of A-C were stained with α-cardiac actin cDNA probe and D-G with anti-sarcomeric actin MoAb. Arrowheads in A indicate anlagen II and III. Arrows in G indicate the interconnected region between neighbouring anlagens. am, abdominal muscle. s, somite. h, heart. e, eye. hd, head muscle. Scale bars=100 μm.

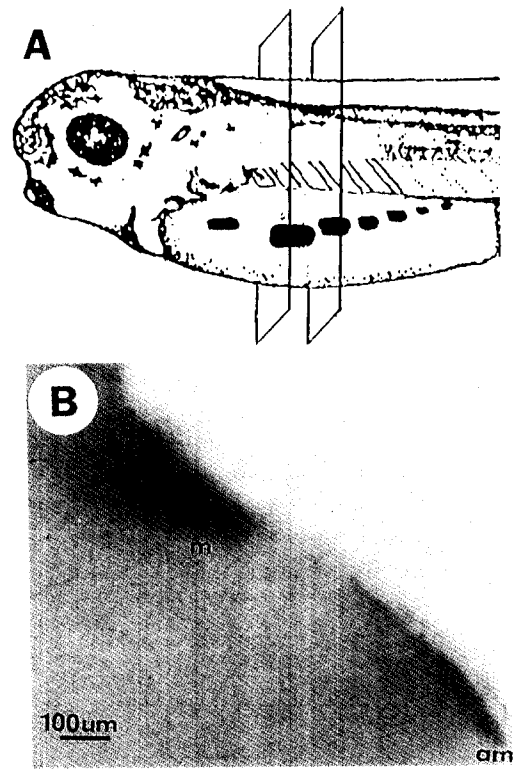

Fig. 3. Anlagens migrate through cell free space as a group of cells. Embryo was stained with α-cardiac actin cDNA probe and was blade-sectioned. A, Diagrammatic drawing of stage 40 embryo with the indication of the sectioned region. B, Frontal view of the section. m, myotome. am, abdominal muscle. ep, epidermis. Scale bar=100 μm.

bered from anterior to posterior direction) (Fig. 2B and G). After stage 37, another two anlagens from the somite VII, and VIII are detached as other previously migrating anlagens. In this way, a total of 7 discrete anlagens detached from myotomes and migrated ventrally. During migration, the anlagens take separate pathways without any contact with neighbouring ones. The anterior and medial anlagens migrate longer distances than those of posterior ones (Fig. 2C and F). Around stage 40, all anlagens reach their final positions and then the individual anlagen starts to be connected each other, side by side, vertically and rostrocaudally forming a complete multisegmented abdominal muscle at stage 45 (Fig. 2G).

Before migration the ventral margin of the myotome has a round shape toward abdomen and the cells of medial margin detach as a group (Fig. 2A and D). *In situ* hybridization and immunocytology show that there is no additional sign of cell detachment from the same myotomes once the initial group detached (Fig. 2). This type of migration is different from that of human's, in which individual myotomal cell continuously migrates away from the same myotome (Ordahl, 1993). The abdominal muscle anlagen migrates ventrally through through cell free space located between epidermis and endoderm of the abdomen. It migrates rather closely attached to the surface of the abdomen than to the epidermis (Fig. 3C).

Initially, the seven anlagens have nearly equal sizes. However, during migration, the abdominal muscle anlagens are progressively enlarged to several tens fold. The medial anlagens (number II to V) become larger than the others and the last anlagen (number VII) has the smallest size. The enlargement continues even after migration until the individual anlagens contact with side by side, as well as with the myotomes from which they migrated away (Figs. 2 and 4).

Abdominal muscle tissues are eventually connected to other muscle tissues

Around stage 40, myofilament-like structures appear simultaneously in the anlagens and myotomes, indicating the cells are initiating final differentiation into mature muscle cells (Fig. 4G and H). After completion of migration (stage 40/41) several cells of each anlagen start to fuse with one another to form myotubes. Tens of myotubes are formed in an anlagen.

A myotube is formed only by cells in the same anlagen. No direct cell fusion occurs between the cells of neighbouring anlagens so that no myotube spans two or more anlagens. There are sharp boundaries in

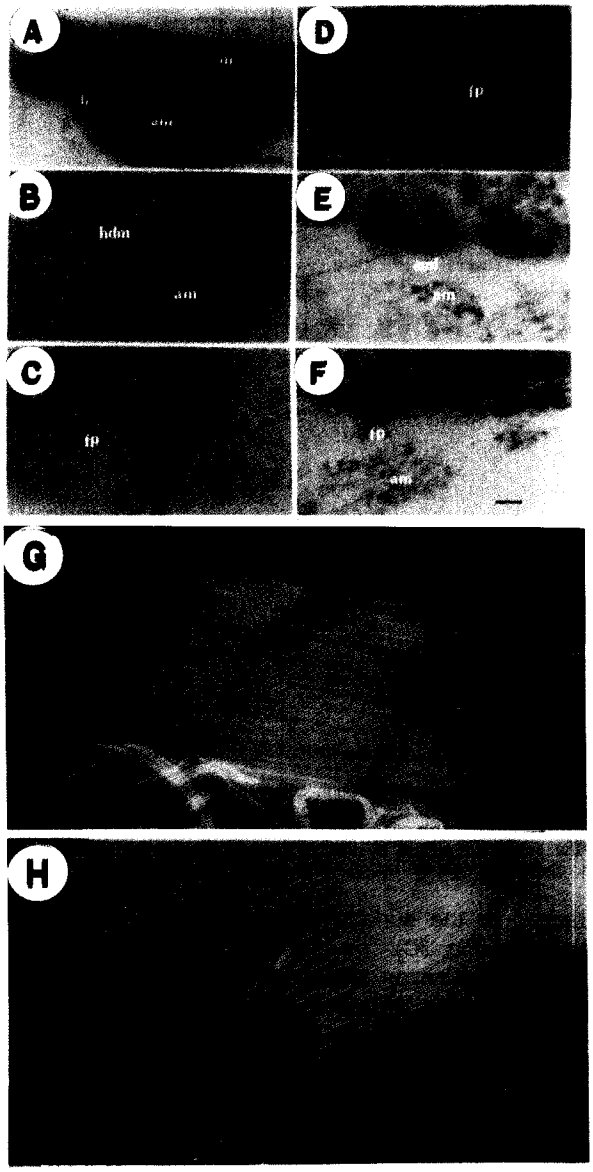

Fig. 4. Connection between abdominal muscle anlagens and to other muscle tissues. Stage 41 embryo was stained with α-cardiac actin cDNA probe (A-F) or anti-sarcomeric actin MoAb (G-H). A, Whole embryo. B-F, Enlarged view of A. B, Connection between head and abdominal muscles. C, Connection between number II and III anlagens. D, Connection between number III and IV anlagens. E, Connection between myotome and number III anlagen. F, Connection between myotome and number VII anlagen. G, Connection of myotubes in somites. H, Whole abdominal muscle. Lower central region of the muscle was disrupted during peeling-off the epidermis. Arrowheads in G indicate inter-somitic region. Arrows in H indicate the interconnected region between neighbouring anlagens. h, heart. m, myotome. hdm, head muscle. am, abdominal muscle anlagen. amf, approaching abdominal muscle. fp, muscle connecting point. Scale bars=100 μm.

the connected regions between neighbouring anlagens, resulting in a multisegmented abdominal muscle on one side of an embryo (Figs. 2G and 4H). No direct cell-cell fusion was detected between neighbouring somites too (Fig. 4G). With the formation of myotubes, cells of anlagens are further connected to neighbouring muscle tissues such as cranial muscle and axial muscle (muscle formed in the somite by the myotomal cells). The anlagen I is connected to cranial muscles (Fig. 4A and B) and the other anlagens are connected to the myotomes from which they originated (Fig. 4E and F). These connections of anlagens to other muscular tissues might be needed to exert an anchoring force for the movement of abdominal muscle at later stages of development. Shortly before abdominal muscle tissues are connected to somitic muscle tissues, fuzzy clusters of muscle cells appear in the lower region of the somites (Fig. 4F). Those fuzzy clusters are thought to mediate the connection between different muscle tissues.

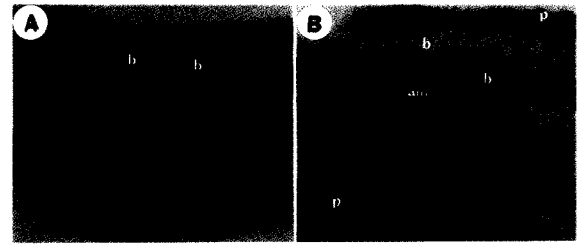

Fig. 5. Heat shock effects on the migration pattern. Stage 40 embryos treated with heat shock at stage 13 at 37℃ for 15 min (A) and 20 min (B). m, myotome. b, blur. am, abdominal muscle. p, pigment cell. Scale bars=100 μm.

Heat shock changes the migration pattern of abdominal muscle anlagen.

In *Xenopus*, somite segmentation begins at stage 17 in the occipital region and progresses in a cranio-caudal direction. By stage 22 somites in the trunk area (somites I-X) have completed segmentation (Jeon and Chung, 1987). To study the relationship between somite segmentation and the migration pattern of the abdominal muscle anlagen, heat shock treatment was adopted. It has been reported that heat shock treatment disrupts the segmentation of somites (Elsdale et al., 1976; Chung et al., 1988; Danker et al., 1992).

Xenopus embryos were given heat shock treatment at stage 13 to 35 and reared until stage 40 or 41. Low to medium dose of heat shock (37℃ for 5 to 15 min) inhibited somite segmentation (blurring). Unexpectedly, anlagens from the blurred myotomes migrated along separate pathways as in normal embryo (Fig. 5A). However, following a heavy dose of heat shock (37℃ for 20 min), both of the somite segmentation and muscle migration showed abnormalities and these embryos lost motility at later stage. Those abnormalities are multifaceted; decrease in the sizes of abdominal muscle anlagens, scattered pattern of migration, and decrease in migration speed (Fig. 5).

Migration speed was measured by RMP value (Figs. 1 and 6). RMP analysis reveals that heat shock shows stage- and dosage- dependent effects on abdominal muscle migration. Embryos were heat shock treated at stage 13, 25, 30, and 35 with medium dose

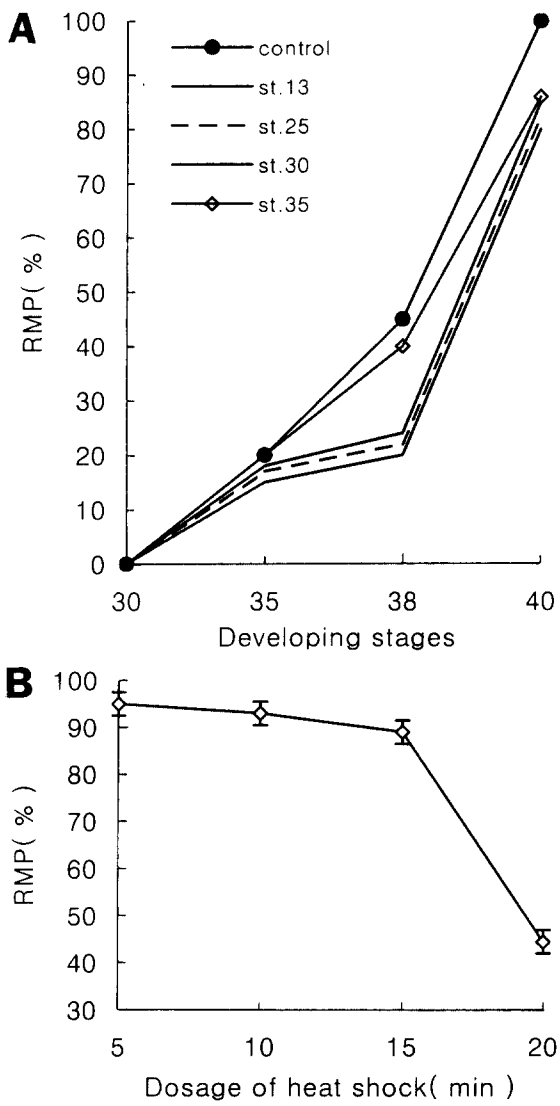

Fig. 6. RMP analysis for abdominal muscle migration. A, Stage dependent heat shock effect on abdominal muscle migration. Heat shock was treated at 37℃ for 15 min at the indicated stages. The standard deviations are smaller than 5%. B, Dosage-dependent heat shock effect on abdominal muscle migration. Heat shock was treated at stage 13 with indicated dosage and RMP was calculated at stage 40. Total 252 embryos were analyzed.

(37℃ for 15 min) and the RMPs were calculated at stage, 35, 38, and 40. Analyzed at stage 38, the RMP value of non-treated embryos was 45, however the values of the heat shock treated embryos were 20, 22, 24, and 40 depending on the treating time; the earlier the treatment the lower the values (Fig. 6A). When embryos were treated at stage 25 to 35, blurs were not formed in the myotomes where abdominal muscle cells would migrate away (in this condition blurs are formed only in the caudal myotomes where no abdominal muscles originate), but the migration showed irregular patterns (data not shown). When embryos were heat shock treated before segmentation begins (stage 13) for 5, 10, 15, 20 min at 37℃, RMP values at stage 40 were 92, 91, 85, and 34, respectively. In the embryos treated for 20 min, the detachment of abdominal muscle anlagen took place as normal embryos, but their migration was severely blocked (Fig. 6B) and eventually lost motility at a later stage.

Discussion

The present lineage tracing study with α-cardiac actin cDNA probe and anti-sarcomeric actin monoclonal antibody confirmed the previous report (Lynch, 1990) that cells of abdominal muscle originated from the myotomes of dorsal mesoderm and clearly showed the migration pattern of abdominal muscle anlagen (Figs. 2 and 3). Although detailed structures of the clusters were not investigated, in this study it is clear that the cells in a migrating anlagen are interconnected with neighbouring cells, not separated as individual cells (Figs. 2 and 3).

As reported, abdominal muscle anlagens migrate ventrally through a cell free space between epidermis and subepidermal layer of pigment cells. Throughout the course of migration, anlagens do not lose their coherent structures. Our study shows that, during migration, the anlagens are tightly attached to the surface of the abdomen (Fig. 3C). This was confirmed by the fact that the epidermis was easily peeled off in 25% ethanol without damaging the structure of abdominal muscle anlagens (Figs. 2F, G, and 4H). Most of the surfaces of the endoderm of vertebrates are coated with basal lamina (consisting of collagen, fibronectin, laminin, and glycosaminoglycan) (Kosher and Lash, 1975; Menko and Boettinger, 1987). And when hybridoma cells, which secrete an anti-integrin antibody that binds to a number of extracellular matrix (ECM), were injected into chick embryo, ventral muscular development was completely blocked (Jaffredo et al., 1988). These reports and our observation suggest that some kinds of ECM on the subepidermal layer do play important roles as an anchoring or substrating material for anlagen migration in *Xenopus*.

During migration the anlagens are progressively enlarged several tens fold compared to their initial sizes. The enlargement might be accomplished by either the increasing cell size, additional detachment of myotomal cells, or by the increased cell population by mitosis of the migrating cells. In our study no additional migrating muscle cells was detected in the region between migrating anlagen and the myotome from which the anlagen has originated and there was no recognizable increase in the size of migrating cells, either (Figs. 2 and 3). Regarding these observations and the report that muscle precursor cells (myoblasts) are still able to divide prior to their differentiation (Konigsberg, 1963), the muscle anlagens in *Xenopus* are more likely to be composed of dividing myoblasts and the cells of the anlagen repeat several rounds of divisions during their migration (Fig. 2).

The mechanism that regulates the spatio-temporal migration pattern of abdominal muscle cells is still unknown. One way to understand the mechanisms of the regulation or coordination of abdominal muscle migration is to give heat shock treatment to the embryos. Heat shock treatment disrupts the segmentation of somites by disturbing the deposition of fibronectin in the segmental border (Danker et al., 1992). In case, heat shock was treated at stage 13 in which deposition of fibronectin initiates to segment somites with small dose (37 ℃ for 5 min) or medium dose (37℃ for 10 or 15 min), segmentation of somite II and III was inhibited resulting in no segmentation in the middle region and partial segmentation in the dorsal most and ventrolateral region of somites (Fig. 5). However, abdominal muscle anlagens migrated away from these nonsegmented somites and migration proceeded almost normally as in control embryo. These results shows that heat shock does not change the fate of abdominal muscle precursor cells and inhibition of somite segmentation in the central region of somites does not affect the migration pattern of abdominal muscle. Somitic tissue exchange experiments might be helpful to understand the selection mechanisms of somite cells as abdominal muscle precursor cells and to study the relationship between somite segmentation and migration pattern.

Considering the report that ECM plays important roles in cell migration, scattered migration in the heat shock treated embryos is believed to be caused by the irregular deposition of ECM in the migration pathway. This idea is supported by the fact that anlagens migrated irregularly in the medium dose of heat shock treated embryos at stages 25 to 35, even though heat shock did not affect segmentation of the somites abdominal muscle cells. Originated Inhibitory effect of heat shock on the deposition of ECM in the migration pathway would decrease the migration speed. RMP analysis reveals that heat shock exerts stage- and dose-dependent effects on the migration pattern, suggessting that ECM deposition for the migration pathway is a rather long lasting event.

The present study could be served as an useful guide for the *in vivo* studies of cell migration and tissue-tissue interaction in muscle development, particularly in the study of myoblast differentiation and the genes which mediates the differentiation process.

Acknowledgements

We thank Drs. Anton Neff and George Malacinski at the Indiana University for their helpful comments on this work. This work was supported by 1994 Non-directed Research Fund, Korea Research Foundation.

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[Received February 3, 1997; accepted April 15, 1997]