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Morphological boundary forms by a novel inductive event mediated by Lunatic fringe and Notch during somitic segmentation

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SUMMARY

Boundary formation plays a central role in differentiating the flanking regions that give rise to discrete tissues and organs during early development. We have studied mechanisms by which a morphological boundary and tissue separation are regulated by examining chicken somite segmentation as a model system. By transplanting a small group of cells taken from a presumptive border into a non-segmentation site, we have found a novel inductive event where posteriorly juxtaposed cells to the next-forming border instruct the anterior cells to become separated and epithelialized. We have further studied the molecular mechanisms underlying these interactions by focusing on Lunatic fringe, a modulator of Notch signaling, which is expressed in the region of the presumptive boundary. By combining DNA in ovo electroporation and embryonic transplantation techniques we have ectopically made a sharp boundary of Lunatic fringe activity in the unsegmented paraxial mesoderm and observed a fissure formed at the interface. In addition, a constitutive active form of Notch mimics this instructive phenomenon. These suggest that the boundary-forming signals emanating from the posterior border cells are mediated by Notch, the action of which is confined to the border region by Lunatic fringe within the area where mRNAs of Notch and its ligand are broadly expressed in the presomitic mesoderm.

Key words: Segmentation, Boundary formation, Notch, Lunatic fringe, Somites, Induction, Chick

INTRODUCTION

Morphogenesis during early development, begins with an originally continuous tissue becoming segregated, followed by the generation of a variety of tissues and organs. Boundary formation is a vital process in the early segregation that is brought about by distinct patterns of gene expression. In many cases this step is followed by a morphological separation by a fissure. If these events fail to occur correctly, there are serious consequences in organogenesis. Mechanisms underlying boundary formation have been extensively studied in *Drosophila*, where body segmentation and anteroposterior (AP) and dorsoventral (DV) separations in the wing disk have often been used as a model system (Dahmann and Basler, 1999; Irvine and Rauskolb, 2001; Lawrence and Struhl, 1996; McNeill, 2000; Sanson, 2001). It is widely accepted that a gradient of signals including morphogens subdivides a tissue field into distinct cell populations by establishing delimited domains of gene expression through cross regulatory interactions, resulting in the formation of a boundary at the level of the gene cascade. Specialized cells localized on one side (border cells) then start to interact with the border cells of the other side. These communications are thought to maintain the boundary and/or to establish a signalling center that organizes further patterning (Dahmann and Basler, 1999; Irvine and Rauskolb, 2001; Lawrence and Struhl, 1996; McNeill, 2000). In vertebrates, mutual suppression of genes has been shown to direct a boundary during regionalization in the early neural tube and brain, including the rhombomeres (Briscoe et al., 2000; Jessell, 2000; Lumsden, 1999; McNeill, 2000), and this border region indeed dictates subsequent events as shown by the midbrain/hindbrain boundary (Joyner et al., 2000; Simeone, 2000; Wurst and Bally-Cuif, 2001). However, it has remained largely unknown how a morphological boundary or separation, the final step of shaping tissues and organs, is manifested in vertebrates. We have used mesodermal segmentation in somitogenesis as a model system to study mechanisms of dynamic morphogenetic movements leading to a boundary formation since the somite forms by pinching off a block of cells from a continuous tissue, thus producing a clear gap and a boundary to the tissue.

The somite in vertebrates is a transient structure that is reiterated along the body axis by segmentation processes and makes a major contribution to the formation of the axial structures including vertebral bones and skeletal muscles (Christ and Ordahl, 1995; Pourquié, 2001; Stern and Vasiliauskas, 2000; Stockdale et al., 2000). We reasoned that the somitic segmentation serves as a useful model because it offers the following advantages: (1) the segmentation repeatedly takes place one pair at a time with regularity in time

and distance in an anterior to posterior order, and (2) overt proliferation or movement of cells does not occur when a fissure forms (Primmett et al., 1989; Stern et al., 1988), allowing an evaluation of consequences of various embryonic manipulations such as we describe in this paper.

In the anterior end of the unsegmented paraxial mesoderm (presomitic mesoderm: PSM), an expression boundary of genes including *MesP2* that coincides with the next border being formed is established prior to a morphological change. The segmental patterns of these genes are thought to be regulated by a "segmentation clock", first demonstrated by wavy and cyclic expression of *c-hairy1* (Maroto and Pourquié, 2001; Palmeirim et al., 1997; Pourquié, 2001). Thus, the segmentation clock operates in the continuous young PSM to establish the segmental patterns of gene expression in the anterior PSM, which eventually implements a morphological fissure formation. Both clock and segmentation genes are tightly related to Notch signaling, as revealed mainly by recent knockout and mutant studies: an animal where Notch signaling is (at least in part) deficient displays perturbed patterns of cyclic and segmental expression of genes in PSM, and also shows its consequent malformation of segmented structures later in development (Bessho et al., 2001; Conlon et al., 1995; Evrard et al., 1998; Holley et al., 2000; Hrabe de Angelis et al., 1997; Jiang et al., 2000; Kusumi et al., 1998; Oka et al., 1995; Saga et al., 1997; Saga and Takeda, 2001; Shen et al., 1997; Wong et al., 1997; Zhang and Gridley, 1998). In general, studies using mutants or knockout animals unveil the 'first stage' where the gene of concern is essential during development. However, if a given gene plays a role in the fissure formation as well as at earlier steps of segmentation, it would be difficult to distinguish between them. This may be the reason why the molecular mechanisms underlying the fissure formation have been poorly addressed.

In this paper we first describe a novel inductive event taking place when a segmentation fissure forms, in which posterior border cells located immediately posterior to the next forming boundary instruct the anterior ones. We next address molecular mechanisms underlying these events by focusing on Notch signals where Lunatic fringe (Lfng) is involved. Lfng is a modulator of the Notch receptor (Bruckner et al., 2000; Moloney et al., 2000) with glycosyltransferase activity, and is expressed in a region coinciding with the segmentation border in PSM. By combining DNA in ovo electroporation with embryological manipulations to make an ectopic boundary of a transgene activity in PSM, we found that Notch signals play major roles in the formation of a fissure. We present a model in which specific localization of *Lfng* determines the site of Notch action relevant to the morphological segmentation. We also discuss a mode of action for Notch in vertebrate somitogenesis using an analogy of that known for *Drosophila*.

MATERIALS AND METHODS

Embryological manipulation

Surgical manipulation was performed with chicken and Japanese quail embryos of Hamburger and Hamilton (HH) stage 13 (about 18-20 somites) (Hamburger and Hamilton, 1951). Transplantation of a donor tissue taken from PSM into a host chicken embryo was performed as previously described (Tonegawa et al., 1997). DiI or DiO (Molecular Probes) was dissolved in dimethylformamide (2.5 mg/ml) and injected into the PSM with a glass capillary.

Histological analyses

Embryos were fixed in Carnoy's solution (60% ethanol, 30% chloroform, 10% acetic acid) dehydrated in ethanol and embedded in paraffin wax. For staining with anti-quail QCPN monoclonal antibody, 7 μm histological sections were incubated with hybridoma supernatant (DHSB) for 2 hours. After washing in phosphate-buffered saline (PBS), they were reincubated for 90 minutes with horseradish peroxidase-conjugated anti-mouse immunoglobulin (Dako) diluted 1:100 with 2% skim milk in PBS. The reaction was developed in 80 μg/ml diaminobenzidine (DAB), 0.004% H_2O_2/PBS. Mayer's Hematoxylin solution (Wako) was used for a background nuclear staining of paraffin sections of embryonic day 4 (E4) embryos. For phalloidin staining, embryos were fixed in 4% paraformaldehyde in PBS and immersed in a graded series of sucrose solutions up to 30%, then embedded in Tissue-Tek (Sakura). Ten μm cryostat sections were incubated for 30 minutes with 5 Units/ml of Alexa Flour™ 568 or 647 phalloidin (Molecular Probes) dissolved in PBS.

RNA probes and whole-mount in situ hybridization

Chicken *Lfng* and *Notch1* were provided by Drs C. Tabin (Laufer et al., 1997) and Y. Wakamatsu (Wakamatsu et al., 1999), respectively. *Delta1* was a 946 bp fragment obtained by the RT-PCR technique using the primers, 5′-TACTGCACTCACCACAAGCC-3′ and 5′-TGATGGAGATGTCCTTCTCG-3′. Preparation of Dig-labeled RNA probes and whole-mount in situ hybridization followed by histological sectioning were performed as previously described (Takahashi et al., 1996).

In ovo DNA microelectroporation

The entire cDNA for chicken *Lfng* tagged with FLAG (a gift from Dr C. Tabin) (Laufer et al., 1997), and mouse *NotchΔE-6myc* and *Notch LNG-6myc* (gifts from Dr R. Kopan) (Schroeter et al., 1998) were subcloned into the pCAGGS expression vector (Niwa et al., 1991). They were co-electroporated with pCAGGS-GFP (green fluorescence protein) into the presumptive somitic mesoderm of HH stage 7-8 chicken embryos. Microelectroporation was carried out essentially according to the method previously reported (Momose et al., 1999; Yasuda et al., 2000) with slight modifications as follows: DNA solution was prepared at 5 μg/μl, colored with 2% Methylgreen (Nakalai) and placed onto the anterior primitive streak using a glass capillary. A plus electrode (platinum) was positioned under the embryo, and a minus electrode (sharpened tungsten) was put near the DNA solution. An electric pulse of 6V, 25 mseconds was charged three times.

Western blotting

COS cells transfected using Lipofectamine (Gibco BRL) were subjected to western blotting analyses as described previously (Kopan et al., 1996). Anti-FLAG M2 monoclonal antibody (Sigma) and anti-Myc (9E10) monoclonal antibody (Santa Cruz Biotechnology) were diluted 1:8000 and 1:1000, respectively, with 5% skim milk in PBST (0.1% Tween 20 in PBS).

RESULTS

Processes of boundary formation and nomenclature of the forming fissure by histological criteria

Presomitic mesoderm basically consists of mesenchymal cells. After the segmentation process is completed, the resulting somites are of a characteristic structure consisting of an outer epithelium enveloping mesenchymal somitocoel cells. The

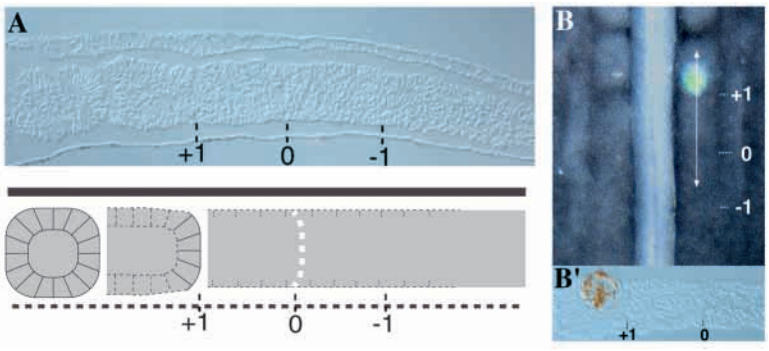

Fig. 1. Definition of the levels of a forming boundary in the anterior PSM as seen in sagittal section of an E2 embryo under Nomarski optics. Anterior is at left. (A) The first feature of morphological segmentation is a gap within mesenchymal PSM, and this position is designated as the line 0. The site located one-somite length posterior to the line 0 is defined as the level –1. The border cells immediately anterior to the gap undergo epithelialization resulting in a solid boundary at the line +1. (B) Identification of the line 0 in living embryos was confirmed by the location of the condensed cell mass posterior to +1. The position of level +1 was judged by combining a landmark made by a GFP/COS implantation and histological criteria. GFP in a paraffin section was visualized by anti-GFP antibody (brown).

epithelialization process that takes place along the axis perpendicular to the AP axis at the next-forming boundary is preceded by a gap within the mesenchymal cell population where overt changes in cell shape are still unrecognizable (Fig. 1). The gap which is initially a straight line seems to progressively become U-shaped. In this report, we designate a position, line 0, where an obvious gap is seen in histological paraffin sections under Nomarsky optics, although it is occasionally difficult to detect this line under a dissecting microscope, and a second position, the prospective line –1 as the site posterior to line 0 by one-somite unit. Thus level –1 is the position where no morphological sign is yet detected. Since the primary aim of this study was to learn what triggers the initial step of morphological boundary formation, we focused on the cellular and molecular events taking place near the prospective line –1. We paid particular attention to identify a prospective boundary level in living embryos: by making a landmark with implanted GFP-positive COS cells or DiI labelling we ensured that the position of a clear separation, which was easily recognized under a dissecting microscope, was line +1 and also that the posterior edge of a condensed cell mass was line 0 (Fig. 1B).

The prospective line –1 was determined to form a boundary

We focused on the differences in regulatory mechanisms between the presumptive boundary and non-boundary sites. We first examined whether the prospective line –1 was already committed to form a fissure by relocating –1-containing tissue into the non-segmentation level (–1.5) tissue of early chicken embryos. As shown in Fig. 2, the size of the graft was largely equivalent to the entire somite unit. Prior to the transplantation, the portions anterior and posterior to –1 of a donor were labelled with DiI and DiO, respectively, and a piece of host tissue corresponding to the size of the graft was removed from the PSM (Fig. 2). The transplanted tissue exhibited a fissure between the DiI and DiO labelled portions (Fig. 2A,B), showing that –1 was already determined to form the fissure. Transplantation of tissue from this same area into a more posterior region (i.e. near –4.5) also gave rise to a fissure within the graft (not shown). In both cases, the timing of fissure formation seemed to correspond to that of the original graft (=–1). In control experiments, an isotopic transplantation of tissue from level –1.5 into the –1.5 level did not show any morphological effects (Fig. 2C,D). Histological sagittal sections of the manipulated embryos further showed that the –1-derived fissure was flanked by epithelialized miniature somites (Fig. 2B).

Boundary-forming activity in the posterior border cells

To further analyze the boundary-forming events assumed to take place near the prospective line –1, we dissected a smaller piece of tissue from level –1 of a quail and inserted it into level –1.5 of a chicken (Fig. 3). As mentioned earlier, the prospective line –1 is the position where no morphological sign is yet

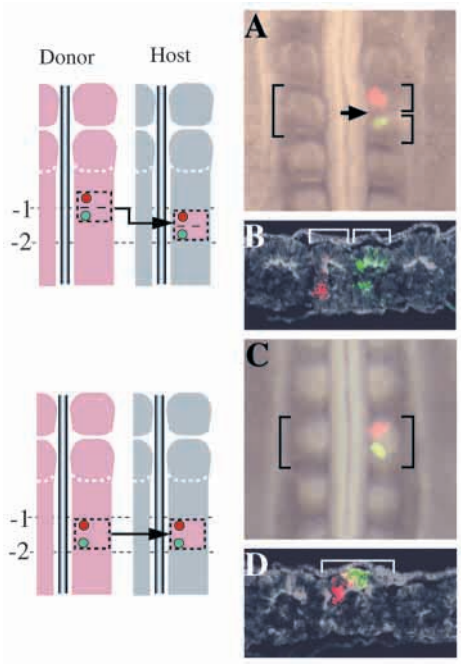

Fig. 2. The prospective line –1 was already determined to make a fissure. (A,B) Transplantation of a piece of PSM containing level –1 into a non-segmented level (–1.5). The size of the graft largely corresponded to a prospective somite. Anterior and posterior halves of the graft were labeled with DiI (red) and DiO (green), respectively. On the operated side, a boundary (arrow) formed between the DiI- and DiO-labeled regions, and along this fissure epithelialized miniature somites were present (bracket). (C,D) In the control, an isotopic transplantation (–1.5 into –1.5) did not produce a boundary between DiI- and DiO-labeled areas. (B,D) Sagittal frozen sections of embryos treated similarly to A and C, respectively. Stained for F-actin with Phalloidin (white).

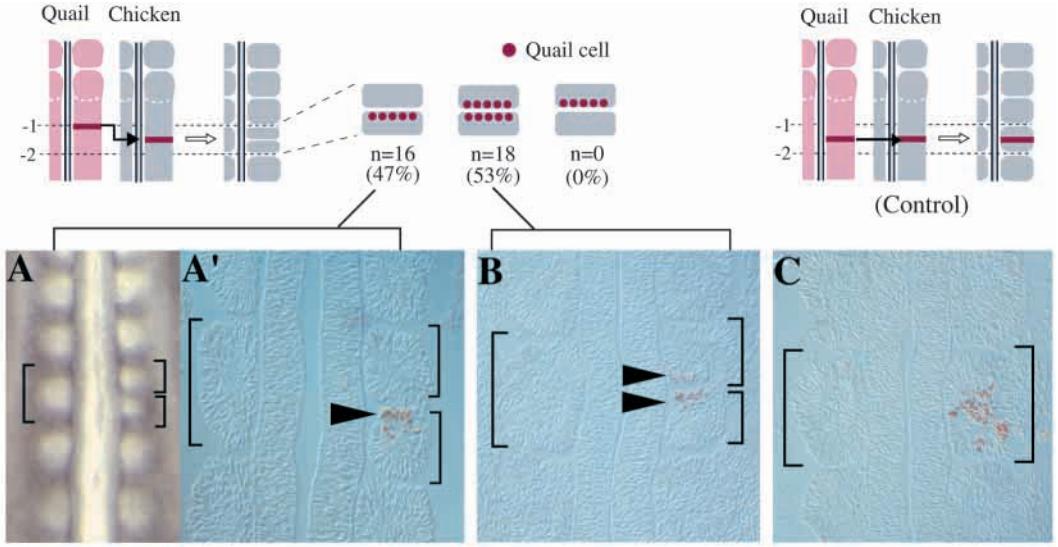

Fig. 3. A fissure was induced to form by the posterior border cells. A group of cells taken from near –1 of a quail donor was inserted into the level –1.5 of a chicken host. This manipulation resulted in the ectopic formation of a fissure with supernumerary somites along it (brackets). Sixteen out of 34 specimens showed distribution of the quail cells confined to the posterior region of the ectopic border (A), whereas 18 embryos had the donor cells spanning the border (B). No embryo was observed where the graft was located only anteriorly to the ectopic fissure. A; A dorsal view under a dissecting microscope. (A′,B,C) A horizontal section stained with QCPN antibody to visualize the quail cells (brown, arrowheads). Anterior at top. (C) Control isotopic graft (–1.5 into –1.5).

recognizable, we therefore located it as being one-somite unit posterior to line 0 in living embryos (Fig. 1B). It is already known that no overt cell proliferation or cell death occurs around this region (Primmett et al., 1989). Since we tried to take the smallest possible piece of tissue, which consisted of 50~100 cells, out of line –1 region, it was difficult to control the original A-P orientation of the graft.

This manipulation resulted in a supernumerary formation of miniature somites with an ectopic fissure intervening between them (n=34). Approximately half of such embryos (n=18) showed quail cells distributed both anteriorly and posteriorly to the fissure (Fig. 3B). This can be accounted for by the fact that –1 possesses an intrinsic segmentation ability as shown above. In the other half of the embryos with an ectopic fissure (n=16) the quail cells were found only in the position posteriorly adjacent to the boundary, and the host-derived cells anterior to that were ectopically epithelialized (Fig. 3A,A′). Importantly, no embryo with supernumerary somites was obtained where grafted quail cells were located only in the region anterior to the fissure. We also carried out a similar manipulation by dissecting a small piece from sites more posterior to –1 (i.e. –1.5, –2.0), and found no ectopic fissure in the host (n=12, Fig. 3C). Again, because of no clear morphological sign at –1, it was virtually impossible to know in this series of experiments whether we took ‘only’ the posterior border cells from –1 of a donor embryo, or whether we took cells straddling the prospective line. Nevertheless, the simplest and most reasonable interpretation of the results is that during normal somitogenesis the cells posteriorly juxtaposed to –1 play essential roles in instructing the fissure formation that leads to a separation and epithelialization of the anterior border cells, and also that this instructive activity is generated just before PSM becomes –1.

Lfng mediated the boundary-forming activity

We next investigated the molecular mechanisms underlying the inductive events that we found by focusing on the roles of Lfng. *Lfng* mRNA in the anterior PSM displays a sharp anterior boundary of expression coinciding with –1 (Aulehla and Johnson, 1999; McGrew and Pourquié, 1998), which was confirmed in histological sagittal sections (Fig. 4A,A′). A clear edge to the signal at –1 contrasts with its variable position in posterior PSM. Lfng is known to modify Notch receptor with the glycosyltransferase activity (Bruckner et al., 2000; Ju et al., 2000; Moloney et al., 2000; Panin et al., 1997). A growing number of reports, mainly using studies of mutants, have shown essential roles of Notch signaling during somite segmentation (Bessho et al., 2001; Conlon et al., 1995; Evrard et al., 1998; Holley et al., 2000; Hrabe de Angelis et al., 1997; Jiang et al., 2000; Kusumi et al., 1998; Oka et al., 1995; Saga et al., 1997; Saga and Takeda, 2001; Shen et al., 1997; Wong et al., 1997; Zhang and Gridley, 1998), although the widely distributed patterns of the transcripts of *Notch* and Notch-related molecules including ligands in PSM (Saga and Takeda,

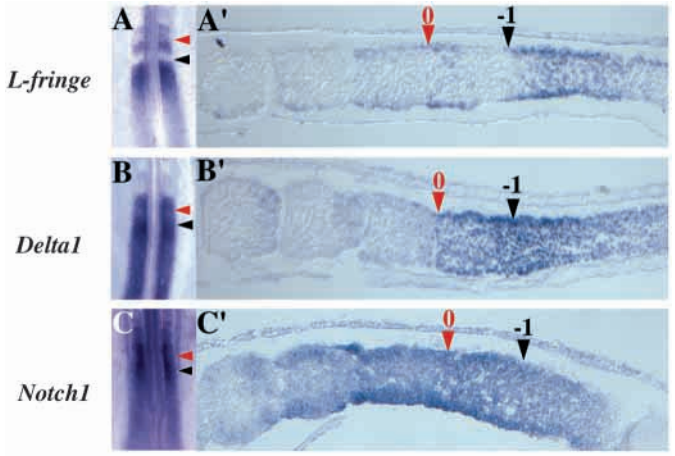

Fig. 4. Expression patterns of *Lfng* (A), *Delta1* (B) and *Notch1* (C) mRNAs in the anterior PSM of E2 chicken embryos. The sharp anterior boundary of the *Lfng*-expressing area demarcates the prospective line –1. Red and black arrowheads indicate levels 0 and –1, respectively. (A-C) A dorsal view with anterior at top. (A′-C′) A sagittal section of the same specimen shown in A-C with anterior at left.

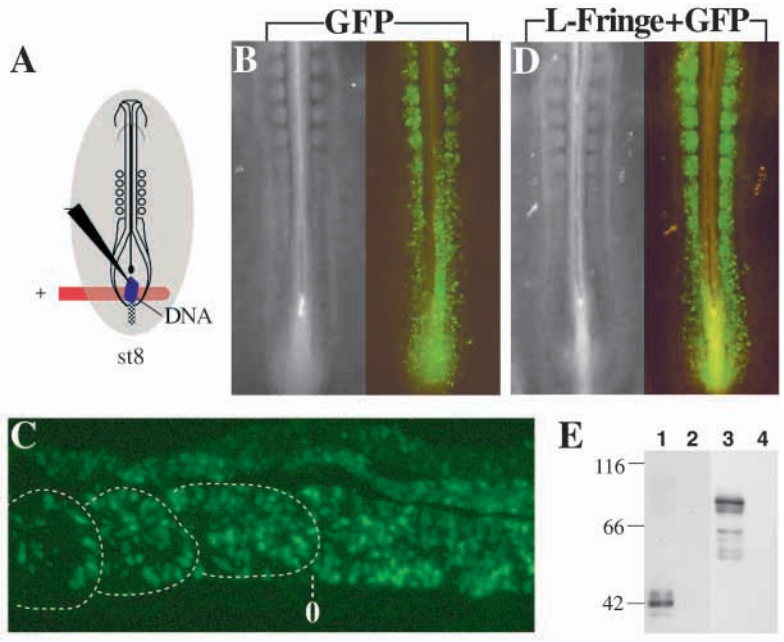

Fig. 5. In ovo DNA electroporation into PSM. (A) A diagram showing positions of a plus electrode (platinum) under the embryo, and a minus electrode (sharpened tungsten) near the DNA solution which was laid over the anterior primitive streak at HH stage 7-8. (B) 24 hours after the electroporation with pCAGGS-GFP, GFP signal was observed in the segmented somites and PSM. (C) A sagittal section of a GFP-electroporated embryo (anterior to the left). (D) An embryo co-electroporated with pCAGGS-Lfng and pCAGGS-GFP showing no gross disturbance in the somites. (E) Western blotting showing that pCAGGS-Lfng and pCAGGS-NotchΔE, used for the electroporation, gave rise to proteins of expected size when transfected into COS cells. Products of Lfng/FLAG (42 kDa, lane 1) and NotchΔE/Myc (83 kDa of an unprocessed form, and 70 kDa and 63 kDa fragments of processed forms translocating to the nucleus (Kopan et al., 1996), lane 3) were, respectively, detected by anti-FLAG and anti-Myc antibodies. Lanes 2 and 4 are controls for lanes 1 and 3, respectively.

2001) (also Fig. 4) have made it difficult to precisely locate the site of Notch actions. We therefore reasoned that Lfng at –1 would have the role in boundary forming activity which we found in the present study.

In ovo DNA electroporation into PSM

We wanted to overexpress *Lfng* cDNA in PSM and examine the effects on boundary formation. To do this, we first determined the optimal condition of in ovo DNA microelectroporation into this tissue. With a slight modification of the previously reported protocol that was primarily developed for the neural tube and optic cup (Momose et al., 1999; Yasuda et al., 2000), we targeted HH stage 7-8 anterior primitive streak (posterior to Hensen's node; Fig. 5A), from where somite precursor cells derive (Catala et al., 1996; Psychoyos and Stern, 1996). An electric pulse of 6 V was given three times for 25 mseconds (see also Materials and Methods). Electroporation performed at earlier or later stages than HH stage 7-8 resulted in, respectively, a more damaged specimen or much less efficiency in gene transfer as far as the PSM was concerned. Under the above condition, approximately 50% of the PSM cells were positive for GFP (used as a control; Fig. 5C), and GFP overexpression had no morphological effects on PSM or somite segmentation (Fig. 5B). The area we targeted for the electroporation is also thought to be presumptive neural tube (Catala et al., 1996), and some of the specimens indeed displayed GFP signals in the neural tube (Fig. 5B).

An Lfng boundary induced a morphological fissure

When we overexpressed the *Lfng* gene in a wide region of the PSM, no significant effects on morphological segmentation were observed (n=107, Fig. 5D; also see Discussion), although we confirmed that the expression vector containing *Lfng* cDNA rendered protein products of expected size when transfected into COS cells (Fig. 5E). We next attempted to make a boundary of Lfng activity: we dissected a small piece of electroporated PSM of a donor embryo from the level of –1.5 or –2.5 and transplanted it into level –1.5 of a non-electroporated host (Fig. 6A). Although the host area between –1 and –2 was also positive for *Lfng* mRNA (Fig. 4), it did not necessarily reflect the presence of Lfng protein (see also Discussion). In addition, the expression vector (pCAGGS) used in this experiment is known to drive a constitutively high level of protein production when electroporated into a chicken embryo (Momose et al., 1999). Therefore, this manipulation was anticipated to create an ectopic interface between on and off (or high and low, at least) regions for the Lfng activity, thus mimicking the prospective line –1. In the manipulated embryos we found an ectopic formation of a fissure coinciding with the confrontation site between the host and donor tissues (18/50, Fig. 6B). Resultant supernumerary somites were epithelialized as revealed by F-actin staining in histological sections (Fig. 6E). The effects for Lfng were specific because the same manipulation with GFP alone as a control did not affect segmentation (n=17, Fig. 6F). These results suggest that during normal segmentation Lfng at –1 plays a role in the boundary instruction.

Lfng-mediated boundary formation was mimicked by a Notch signal

We reasoned that the formation of an ectopic boundary by Lfng could be attributed to the Notch signal since Lfng modulates Notch receptor as mentioned. We therefore performed a similar manipulation using a constitutively active form of Notch 1 (NotchΔE) (Schroeter et al., 1998) after confirming that the expression vector produced the protein of correct size in COS cells (Fig. 5E). As expected, an ectopic boundary was observed only when a sharp interface of Notch activity was made, as was the case for Lfng (18/27, Fig. 6C). In control, Notch LNG, an inactive form of Notch receptor (Schroeter et al., 1998), showed no effects (n=5, Fig. 6D).

Given these results, we conclude that during normal segmentation Lfng activity demarcating the prospective line –1 influences Notch receptor so that Notch signals become active at this site to mediate a morphological segmentation. Thus, establishment of the Lfng boundary in the anterior PSM

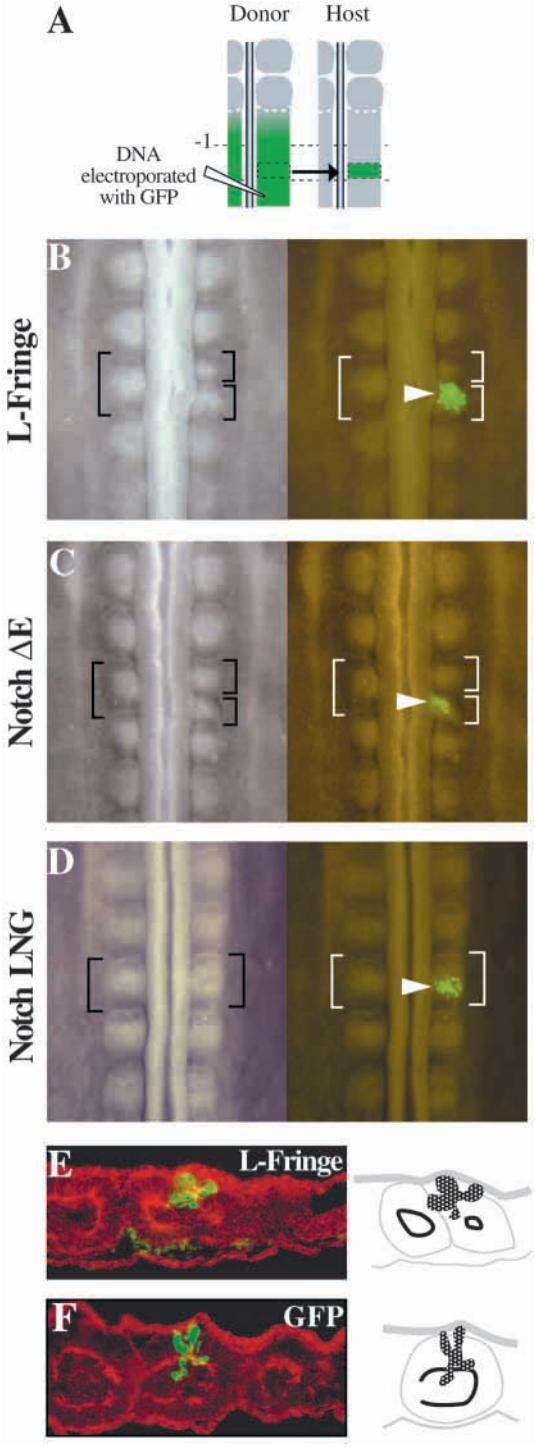

Fig. 6. A morphological boundary was induced to form at an interface between host and Lfng/Notch-electroporated donor tissues. (A) A diagram showing a manipulation to create a sharp border of Lfng activity. *Lfng* or *Notch ΔE* DNA was electroporated into PSM of a donor embryo together with GFP, and subsequently a small piece was dissected from a donor PSM, and transplanted into a host. (B) An ectopic fissure was formed at the boundary between the donor (GFP-positive cells in the right panel; arrowhead) and host regions. (C) A confrontation between NotchΔE-positive and -negative cells gave a similar result to that in B, whereas Notch LNG did not show such effects (D). The left and right panels in B-D show the same specimen with the position of a fissure and electroporated donor-derived cells (green), respectively, indicated. (E) A sagittal section of the supernumerary somites resulting from a Lfng boundary. Anterior is at left. Epithelial cells visualized by phallodin stainin (red) were present anterior to the Lfng-producing cells (green), whereas control GFP-expressing cells did not affect the epithelialization (F). The diagrams on the right of E and F show the position of the boundaries more clearly.

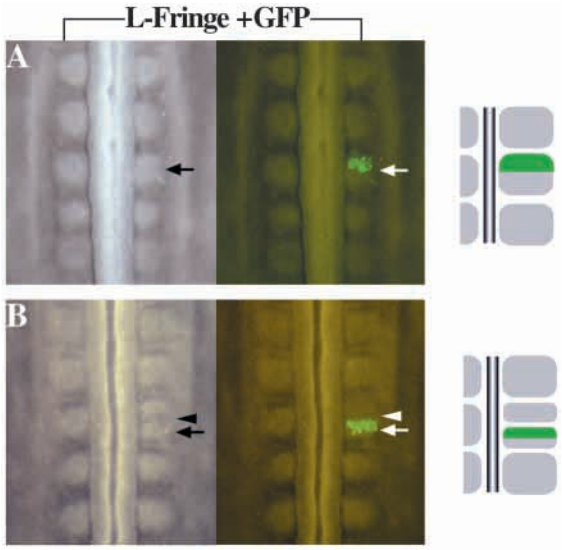

Fig. 7. Notch/Lfng acts directionally on the anteriorly located cells. (A) When Lfng-producing cells were located in the anterior half of a host somite, the interface (arrow) did not induce a fissure. (B) When a 'strip' of Lfng-producing cells was positioned in the middle of a host somite, only the anterior (arrowhead) but not the posterior (arrow) interface produced an ectopic fissure.

appears to determine the site of Notch action within the area where mRNAs of *Notch* and its ligand are widely distributed (Fig. 4, see also Fig. 10).

Directional action of Notch/Lfng activity

The action of Notch/Lfng observed in the present study appeared to mediate signals in a directional fashion. As shown above, an ectopic fissure formed when a piece of tissue electroporated with *NotchΔE/Lfng* was grafted into the posterior half of a somite unit of a host. In contrast, when it was grafted into the anterior half, no fissure was observed (n=45, Fig. 7A), despite an equal possibility of an AP or PA orientation of the graft (we could not control the AP orientation since we wanted to take the smallest possible graft). The unidirectional action of Notch/Lfng was further supported by the observation that a host somite receiving a "strip" of *NotchΔE*- or *Lfng*-electroporated PSM midway along the AP axis exhibited an ectopic fissure only at the anterior interface of the strip (n=36, Fig. 7B). Thus, during normal segmentation NotchΔE/Lfng-mediated signals appear to be transmitted only from posterior to anterior at the next-forming boundary. This is consistent with only the anterior margin (–1) but not the posterior one of the *Lfng*-expressing area in normal PSM being relevant to the morphological segmentation.

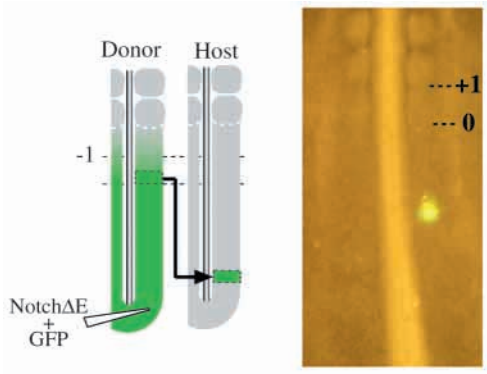

Fig. 8. A Notch boundary did not induce a fissure in posterior PSM. A piece of NotchΔE/GFP-electroporated PSM was taken from level –1.5 and transplanted into posterior PSM near the level –4.5.

An interface of *NotchΔE* did not induce a fissure in posterior PSM

To study whether an interface of the Notch activity is sufficient to make a morphological boundary, even in posterior PSM that would normally never segment, we grafted a piece of *NotchΔE*-electroporated donor, taken from the same region as the experiments above, into the region near –4.5 of a host. This manipulation showed no morphological effects (n=4, Fig. 8), suggesting that manifestation of the segmentation fissure mediated by Notch signaling depends on the extent of 'maturation' of PSM, involving several segmentation genes such as *MesP2* (Buchberger et al., 1998; Saga et al., 1997; Sawada et al., 2000; Sparrow et al., 1998). These observations are also consistent with the fact that although a domain of *Lfng* mRNA moves from posterior to anterior in young PSM during each segmentation cycle (Aulehla and Johnson, 1999; Forsberg et al., 1998; McGrew et al., 1998), only a sharp border of the expression at –1 is relevant to the morphological boundary.

AP polarity of the miniature somites separated by an ectopic boundary

We examined whether two half-sized somites separated by an ectopic fissure acquired a new AP polarity for each, or simply maintained the original AP characters as shown in double knockout mice for N-cadherin and cadherin 11 (Horikawa et al., 1999). *Delta1* is widely used as a marker to identify the posterior of a young somite in chicken, and a dorsal root ganglion is known to form in the anterior somite-derived sclerotome. In two specimens where a fissure was ectopically formed by a NotchΔE boundary, a half-sized somite anterior to the fissure exhibited expression of *Delta1* in its posterior margin (Fig. 9A,A′), showing that this region rearranged the A-P polarity since it was originally of anterior character. The NotchΔE-expressing graft itself also showed intense *Delta1* expression (asterisk in Fig. 9A′), but this strong signal was always observed even in a graft located far from the somite (Fig. 9B), suggesting that this *Delta1* signal does not reflect the posterior identity of the segmented somite, but rather implies upregulation of *Delta1* by Notch, a gene cascade that might take place in young PSM (Takahashi et al., 2000). When a large piece containing –1 level tissue was grafted into level –1.5 (the same experiment as Fig. 2A), the resulting supernumerary somites also displayed *Delta1* expression in the posterior region of each compartment observed at E 2.0 (Fig. 9D); likewise, a host-derived dorsal root ganglion formed in the anterior portion of each small segment (Fig. 9E).

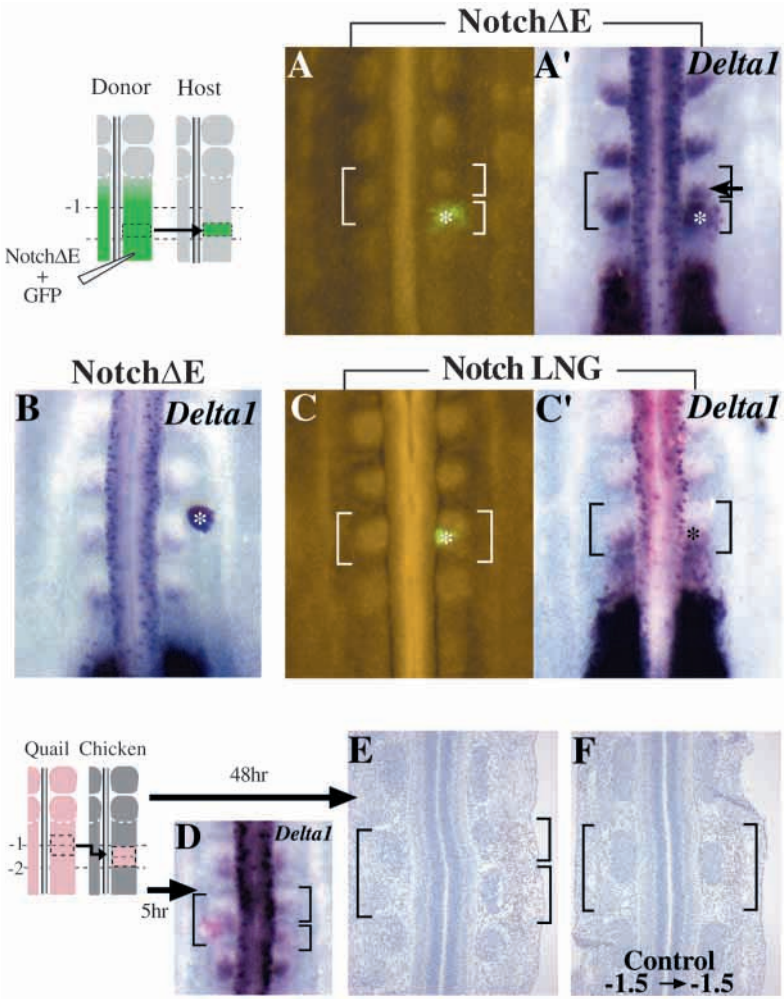

Fig. 9. The AP polarity in the supernumerary somites. (A,A′) When an ectopic fissure was induced to form by NotchΔE-producing cells (asterisk), the small somite located anterior to the fissure expressed *Delta1* mRNA at its posterior margin (arrow). (B) NotchΔE-producing cells themselves showed an intense signal even when they were located outside the somite. (C,C′) Notch LNG did not affect the AP polarity of the host somite. (D) When a large graft of level –1 was placed into level –1.5 (the same transplantation as Fig. 2A), the resulting miniature somites, after 5 hours of incubation, expressed *Delta1* mRNA in the posterior margin of each somite. DiI was used to mark the grafted level along the AP axis of a host (pink in the control side). (E,F) QCPN staining of horizontal sections. Embryos with the same manipulation that were incubated for another 2 days formed supernumerary DRGs in the anterior part of each segment (E), whereas a control isotopic transplantation had no effect on DRG (F).

Thus, we recognized a correlation between an ectopically induced fissure and rearrangement of the A-P polarity of a segmented somite. The efficiency of a complete rearrangement was low, however, and the reason is unknown at present.

DISCUSSION

We have demonstrated in this report that a fissure formation at the final step of somitic segmentation is controlled by novel inductive events near the next-forming border, and also that cells immediately posterior to this line appear to have primary roles involving Notch signaling. Mechanisms underlying segmentation during vertebrate somitogenesis have long attracted attention, and recent outstanding advances, mainly from mutational studies, have shown that a variety of genes are involved during these processes, with Notch signaling being pivotal (Bessho et al., 2001; Conlon et al., 1995; Evrard et al., 1998; Holley et al., 2000; Hrabe de Angelis et al., 1997; Jiang et al., 2000; Kusumi et al., 1998; Oka et al., 1995; Saga et al., 1997; Saga and Takeda, 2001; Shen et al., 1997; Wong et al., 1997; Zhang and Gridley, 1998). However, the widely distributed patterns of the transcripts of *Notch* and Notch-related molecules in PSM (Saga and Takeda, 2001) and inability to directly visualize Notch activity in vivo has hindered the analysis of when and where Notch acts. In this study, by combining embryological manipulations and DNA in ovo electroporation, we were able to address this question and clarify at least one site of Notch action.

Inductive events occur near the prospective line –1

PSM is known to possess an intrinsic ability to establish a segmented pattern from specific gene expression since, deprived of its surrounding tissues, including the ectoderm, it still displays a normal pattern of reiterated *Delta1* and *Delta-like1 (Dll1)* expression (Correia and Conlon, 2000; Palmeirim et al., 1998). As for the mechanisms by which the morphological fissure forms, however, up to now it was known only that the PSM requires the ectoderm (Correia and Conlon, 2000; Palmeirim et al., 1998; Schmidt et al., 2001). In this report we have for the first time demonstrated that an inductive event takes place instructed by the cells located posterior to –1 (posterior border cells). This instructive phenomenon contrasts with a permissive effect of the surface ectoderm which supports but apparently does not instruct the fissure formation (Correia and Conlon, 2000; Palmeirim et al., 1998; Schmidt et al., 2001). In our experimental system in which a small group of cells (50 ~100 cells) were dissected from a quail donor embryo, it was virtually impossible to identify whether they were only posterior border cells, or whether they included cells straddling the prospective line. Nevertheless, the simplest and most reasonable interpretation of the results we obtained is that during normal segmentation the posterior border cells play primary roles in the instruction to make a fissure. This is further supported by another line of evidence shown in this study: an interface of Lfng activity, mRNA of which is normally expressed with a sharp anterior boundary coinciding with –1, mimics the actions of the posterior border cells. We designate this boundary-forming activity as a "segmenter" (Fig. 10A).

Segmenter activity is mediated by Lfng and Notch signals

Lfng has been shown to modify Notch by its glycosyltransferase activity in the same cells that express Notch (Bruckner et al., 2000; Moloney et al., 2000; Panin et al., 1997). Thus, the segmenter appears to be mediated by Notch actions. Given these facts, we present a model as shown in Fig. 10A that shows how the segmenter activity is generated. Within anterior PSM where *Notch1* and *Delta1* mRNAs are widely distributed, a specific localization of Lfng activity restricts the site of Notch action to posterior to –1. The Notch-activated cells then signal to the cells anterior to them which become separated and eventually epithelialized. The molecular nature of this signal(s) remains to be determined, but could be direct cell-cell interactions and/or some secretory factors. Candidates for the former signals involve Eph/ephirin-directed repulsion and also cadherin-mediated segregation between two

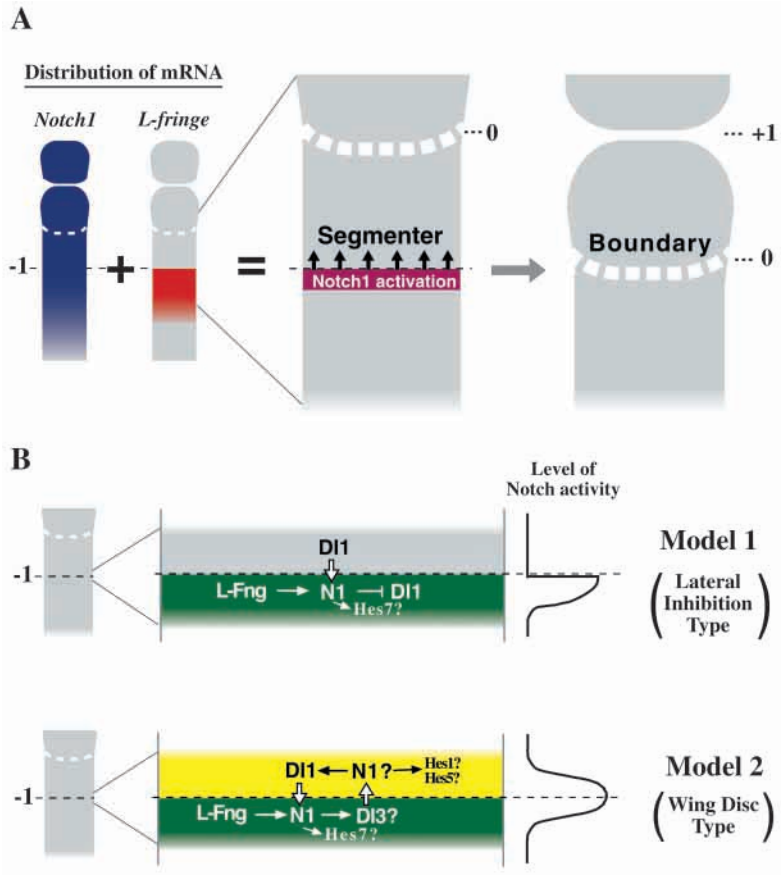

Fig. 10. Summary and model for the action of Notch signaling. (A) The signal (arrows) emanating from the posterior border cells and acting on the anterior border cells is designated as the 'segmenter'. See text for detailed explanation. *Lfng* mRNA normally located posterior to the line 0 is not shown to allow the molecular events at –1 to be clearly represented. (B) Two models proposing a mode of Notch action in somite boundary formation analogous to its action in *Drosophila*. See text for details.

types of cells (Durbin et al., 1998; Durbin et al., 2000; Holder and Klein, 1999; Inoue et al., 2001; Nose et al., 1988; Schmidt et al., 2001; Takeichi, 1995; Wilkinson, 2001). Notch-Delta signals themselves might directly contribute to this segregation as shown in more detail below. This does not exclude the possibility that the posterior border cells simply produce some extracellular matrix so that a mechanical gap forms between the anterior cells. Nevertheless, since in several specimen we have observed that segmenter- or NotchΔE/Lfng-producing cells caused the anterior cells to rearrange their AP polarity, although with a relatively low efficiency, it is reasonable to propose that the posterior border cells possess an instructive capability over the anterior ones. Although *Lfng* knockout mice show a severe phenotype in segmentation where the proper spacing and segmented border of somites are lost (Evrard et al., 1998; Zhang and Gridley, 1998), it has been unclear whether the deficiency was attributable to *Lfng* that cycles in the posterior PSM or to *Lfng* restricted to –1 (Aulehla and Johnson, 1999; Forsberg et al., 1998; McGrew et al., 1998). In this study we were able to distinguish between them by determining the role of Lfng at the prospective line –1.

The Notch-mediated segmenter appears to act only anteriorly. This was shown by the fact that NotchΔE/Lfng-expressing cells located in the anterior half of the presumptive somite did not make a fissure even though the interface of the transgene activity was midway along the AP axis of the somite (Fig. 7A). It was also supported by the finding that in the embryos that made supernumerary somites by receiving –1-derived cells, the grafted cells were located either posterior to- or on both sides of the ectopic fissure, but never confined only to the anterior region (Fig. 3). Although the precise mechanisms of this unidirectional action are unknown, the presence of a global gradient of morphogen-like signaling cannot account for it. It is possible that AP polarity is present in each single cell of the PSM (=recipient cells in our experiments) so that the cells can 'detect' where the segmenter signal comes from. This polarization may include planar cell polarity as seen in epithelial cells in the *Drosophila* wing (Usui et al., 1999). The directional signaling in fissure formation shown in the present study is also consistent with the finding that zebrafish double mutant for *knypek* and *trilobite* have a single somite unit consisting of only two rows of cells (Henry et al., 2000), indicating that two types of cells suffice in boundary formation. Thus, these findings argue against the model in which the existence of a third cell state alternating with the anterior (A) and posterior (P) ones was proposed to explain why a fissure forms at alternate confrontations between the A and P (Meinhardt, 1986).

In our experiments, making an interface of pCAGGS/Lfng-electroporated cells at level –1.5 resulted in an ectopic fissure. Level –1.5 of the normal embryo is the site where *Lfng* mRNA is also present (Fig. 4A and Fig. 10A). Since the presence of transcripts of a given gene does not necessarily reflect protein activity, we interpret our results as being brought about by the creation of a boundary between Lfng on/off, if not, high/low regions. Thus, during normal segmentation Lfng activity affecting Notch appears highly confined to –1 (posterior border cells). It is also conceivable that since *Lfng* mRNA is expressed in waves that stops at –1 during each cycle, the level of Lfng protein would be highest at this point, and this accumulation might be a requisite for fissure formation. The precise localization of Lfng protein/activity in normal PSM needs to be determined in order to distinguish between these possibilities.

We have shown that only a sharp interface between Lfng on and off (or high and low) regions resulted in an ectopic fissure, whereas widely distributed Lfng in PSM did not significantly affect the morphological segmentation. This could be due to a mosaic pattern of transgene activity (~50%) in PSM, and therefore, widely electroporated Lfng was not sufficient to interfere with the endogenous boundary of Lfng at –1. It is apparent that Notch signaling needs to be precisely regulated in PSM to manifest segmentation: expression of some Notch-related genes oscillates in a coordinated manner during the segmentation cycle, and perturbation of these stereotyped patterns leads to defects in segmentation (Barrantes et al., 1999; Holley et al., 2000; Holley et al., 2002; Jiang et al., 2000; Jouve et al., 2000; Takke and Campos-Ortega, 1999).

How does Notch/Lfng make a morphological boundary?

The mechanisms by which Notch signaling establishes a boundary between cells and tissues have been extensively studied in *Drosophila*, where two modes of actions have been proposed, the 'lateral inhibition type' and the 'wing disk type'. At present both models fit the interpretation of our results (Fig. 10B). In the lateral inhibition type, confrontation between Notch-active and -inactive cells produces two types of cells, which exclude each other on either side of the interface (Artavanis-Tsakonas et al., 1999). One can depict a possible mechanism for vertebrate segmentation by analogy with this model (Fig. 10B): in the posterior border cells (green) Lfng modifies Notch so that it efficiently binds to Delta1 and transmits signals intracellularly and Notch-activated cells produce the segmenter that acts on the anterior cells. In this model, the posterior and anterior cells are negative for actions of Delta1 and Notch1, respectively, generating a sharp interface for Notch activity at –1. A good candidate for an effecter working downstream of Notch signaling is Hes7 since *Hes7* knockout mice are affected in somite segmentation. In addition, the expression of *Hes7* mRNA is almost identical to *Lfng* with a sharp anterior boundary at –1 (Bessho et al., 2001).

In the wing disk of *Drosophila*, Fringe which is expressed in the dorsal region modifies Notch so that it can efficiently transmit a signal with Delta, expressed in the ventral region (negative for Fringe), whereas Fringe-modified Notch cannot interact with another ligand, Serrate, present in the dorsal half. These interactions between Notch, Fringe, Delta and Serrate result in a sharp peak of Notch activity that straddles the boundary between the dorsal and ventral regions (Panin et al., 1997). A similar relationship could occur at the next forming boundary of vertebrate segmentation (Fig. 10B): Lfng-modified Notch in the posterior border cells (green) transmits a signal with Delta1, presumed to be active in the anterior border cells (yellow), and another ligand Delta3, known to be localized in the posterior border cells in mice and essential for segmentation (Dunwoodie et al., 1997; Kusumi et al., 1998) might activate Notch signal in the anterior border cells, leading to a sharp peak of Notch activation straddling –1. In this case Lfng might confer differential susceptibility on Notch receptor to Delta1 and Delta3 as has been shown for Delta1 and Jagged1 (Hicks et al., 2000). Hes1 and Hes5, presumed downstream

effectors of Notch pathway, are indeed expressed in the anterior border cells (Ishibashi et al., 1995; Ohtsuka et al., 1999). It remains uncertain, however, whether they really act as an effecter since no defects have been reported even in double knockout mice for the *Hes1* and *Hes5* (Ohtsuka et al., 1999). It is of interest to learn whether other Hes or Hes-related members are localized in this region.

The posterior and anterior border cells subsequently follow distinct morphogenetic pathways: the former remain mesenchymal during a period when two somites form, whereas the latter become epithelialized immediately after a gap forms (Fig. 1) (Duband et al., 1987). This distinction between the posterior and anterior border cells would be attributed, according to the lateral inhibition type model, to the presence or absence of Notch activity, and in the wing disk type model other molecules would work in concert with Notch signaling to differentiate between these cells. Whatever the mode of Notch action is, it is still unknown how the border cells manifest dynamic changes in morphology. Studies that link Notch signaling to cytoskeletal dynamism will be required.

Recently, another role for Notch has been reported in zebrafish, in which Notch signals are essential for coordination of oscillating expression in the posterior PSM during each cycle of segmentation (Holley et al., 2002; Jiang et al., 2000), but at present this role seems distinct from those determined in this study. Thus, the role of Notch signaling and its contribution to the boundary formation may differ in each morphogenetic process.

Relationships between the segmenter and other factors important for morphological boundary

It has previously been shown that the *paraxis* gene (*Tcf15* – Mouse Genome Informatics), which encodes a bHLH molecule, plays essential roles in epithelialization during somite segmentation. In *paraxis* knockout mice, a gap forms between segments but a subsequent epithelialization does not proceed, resulting in malformation of somite-derived organs (Burgess et al., 1996). Since in normal segmentation processes the gap formation is immediately followed by the epithelialization of the anterior border cells, the segmenter seems to act close to the functional pathway of Paraxis. It remains to be clarified whether the ectoderm-derived signals, known to support the morphological segmentation, act during the gap formation or the epithelialization process. It is also important to consider the dorsoventral and mediolateral axes to further understand the mechanisms underlying generation and action of the segmenter, and this is currently underway.

Lastly, Fringe-mediated formation of a tissue boundary has previously been shown in vertebrates: in the limb bud Radical fringe determines the position of the apical ectodermal ridge (AER), and Lfng is involved in barrier formation in zona limitans intrathalamica (zli) of the diencephalon (Irvine, 1999; Laufer et al., 1997; Rodriguez-Esteban et al., 1997; Zeltser et al., 2001). It is of interest to know whether these morphogenetic movements share mechanisms similar to those shown in this study. Unlike the cases for AER or zli, somite segmentation involves an overt morphological separation between cells followed by dynamic changes in cell shape. In combination with the advantage that somite segmentation proceeds in a geometrically simple reiterated structure, our finding of the segmenter opens a way to unravel complex events occurring during border formation at the molecular and cellular level, and to depict a general picture of morphogenesis in vertebrates.

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