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Anteroposterior patterning is required within segments for somite boundary formation in developing zebrafish

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This paper is dedicated to the memory of Nigel Holder who tragically died during the course of the work

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SUMMARY

Somite formation involves the establishment of a segmental prepattern in the presomitic mesoderm, anteroposterior patterning of each segmental primordium and formation of boundaries between adjacent segments. How these events are co-ordinated remains uncertain. In this study, analysis of expression of zebrafish *mesp-a* reveals that each segment acquires anteroposterior regionalisation when located in the anterior presomitic mesoderm. Thus anteroposterior patterning is occurring after the establishment of a segmental prepattern in the paraxial mesoderm and prior to somite boundary formation.

Zebrafish *fss*$^{-}$, *bea*$^{-}$, *des*$^{-}$ and *aei*$^{-}$ embryos all fail to form somites, yet we demonstrate that a segmental prepattern is established in the presomitic mesoderm of all these mutants and *hox* gene expression shows that overall anteroposterior patterning of the mesoderm is also normal. However, analysis of various molecular markers reveals that anteroposterior regionalisation within each segment is disturbed in the mutants. In *fss*$^{-}$, there is a loss of anterior segment markers, such that all segments appear posteriorized, whereas in *bea*$^{-}$, *des*$^{-}$ and *aei*$^{-}$, anterior and posterior markers are expressed throughout each segment. Since somite formation is disrupted in these mutants, correct anteroposterior patterning within segments may be a prerequisite for somite boundary formation. In support of this hypothesis, we show that it is possible to rescue boundary formation in *fss*$^{-}$ through the ectopic expression of EphA4, an anterior segment marker, in the paraxial mesoderm. These observations indicate that a key consequence of the anteroposterior regionalisation of segments may be the induction of Eph and ephrin expression at segment interfaces and that Eph/ephrin signalling subsequently contributes to the formation of somite boundaries.

Key words: Somitogenesis, Segmentation, Eph signalling, Zebrafish, *mesp-a*

INTRODUCTION

The mechanisms underlying the establishment of segmental prepattern in vertebrate embryos is not well characterised at a molecular level. This is in contrast to the situation in *Drosophila*, in which the molecular basis of segmentation has been shown to involve a hierarchy of gene interactions initiated by maternal factors that pattern the egg and early embryo. These maternal factors control the expression of gap genes in domains that constitute several adjacent presumptive segments. Gap genes subsequently regulate the expression of pair-rule genes that are transcribed in overlapping regions corresponding to alternating segments. In turn, pair-rule genes control the expression of segment polarity genes that generate anterior and posterior regions within each segment. Finally segments acquire individual identities through the function of *hox* genes (reviewed by Lawrence, 1992).

Some of the mechanisms necessary to achieve segmentation of the body axis in *Drosophila* may be conserved and utilised during vertebrate development. For instance, vertebrate homologues of the *Drosophila* pair-rule gene *hairy* are involved in somite segmentation (Muller et al., 1996; Palmeirim et al., 1997). The hox genes are also highly conserved and may regulate segmental identity in both vertebrates and invertebrates (Krumlauf, 1994). However, genes that are involved in the establishment of segment polarity in the *Drosophila* embryo, such as *hedgehog*, do not appear to be involved in somite segmentation (Ingham, 1995). Thus, while there are similarities between the mechanisms that establish segments and assign their identities in vertebrates and invertebrates, it is not yet clear how anteroposterior (AP) regionalisation of segments occurs in vertebrates.

The establishment of anterior and posterior differences within segments is essential for the development of somites and the correct patterning of a number of other structures. The vertebrae form from the somites, and the peripheral nervous

system is also segmentally patterned, with migrating neural crest cells and outgrowing sensory and motor axons restricted to the anterior half of each somite (Keynes and Stern, 1984). The AP polarity of somites is also necessary for the maintenance of segment boundaries, as when half somites are juxtaposed, boundaries only form when anterior and posterior halves are confronted (Stern and Keynes, 1987).

Several genes expressed in the paraxial mesoderm of the vertebrate embryo are known to regulate AP polarity within somites. These include the signalling proteins Notch (Conlon et al., 1995), Delta (Hrabe de Angelis et al., 1997) and lunatic fringe (Evrard et al., 1998; Zhang and Gridley, 1998), and the bHLH transcription factor MesP2 (Saga et al., 1997). Other molecules may also be involved in the processes of morphological boundary formation. These include members of the Eph family of receptor tyrosine kinases and their ligands, the ephrins (Durbin et al., 1998). The expression patterns of these and other genes also show that a segmental pattern is established within the paraxial mesoderm before morphological boundaries are evident. Although rigorous fate mapping of the presomitic mesoderm has not been carried out, it appears that segments defined by the expression of genes in the presomitic mesoderm subsequently correspond to somites (Muller et al., 1996).

There are a number of zebrafish mutations that lead to defects in somite boundary formation (van Eeden et al., 1996). No somite boundaries form early during development in *fused somites* (*fss*$^{-}$); whereas in *beamter* (*bea*$^{-}$), *deadly seven* (*des*$^{-}$) and *after eight* (*aei*$^{-}$) only the first four, seven and eight somites form respectively. Homozygous mutants are viable for all four of these mutant loci. Since vertebrae form with a relatively normal periodic pattern in these mutants (van Eeden et al., 1996), it is presumed that the genes function downstream of the processes that establish a segmental pattern in the paraxial mesoderm. Instead the mutations could affect AP patterning events within each segment (van Eeden et al., 1998).

In this paper, we describe the expression pattern of a zebrafish *MesP*-related gene, *mesp-a*, which reveals that each segment is AP regionalised following the establishment of a segmental prepattern in the paraxial mesoderm and before somite boundaries form. We show molecular evidence for the establishment of a segmental prepattern in *fss*$^{-}$, *bea*$^{-}$, *des*$^{-}$ and *aei*$^{-}$ embryos. However, AP regionalisation within segments is disturbed in these mutants. In *fss*$^{-}$, segments appear posteriorized whereas in *bea*$^{-}$, *des*$^{-}$ and *aei*$^{-}$ there is a loss of polarity, with markers of anterior and posterior segment identity expressed throughout each segment. Since somite formation is disrupted in these mutants, AP regionalisation within segments would appear to be required for somite boundary formation. In support of this, we rescue boundary formation in *fss*$^{-}$ embryos through ectopic expression of EphA4, an anterior segment marker, within the paraxial mesoderm, demonstrating that boundary formation requires an interface between cells of anterior and posterior segment identity, and that Eph signalling molecules may underlie this AP character.

MATERIALS AND METHODS

Maintenance of fish

Breeding fish were maintained at 28.5°C on a 14 hour light/10 hour dark cycle. Embryos were collected by natural spawning and staged according to Kimmel et al. (1995). The adult viable recessive mutants *after eight*^{tm223} (*aei*$^{-}$), *beamter*^{tm98} (*bea*$^{-}$), *deadly seven*^{tw239} (*des*$^{-}$) and *fused somites*^{te314a} (*fss*$^{-}$) were used (van Eeden et al., 1996).

Cloning of *mesp-a* cDNA

mesp-a cDNA was isolated from a zebrafish cDNA library (provided by Dr David Grunwald) in a screen for bHLH genes (Gering et al., 1998). The probe used was a 160 bp DNA fragment amplified from zebrafish genomic DNA using primers 5′ GTCTTCACCAACAGCCG 3′ and 5′ CCAGGAAGTTGATGTACTT 3′, defined according to the 5′ and 3′ ends of the *SCL*-related bHLH gene *SLP1* (Gottgens et al., 1998). Three different cDNAs were isolated, one of which encoded a protein that possessed a bHLH region similar to that of the murine MesP proteins and was named Mesp-a. The DNA sequence is available from GenBank under accession number AF188833 (also assigned AB037939).

Whole-mount in situ hybridisation

Single whole-mount in situ hybridisations were carried out following the protocol of Thisse et al. (1993). Double whole-mount in situ hybridisations were carried out according to the method of Hauptmann and Gerster (1994).

Whole-mount biotin detection

Detection of biotin-dextran in transplanted embryos was carried out as described in Westerfield (1993) using the ABC Vectastain Kit (Vector Laboratories, Inc.).

Mosaic analysis

Embryos from a cross between *fss*$^{+/-}$ × *fss*$^{+/-}$ fish were labelled at the 1- to 4-cell stage with a mixture of rhodamine-dextran and biotinylated-dextran (Molecular Probes). Transplants were carried out as described in Ho and Kane (1990) using unlabelled host embryos, also from a *fss*$^{+/-}$ × *fss*$^{+/-}$ cross. Donor and host embryos were allowed to develop until between 8 and 12 somites, when they were genotyped morphologically. Host embryos were in situ hybridised with an *fgf8* probe and processed for biotin detection.

In other experiments, wild-type donor embryos were injected at the 1- to 2-cell stage with 200 pl of mRNA encoding an EphA4-GFP fusion construct at 1000 ng/μl or GFP at 200 ng/μl. The fusion construct was made such that the GFP was in-frame at the 3′ end of the receptor. Transplantations were carried out as described above, using wild-type embryos and embryos generated from a cross of *fss*$^{-/-}$ × *fss*$^{-/-}$ fish as hosts. Only cells positive for EphA4-GFP or GFP, as determined by fluorescence, were transplanted. Host embryos were allowed to develop to 8-10 somites. Those in which there were cells positive for EphA4-GFP or GFP in the paraxial mesoderm were examined morphologically by DIC optics.

RESULTS

Cloning of a zebrafish *MesP*-related gene, *mesp-a*

In order to isolate *MesP*-related genes involved in somite patterning, we performed a low-stringency screen of a zebrafish cDNA library using a PCR-amplified fragment of a zebrafish bHLH gene. One cDNA was isolated, the bHLH region of which showed homology to the murine proteins MesP1 (Saga et al., 1996) and MesP2 (Saga et al., 1997). This gene was named *mesp-a*.

The bHLH domain of Mesp-a is 76% identical at the amino acid level to equivalent domains of mouse MesP1 and MesP2, and shows 73%, 71% and 67% identity to equivalent domains of the related proteins Thylacine 1, Thylacine 2 (Sparrow et al., 1998) and cMeso-1 (Buchberger et al., 1998), respectively.

None of these MesP-related genes show significant homology outside of the bHLH domain, unlike other families of bHLH proteins (Fig. 1), making it difficult to determine orthology within the MesP family.

mesp-a expression is restricted to an anterior region within presumptive somites

Expression of *mesp-a* is initiated at 40% epiboly around the edge of the advancing blastoderm with the exception of cells at the future dorsal region of the embryo (Fig. 2A). Following the start of gastrulation, cells expressing *mesp-a* involute and expression is seen in the presumptive paraxial mesoderm. At 70% epiboly expression starts to be restricted anteriorly, as *mesp-a* is lost from the posterior paraxial mesoderm (Fig. 2B,C). By bud stage, narrow stripes of *mesp-a* expression are present in the anterior presomitic mesoderm (Fig. 2D).

Throughout somitogenesis, expression of *mesp-a* is highly dynamic, with one or two stripes of expression in the anterior presomitic mesoderm of embryos at morphologically equivalent stages of development (Fig. 2E-H). When two stripes of *mesp-a* expression are visible, the most anterior stripe is predicted to be in somite –I (S–I), where SI is the newest formed somite, S0 is the somite in the process of forming, and S–I is the next most posterior presumptive somite, as determined by the position of the last formed somite boundary (Christ and Ordahl, 1995). The posterior stripe of *mesp-a* expression is broader than the anterior one, suggesting that *mesp-a* expression may be restricted within segments as they mature (Fig. 2F). Indeed, *mesp-a* is initially expressed in a stripe of 5-6 cell diameters along the AP axis, which may encompass all of S–II (Fig. 2F,G). During the time that it takes one somite to form, this stripe of expression is restricted rostrally within the segment to a stripe of 2-3 cell diameters along the AP axis (Fig. 2E). These narrow stripes of *mesp-a* are seen in S–I (Fig. 2E-G). During the formation of the next somite, this anterior stripe of expression is lost, with expression persisting for the longest time in the adaxial cells in the anterior of the segment (Fig. 2H). These observations suggest that the anterior stripe of *mesp-a* constitutes a subset of anterior cells of S–I, whereas the posterior stripe constitutes most or all cells in S–II.

Analysis of double in situ hybridisations between *mesp-a*, *her1* and *delta D* support the interpretation that *mesp-a* expression is restricted to the anterior region of S–I. *her1* has been described as being expressed in alternating segments (Muller et al., 1996). When two stripes of *mesp-a* expression are visible, the anterior stripe is a subdomain of the most

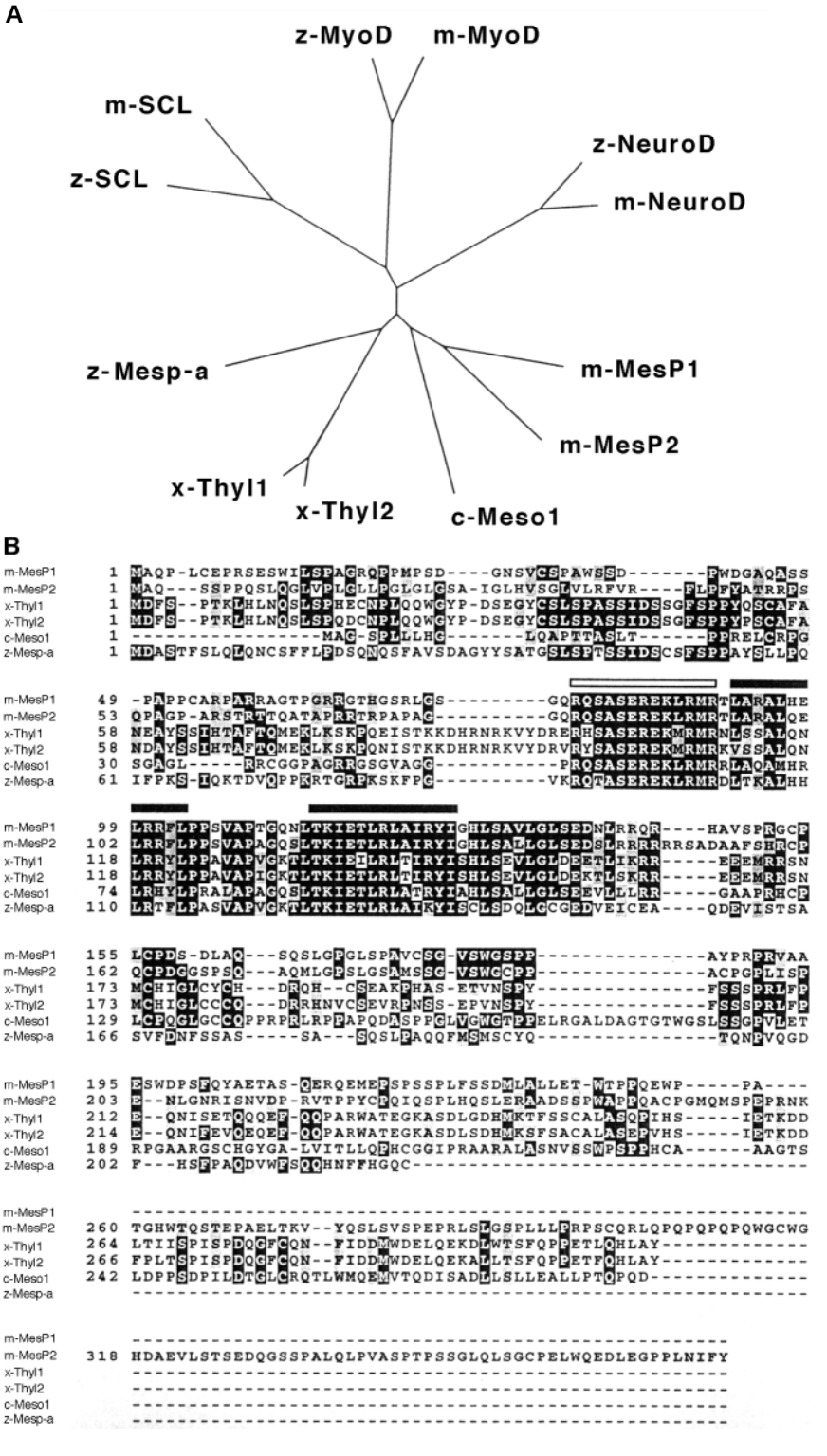

Fig. 1. (A) A radial phylogenetic tree illustrates that Mesp-a falls into the group of MesP1/2-related bHLH proteins. The phylogenetic tree was generated with the PHYLIP program (version 3.572) based on a ClustalX alignment. All sequences used were taken from GenBank. (B) Alignment of MesP-related proteins. Within the bHLH domain the basic sequence is marked with a white bar and the helices with black bars. Dashes indicate gaps introduced to optimise alignment.

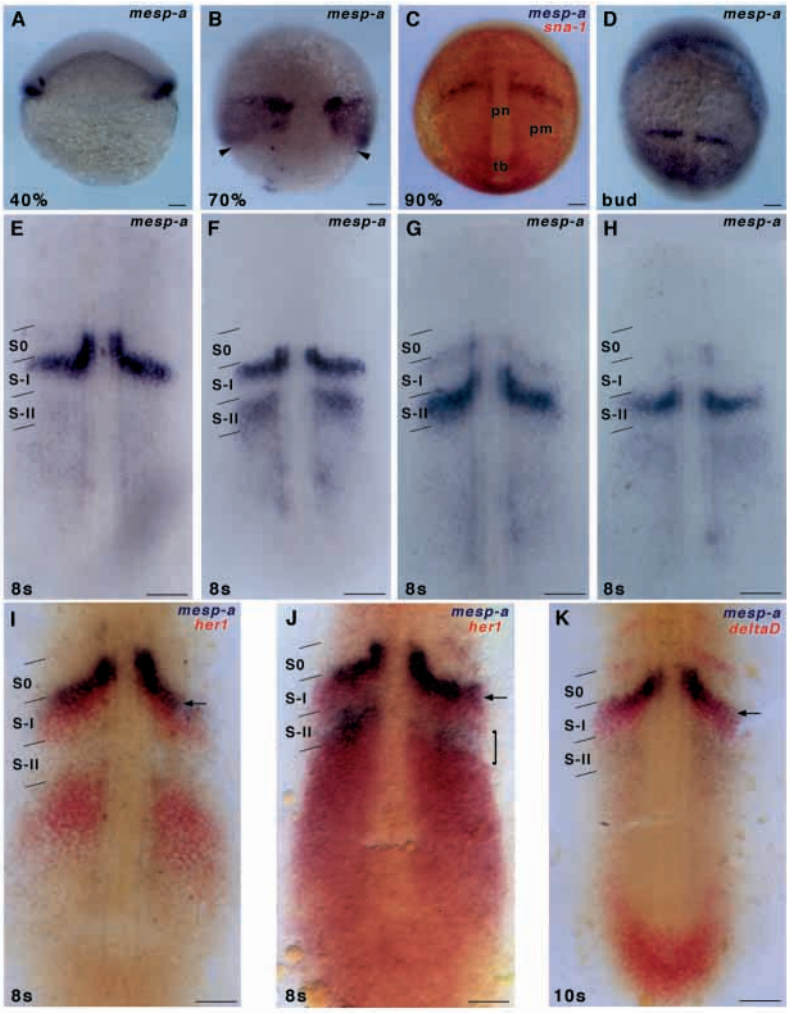

Fig. 2. *mesp-a* is expressed dynamically in segmenting mesoderm. Dorsal views, anterior to the top in this and subsequent figures. (A) 40% epiboly. *mesp-a* expression is initiated in the margin of the blastoderm, except in the future dorsal region. (B) 70% epiboly. *mesp-a* is expressed in the presumptive paraxial mesoderm. Expression is strongest in the anterior region of this domain. Arrowheads mark the germ ring. (C) 95% epiboly. Double in situ hybridisation with *mesp-a* (blue) and *sna-1* (red). *mesp-a* is expressed in bilateral stripes in the anterior paraxial mesoderm, *sna-1* expression can be seen around the germ ring. (D) Bud stage. Stripes of *mesp-a* expression are present in the anterior presomitic mesoderm. (E-H) 8 somites. Expression of *mesp-a* is dynamic, one or two stripes are visible in the anterior presomitic mesoderm of embryos at the same morphological stage. Lines mark the presumed position of segment boundaries in these and I-K. (I,J) 8 somites. Double in situ hybridisation with *mesp-a* (blue) and *her1* (red). (I) *mesp-a* is co-expressed with *her1* in the anterior region of S–I (arrow). (J) *mesp-a* expression overlaps with *her1* in the anterior region of S–I (arrow), and is found throughout S–II between the two stripes of *her1* (bracket). (K) 10 somites. Double in situ hybridisation with *mesp-a* (blue) and *delta D* (red). *mesp-a* expression overlaps with *delta D* expression in the anterior region of S-I (arrow). pm, paraxial mesoderm; pn, prospective notochord; tb, tail bud. Scale bars, 100 μm.

anterior domain of *her1* expression; whereas the posterior stripe of *mesp-a* is in the segmental domain which is devoid of *her1* expression, S–II (Fig. 2J). When one stripe of *mesp-a* is visible, this constitutes an anterior subset of the cells expressing *her1* and *delta D* in S–I (Fig. 2I,K).

In summary, our data suggest that *mesp-a* is initially expressed throughout S–II, then is restricted to anterior cells as the domain becomes S–I, and expression is lost by the stage that the cells are in S0. The restriction of *mesp-a* expression from throughout S–II to an anterior domain within S–I suggests that each segment is regionalised along its AP axis at this time in their development.

A segmental prepattern is established in zebrafish mutants that show defects in somite boundary formation

The expression pattern of *mesp-a* during somitogenesis indicates that each segment is patterned along its AP axis following the establishment of a segmental prepattern in the paraxial mesoderm. In zebrafish mutants that show a disruption of somite boundary formation (van Eeden et al., 1996) the defects must lie in the establishment of a segmental prepattern, in the AP regionalisation of each segment, or in the later step of boundary formation. Homozygous *fss*$^{-}$, *bea*$^{-}$, *des*$^{-}$ and *aei*$^{-}$ mutants form vertebrae with an almost normal periodicity (van Eeden et al., 1996), suggesting that the mutated genes function downstream of the processes in which a segmental prepattern is established in the paraxial mesoderm. To determine whether there is any molecular evidence of the establishment of a segmental prepattern in these mutants during embryogenesis, we examined the expression patterns of mesodermal marker genes in *fss*$^{-}$, *bea*$^{-}$, *des*$^{-}$ and *aei*$^{-}$.

In agreement with van Eeden et al (1998) we found in *fss*$^{-}$, as in wild-type embryos, that *her1* is expressed in a segmental pattern (Fig. 3B). However, the two stripes of *her1* expression present in wild-type embryos, were not seen in *fss*$^{-}$ embryos. This suggests that a segmental prepattern is initially established in *fss*$^{-}$, but that patterning in the presomitic mesoderm is disturbed. In contrast to *fss*$^{-}$, *her1* was not obviously segmentally expressed in *bea*$^{-}$, *des*$^{-}$ or *aei*$^{-}$. In *bea*$^{-}$, *her1* was expressed throughout the tail bud and posterior presomitic mesoderm (Fig. 3C), whereas in *des*$^{-}$ and *aei*$^{-}$, *her1* was expressed strongly in the tailbud, more weakly in the posterior presomitic mesoderm, and in a “salt and pepper” fashion in the anterior presomitic mesoderm (Fig. 3D,E). Although segmental expression of *her1* is lost in *bea*$^{-}$, *des*$^{-}$ and *aei*$^{-}$, from the pattern of the vertebrae that form in these mutants, we know that this does not equate to a complete loss of a segmental prepattern.

Analysis of *CS131* expression confirmed decisively that a segmental prepattern is established in all of these somite

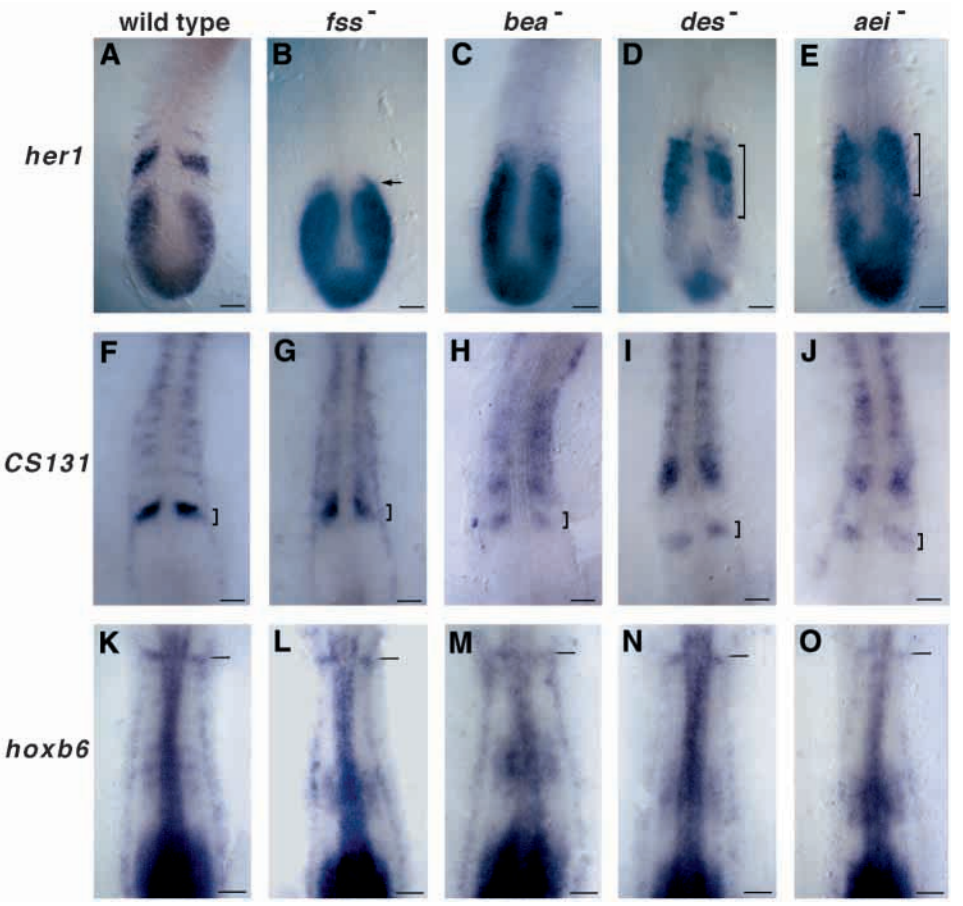

Fig. 3. A segmental prepattern is established in the paraxial mesoderm of *fss*⁻, *bea*⁻, *des*⁻ and *aei*⁻ embryos. (A-E) *her1* expression in 10-somite wild-type (A), *fss*⁻ (B), *bea*⁻ (C), *des*⁻ (D) and *aei*⁻ (E) embryos. (A) *her1* is expressed in segmental stripes in the wild-type embryo. (B) Stripes of *her1* are seen in *fss*⁻ (arrow). (C) *her1* is expressed throughout the presomitic mesoderm in *bea*⁻. (D,E) In *des*⁻ and *aei*⁻, *her1* is seen in the tailbud and in a "salt and pepper" pattern in the anterior presomitic mesoderm (brackets). (F-J) *CS131* expression in 10-somite wild-type (F), *fss*⁻ (G), *bea*⁻ (H), *des*⁻ (I) and *aei*⁻ (J) embryos. (F) *CS131* is expressed in a stripe in the presomitic mesoderm of the wild-type embryo (bracket). (G-J) In the mutants, stripes of *CS131* expression are still present in the presomitic mesoderm (brackets). (K-O) *hoxb6* expression in 10-somite wild-type (K), *fss*⁻ (L), *bea*⁻ (M), *des*⁻ (N) and *aei*⁻ (O) embryos. (K) *hoxb6* is expressed in the paraxial mesoderm posterior to the somite 3/4 boundary (lines) in the wild-type embryo (K). The anterior limit of expression of *hoxb6* is maintained in the somitic mesoderm of the mutants at the correct level, as determined by distance from the anterior end of the somitic mesoderm (L-O). Scale bars, 100 μm.

mutants. CS131 is a member of the group of small acidic proteins, which include GADD45 and myd118, that cause cell cycle arrest (reviewed by Fornace, 1992). During somitogenesis *CS131* is expressed in a posterior domain of formed somites, and in a stripe in the presomitic mesoderm which, from its position, appears to encompass S–II (Fig. 3F). In *fss*⁻, *bea*⁻, *des*⁻ and *aei*⁻ a stripe of *CS131* expression is present in the paraxial mesoderm in a position that corresponds to the presomitic stripe in wild-type embryos (Fig. 3G-J), showing that a segmental prepattern is established in the presomitic mesoderm of all of these mutants.

Analysis of *hox* gene expression revealed that AP identities are maintained along the AP axis of *fss*⁻, *bea*⁻, *des*⁻ and *aei*⁻ embryos. In wild-type embryos, *hoxb6* is expressed posterior to the boundary between somites 3 and 4 (Prince et al., 1998; Fig. 3K) and *hoxa10* is expressed caudal to the somite 11/12 border (Sordino et al., 1996). In *fss*⁻, *bea*⁻, *des*⁻ and *aei*⁻, both *hoxb6* and *hoxa10* are expressed in the paraxial mesoderm in domains that correspond to the wild-type expression domains, despite the lack of somite boundaries (Fig. 3L-O and data not shown). This suggests that patterning along the AP body axis respects segment borders in the paraxial mesoderm of these mutants, even when somite boundaries are not present.

The regionalisation of segments along their anteroposterior axis is disturbed in *fss*⁻, *bea*⁻, *des*⁻ and *aei*⁻

As the establishment of a segmental prepattern in the presomitic mesoderm does not appear to be disturbed in *fss*⁻, *bea*⁻, *des*⁻ and *aei*⁻ mutants, it is possible that segments are not correctly patterned following their specification. To address this possibility, we examined the expression of *mesp-a*, *EphA4* (Durbin et al., 1998), *delta D* (Dornseifer et al., 1997) and *fgf8/ace* (Furthauer et al., 1997; Reifers et al., 1998) markers of anterior segment identity, and *ephrin-B2* (Durbin et al., 1998) and *myoD* (Weinberg et al., 1996) markers of posterior segment character, in the mutants.

A loss of anterior markers and expression of posterior markers throughout the anterior presomitic mesoderm suggests that the somitic mesoderm of *fss*⁻ is posteriorized. *mesp-a* and *EphA4* expression were absent in anterior segmental domains in *fss*⁻ (Fig. 4B,F). Weak, diffuse bands of *delta D* and *fgf8* expression were observed in the anterior presomitic mesoderm, and expression of both was lost in the region equivalent to that where somites have formed in wild-type embryos (Fig. 4D,J). As *delta D* and *fgf8* are only anteriorly restricted in wild-type embryos as the segments mature (Fig. 4C,I), the residual expression in *fss*⁻ may correspond to this unrestricted early expression domain. In contrast, markers of posterior segment identity, *ephrin-B2* and *myoD*, were expressed throughout the somitic mesoderm of *fss*⁻ (Fig. 4H,L). Thus the AP regionalisation of segments is disrupted in *fss*⁻ and the somitic mesoderm appears posteriorized.

The regionalisation of segments along the AP axis is also disrupted in *bea*⁻, *des*⁻ and *aei*⁻, but unlike in *fss*⁻, both anterior and posterior segment markers are expressed throughout the

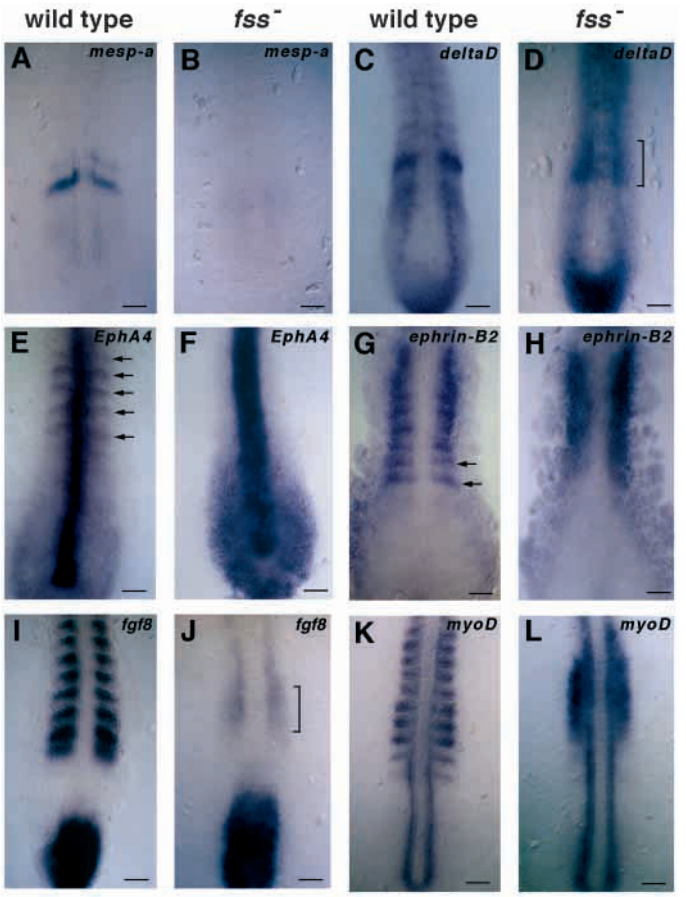

Fig. 4. Expression of anterior segment markers are lost from the somitic mesoderm of *fss*⁻ embryos. (A-B) *mesp-a* is expressed in 6-somite wild-type (A) embryos but not in *fss*⁻ (B) embryos. (C-D) *delta D* expression in 8-somite wild-type (C) and *fss*⁻ (D) embryos. A faint band of *delta D* expression is seen in the *fss*⁻ embryo (bracket, D), but overall expression is significantly reduced. (E-F) *EphA4* expression in 6-somite wild-type (E) and *fss*⁻ (F) embryos. *EphA4* is expressed in an anterior domain of forming somites in the wild-type embryo (arrows, E). This segmental expression of *EphA4* is lost in *fss*⁻. Expression is still present in the posterior presomitic mesoderm, tailbud and notochord of *fss*⁻. Expression appears stronger in these regions of the *fss*⁻ embryo (F) since the in situ was over-developed to confirm that there was no expression of *EphA4* in the anterior somitic mesoderm. (G-H) *ephrin-B2* expression in 6-somite wild-type (G) and *fss*⁻ (H) embryos. Transcripts are detected in a posterior region of presumptive somites in the wild-type embryo (arrows, G). No segmental restriction of *ephrin-B2* expression is seen in the somitic mesoderm of the *fss*⁻ embryo. (I-J) *fgf8* expression in 8-somite wild-type (I) and *fss*⁻ (J) embryos. In the *fss*⁻ embryo the segmentally restricted expression in the somitic mesoderm is lost, and only faint non-segmental expression is observed (bracket, J). (K,L) *myoD* expression in 10-somite wild-type (K) and *fss*⁻ (L) embryos. The segmental restriction of *myoD* expression is lost in the *fss*⁻ embryo. Scale bars, 100 μm.

somitic mesoderm. *mesp-a* expression was dramatically reduced and present in broad diffuse stripes in these mutants (Fig. 5B-D). The segmental restriction of other anterior and posterior segment markers was also lost. *EphA4*, *delta D* and *ephrin-B2* were expressed throughout the region in which they are normally AP restricted within segments (Fig. 5F-H,J-L, N-P). Double in situ hybridisations with *EphA4* and *ephrin-B2* showed that cells in this area were expressing markers of both anterior and posterior segment identity (Fig. 5R). Thus AP regionalisation within segments is disturbed in *bea*⁻, *des*⁻ and *aei*⁻, resulting in a loss of polarisation of segments.

fss appears to act in a cell non-autonomous manner

To determine whether fss acts in a cell autonomous manner, we transplanted labelled wild-type or *fss*⁻ cells into wild-type and mutant embryos. *fgf8* expression, boundary formation and the location of transplanted cells were subsequently determined. Wild-type cells transplanted into a wild-type host dispersed throughout the somitic mesoderm and contributed normally to anterior and posterior domains within somites, as expected (Fig. 6A). *fss*⁻ cells transplanted into a wild-type host also dispersed throughout the paraxial mesoderm and contributed to all regions of the somites, including the boundaries as determined by the position and epithelial morphology of cells (Fig. 6B). This suggests that fss acts in a cell non-autonomous manner and the *fss*⁻ cells were rescued through being in a wild-type environment. This raised the possibility that the presence of wild-type cells in a *fss*⁻ mutant host may be able to rescue the mutant phenotype. However, no obvious rescue was visible when wild-type cells were transplanted into mutant hosts, as detected by *fgf8* expression or morphological boundaries (Fig. 6C). Of course it remains possible that there were not enough wild-type cells or they were positioned in an inappropriate place to bring about rescue. It does suggest, however, that fss acts in a cell non-autonomous manner in the patterning of segments along their AP axis.

Boundary formation can be rescued in *fss*⁻ by groups of cells expressing EphA4

The loss of somite boundary formation in *fss*⁻ correlates with a loss of cells with anterior segment identity. We have shown previously that the juxtaposition of cells expressing *EphA4* and *ephrin-B2* is required for somite boundary formation (Durbin et al., 1998). This interface is lost in *fss*⁻ (Fig. 4). Therefore, we attempted to rescue boundary formation in *fss*⁻ by ectopically expressing EphA4 in the paraxial mesoderm.

Wild-type donor embryos were injected with RNA encoding an EphA4-GFP fusion construct. To test that the EphA4-GFP protein was functional, we showed that it could rescue the effects of overexpression of a co-injected dominant negative receptor (data not shown). Cells expressing ectopic EphA4, as detected by fluorescence, were transplanted into wild-type and *fss*⁻ hosts. Host embryos were allowed to develop to 8-10 somites, when the morphology of their somitic mesoderm in relation to the presence of transplanted cells was examined.

When cells expressing ectopic EphA4 were scattered through the paraxial mesoderm in *fss*⁻ embryos, the somitic mesoderm appeared unsegmented as it typically does in the mutants (n=15; Fig. 7A). However, when clusters of transplanted cells were present, morphological boundaries were visible where the cells expressing ectopic EphA4 were in contact with the host cells (n=30; Fig. 7B-D). Only two *fss*⁻ embryos with small clumps of cells expressing EphA4 in the paraxial mesoderm did not form ectopic boundaries. Boundaries were sometimes only visible on one side of the group of transplanted cells (Fig. 7B), suggesting that boundaries may only form on one side of the polarised cells.

No ectopic boundaries were seen when cells expressing ectopic EphA4 were transplanted into wild-type embryos (*n*=22; Fig. 7E). Boundaries also did not form in control experiments, when wild-type cells expressing GFP were transplanted into *fss*$^{-}$ hosts (*n*=14; Fig. 7F). Thus it is possible to rescue boundary formation in the paraxial mesoderm of *fss*$^{-}$ embryos by the apposition of adjacent domains of cells expressing EphA4 and ephrin-B2.

DISCUSSION

The expression of *mesp-a* shows that segments are internally regionalised along their AP axis by the stage that cells have arrived in the anterior presomitic mesoderm. This precedes somite boundary formation, with borders subsequently forming between cells of anterior and posterior character in adjacent segments. In *fss*$^{-}$, *bea*$^{-}$, *des*$^{-}$ and *aei*$^{-}$ mutants, we show that a segmental plan is established in the paraxial mesoderm, but that patterning within segments is disturbed and somite boundary formation fails to occur. This suggests that the AP regionalisation of each segment is required for somite boundary formation. In support of this, we were able to rescue boundary formation in *fss*$^{-}$ embryos by the reconstitution of adjacent domains of cells expressing anterior and posterior segment markers within the paraxial mesoderm, by ectopically expressing EphA4. This suggests that signalling through Eph receptors is sufficient to bring about morphological boundary formation in the paraxial mesoderm.

AP regionalisation of segments is required for somite boundary formation

Several models have been proposed to explain how a segmental pattern is established within the paraxial mesoderm. One of these, the clock and wavefront model, proposes that cells in the mesoderm are continuously oscillating, this being the clock. The interaction of this clock with a wavefront moving along the axis of the embryo results in the definition of regularly sized segments (Cooke and Zeeman, 1976). The identification of genes, *c-hairy1* (Palmeirim et al., 1997) and *lunatic fringe* (Forsberg et al., 1998; McGrew et al., 1998), which show cyclical expression in the presomitic mesoderm in chick and mouse, suggests the existence of a developmental clock linked to

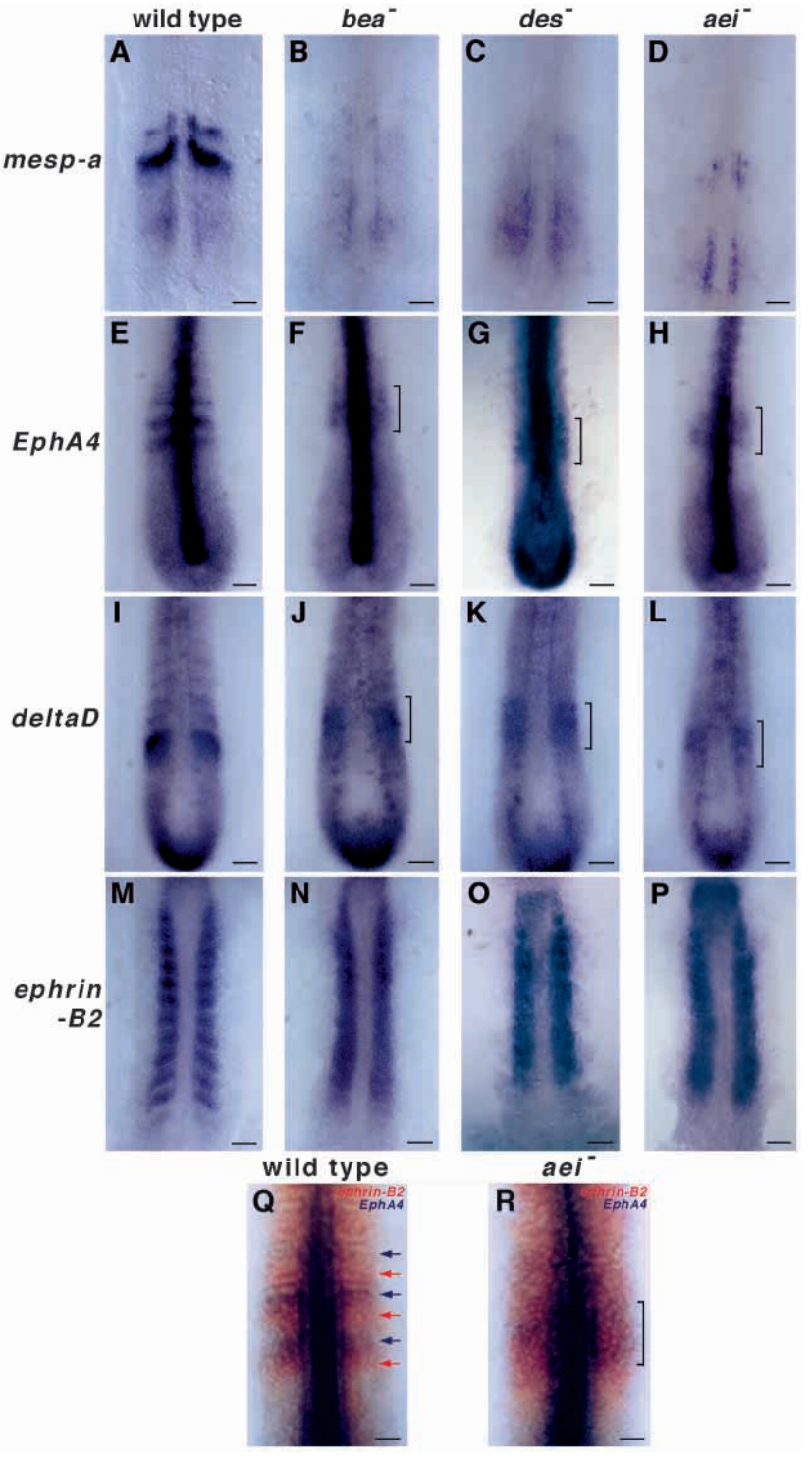

Fig. 5. The polarity of segments is lost in *bea*$^{-}$, *des*$^{-}$ and *aei*$^{-}$ embryos. (A-D) *mesp-a* expression in 10-somite wild-type (A), *bea*$^{-}$ (B), *des*$^{-}$ (C) and *aei*$^{-}$ (D) embryos. The segmental restriction of expression is lost in *bea*$^{-}$, *des*$^{-}$ and *aei*$^{-}$ embryos. (E-H) *EphA4* expression in 10 somite wild-type (E), *bea*$^{-}$ (F), *des*$^{-}$ (G) and *aei*$^{-}$ (H) embryos. In the mutants the segmental restriction of expression of *EphA4* is lost (brackets F-H). (I-L) *delta D* expression in 10 somite wild-type (I), *bea*$^{-}$ (J), *des*$^{-}$ (K) and *aei*$^{-}$ (L) embryos. The segmental restriction of expression is lost in *bea*$^{-}$, *des*$^{-}$ and *aei*$^{-}$ (brackets J-L). (M-P) *ephrin-B2* expression in 10-somite wild-type (M), *bea*$^{-}$ (N), *des*$^{-}$ (O) and *aei*$^{-}$ (P) embryos. In the mutants, *ephrin-B2* is expressed throughout the somitic mesoderm. (Q,R) *EphA4* (blue) and *ephrin-B2* (red) expression in 10 somite wild-type (Q) and *aei*$^{-}$ (R) embryos. In the wild-type embryo *EphA4* and *ephrin-B2* are expressed in complementary anterior (blue arrows Q) and posterior domains (red arrows Q) respectively within somites as they form. In the presomitic mesoderm of the *aei*$^{-}$ embryo cells express both *EphA4* and *ephrin-B2* (bracket, R). Scale bars, 100 μm.

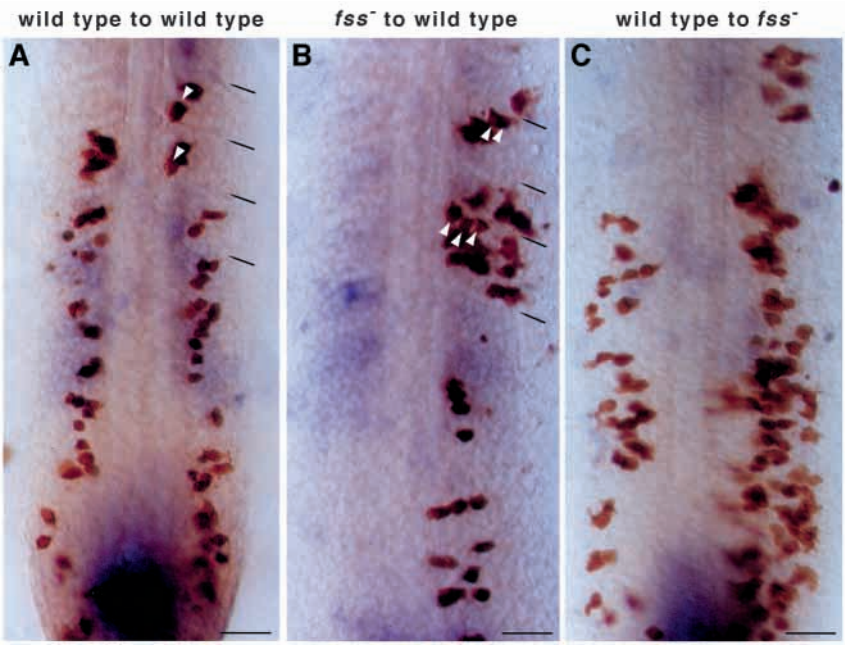

Fig. 6. fss functions in a cell non-autonomous manner. (A) Wild-type transplanted cells (brown) in a 10 somite wild-type host embryo. *fgf8* expression can be seen in the somitic mesoderm (purple staining). The transplanted cells have dispersed throughout the paraxial mesoderm, and contribute to anterior and posterior domains within somites as well as to the boundaries (arrowheads). Lines mark the intersomitic furrows in this and B. (B) *fss*$^{-}$ cells (brown) in a 10 somite wild-type host embryo. *fgf8* is expressed normally (purple staining). The transplanted *fss*$^{-}$ cells have dispersed throughout the paraxial mesoderm, and contribute normally to both anterior and posterior regions within somites, including somite boundaries as determined by position and morphology (arrowheads). (C) Wild-type cells (brown) in a 10 somite stage *fss*$^{-}$ host embryo. *fgf8* expression is absent, as is typical of *fss*$^{-}$ embryos, this has not been rescued by the presence of wild-type cells. The wild-type cells have dispersed throughout the somitic mesoderm and do not form any boundary-like structures. Scale bars, 100 μm.

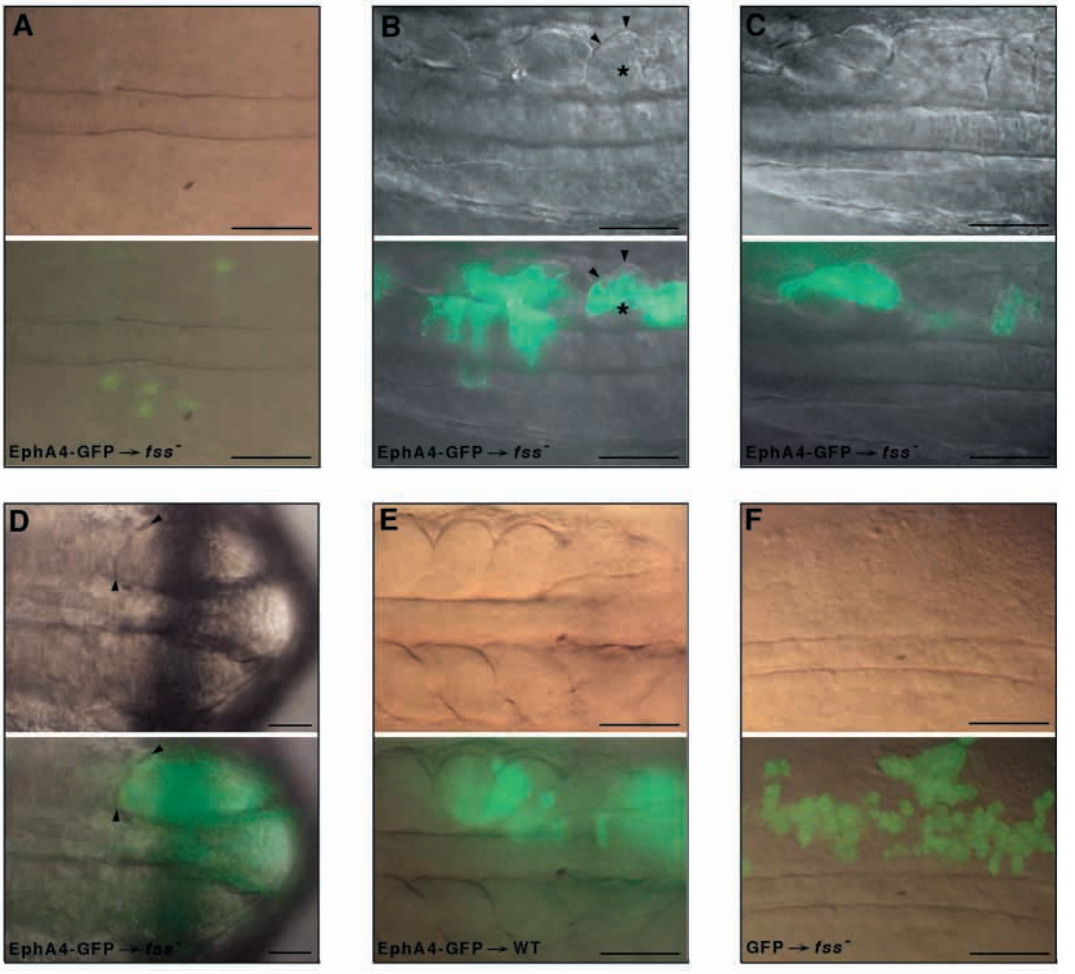

Fig. 7. Ectopic expression of EphA4 rescues boundary formation in *fss*$^{-}$ embryos. Dorsal views of embryos, anterior to the left. The top row of each set of panels is a DIC image of the host embryo, and the bottom row is the superimposition of this and a fluorescent image of the embryo. (A) An 8-somite stage *fss*$^{-}$ embryo into which cells ectopically expressing EphA4-GFP has been transplanted. No boundaries have formed in the paraxial mesoderm, as only a few scattered cells expressing EphA4-GFP are present. (B-D) 10-somite stage *fss*$^{-}$ embryos into which cells ectopically expressing EphA4-GFP have been transplanted. Morphological boundaries are seen in the paraxial mesoderm at the interfaces between cells ectopically expressing EphA4 and host mesodermal cells. A boundary is seen on one side of a clump of transplanted cells (arrowheads) but not on the other side (asterisk) in B. Arrowheads in D indicate position of boundary. (E) A 10-somite wild-type embryo into which cells ectopically expressing EphA4-GFP has been transplanted. The somite boundaries have formed in the correct position, although there are groups of cells expressing ectopic EphA4 in the mesoderm. (F) A 6 somite stage *fss*$^{-}$ embryo into which cells ectopically expressing GFP has been transplanted. A control experiment showing that boundaries do not form in the mutant paraxial mesoderm when large groups of cells expressing GFP are present. Scale bars, 100 μm.

segmentation. Until recently no zebrafish genes had been convincingly shown to exhibit cyclical expression, although there is now evidence to suggest that some elements of the expression of *her1* are cyclical (Sawada et al., 2000). This raises the possibility that a clock linked to somitogenesis also exists in zebrafish.

A variation of the clock and wavefront model was put forward by Primmet et al. (1989), in which the hypothetical oscillator is proposed to be the cell cycle. In this paper, we have described the expression pattern of *CS131*, a gene belonging to a family of proteins involved in regulating the cell cycle, in a restricted segmental pattern in the presomitic mesoderm during somitogenesis. This suggests that there could be a link between the cell cycle and segmentation. The expression pattern of *CS131* is the first molecular evidence that supports the involvement of the cell cycle in the control of segmentation, although functional experiments will be required to address if the gene truly functions during the initial segmentation process or in events downstream of this. However, the segmental pattern of *CS131* is not significantly disturbed in *fss*$^{-}$, *bea*$^{-}$, *des*$^{-}$ and *aei*$^{-}$ mutants, suggesting that any involvement of the cell cycle in somitogenesis is upstream of the AP regionalisation of segments.

A further model that addresses the question of how and where somite boundaries form, rather than how a segmental pattern is established in the presomitic mesoderm, is Meinhardt's positional information model. This proposes that gene expression in the presomitic mesoderm is initially oscillating between two states, anterior and posterior, before the fate of cells is fixed such that there are groups of anterior cells adjacent to groups of posterior cells. Boundaries consequently form where cells with different identities are opposed (Meinhardt, 1986). Our results confirm that somite boundary formation does indeed occur as a result of cells with anterior segment identity being juxtaposed to cells of posterior character. However, we have found no evidence to suggest that cells are initially oscillating between these two fates. The expression pattern of *mesp-a* suggests that this anterior segment marker is initially expressed throughout prospective segments, and the fact that the somitic mesoderm appears posteriorized in *fss*$^{-}$ shows that all cells are also capable of adopting the posterior fate. This suggests that the AP regionalisation of segments involves a determination event in which cells differentiate from a precursor state to either an anterior or posterior identity, all cells being competent to adopt either fate.

Involvement of intercellular signals in the AP regionalisation of segments

The somitic mesoderm of *fss*$^{-}$ embryos appears posteriorized and transplantation experiments have suggested that fss functions in a cell non-autonomous manner, suggesting that an intercellular signal is required for the AP regionalisation of segments. The involvement of the Notch signalling system in somitogenesis has been known since mice homozygous for a null mutation in *Notch1* were found to have severe somitic defects (Conlon et al., 1995). More recently, analyses of mice mutant in the Notch ligands *Dll1* and *Dll3*, and in *lunatic fringe*, have shown that the AP polarity of somites is disturbed in all of these (Hrabe de Angelis et al., 1997; Kusumi et al., 1998; Evrard et al., 1998; Zhang and Gridley, 1998). This suggests an involvement of the Notch signalling system in AP regionalisation within segments, and provides a candidate for the intercellular signalling system that is disrupted in *fss*$^{-}$, and the other mutants that exhibit defects in the AP patterning of segments, *bea*$^{-}$, *des*$^{-}$ and *aei*$^{-}$. Analysis of double mutant combinations between *fss*$^{-}$, *bea*$^{-}$, *des*$^{-}$ and *aei*$^{-}$ has shown that there is no redundancy between them, and this also suggests that all of these genes function within the same pathway (van Eeden et al., 1998). Recent analysis has suggested that a Notch-mediated signal may be required early in the process of somite formation, possibly in the establishment of the segmental prepattern (Jen et al., 1999; Takke and Campos-Ortega, 1999), whereas Delta and Fringe homologues appear to act downstream of this in the AP regionalisation within segments (del Barco Barrantes et al., 1999; Takke and Campos-Ortega, 1999). This suggests that Delta or Fringe signals could be disrupted in *fss*$^{-}$, *bea*$^{-}$, *des*$^{-}$ and *aei*$^{-}$ mutants, and indeed the expression of *delta D* is disturbed in all of these mutants.

The molecular identity of the *fss*, *bea*, *des* and *aei* genes remains unknown at present. It is possible that one or more of these genes are the zebrafish orthologues of the genes that when disrupted in mouse have resulted in related or comparable somite phenotypes. These mammalian genes include *Notch1* (Conlon et al., 1995), *Dll1* (Hrabe de Angelis et al., 1997), *lunatic fringe* (Evrard et al., 1998; Zhang and Gridley, 1998) and *MesP2* (Saga et al., 1997). In mice homozygous for null mutations in any of these genes, the anterior somites form but the posterior ones fail to segment from the paraxial mesoderm. Thus, morphologically, these mouse mutants resemble the zebrafish mutants *bea*$^{-}$, *des*$^{-}$ and *aei*$^{-}$. At a molecular level, disruptions in *Dll1*, *lunatic fringe* and *MesP2* all result in defects in the AP patterning of segments, similar to the zebrafish mutants studied here. In mice homozygous for a null mutations in *Dll1* or *lunatic fringe*, markers of anterior and posterior segment identity are expressed in broad, diffuse regions (Hrabe de Angelis et al., 1997; Evrard et al., 1998; Zhang and Gridley, 1998; del Barco Barrantes et al., 1999), similar to what is seen in *bea*$^{-}$, *des*$^{-}$ and *aei*$^{-}$. In mice in which *MesP2* has been disrupted, anterior segment markers are lost (Saga et al., 1997), similar to what has been described here for *fss*$^{-}$.

The role of the Eph signalling system in somitogenesis

We have previously demonstrated that disturbance of Eph signalling results in loss or disruption of somite boundary formation (Durbin et al., 1998). In *fss*$^{-}$, the somitic mesoderm appears posteriorized, and expression of *EphA4* is lost whilst *ephrin-B2* is expressed throughout this region. Clusters of cells ectopically expressing EphA4 in the paraxial mesoderm of *fss*$^{-}$ embryos rescued boundary formation. Thus signalling through the Eph system is sufficient to cause morphological boundary formation in the mesoderm. Ectopic boundaries were sometimes only seen on one side of a group of transplanted cells, suggesting that cells may become polarised during boundary formation, with morphological boundaries only forming on one side of the polarised cells.

Experiments using cultured explants of chick presomitic mesoderm have shown that the establishment of a segmental prepattern and the patterning of segments along their AP axis can occur in the absence of somite boundary formation

(Palmeirim et al., 1998). Thus there must be molecules that link these events of pattern formation and morphological segmentation in vivo. The receptor EphA4 and its ligand ephrin-B2, are good candidates to fulfil this role. Their expression in anterior and posterior segment halves, respectively, indicates that these molecules lie downstream of the signals that lead to AP polarisation of segments.

When cells expressing ectopic EphA4 were transplanted into the paraxial mesoderm of wild-type embryos no ectopic boundaries formed, although it might be predicted that boundaries should form between transplanted cells and the host's posterior segment cells. This is most probably because the ectopic EphA4 could not overcome the endogenous patterning mechanisms that specify boundary formation at every alternate interface of anterior and posterior segment cells. In contrast, in *fss*$^{-}$ embryos, ectopic boundaries formed wherever the transplanted cells were located, not just at the positions of segment borders. Thus the paraxial mesoderm remains more responsive to somite-boundary-forming signals in *fss*$^{-}$ than wild-type embryos.

The anterior somites appear to be patterned differently to the more posterior ones

The isolation of mutants such as *bea*$^{-}$, *des*$^{-}$ and *aei*$^{-}$ in which only the most anterior somites form, suggests that the first few somites could be specified by a different mechanism to the more posterior ones. We have shown here that a segmental prepattern is correctly established in the paraxial mesoderm of all of these mutants, and it is patterning events within segments that is disturbed in each case. This suggests that the most anterior somites could be patterned internally by a different mechanism to the more posterior ones. However, as AP regionalisation within every segment is disrupted in *fss*$^{-}$, there must be some underlying similarities between the patterning mechanisms in all somites.

The expression pattern of *mesp-a* also supports the idea that anterior and posterior segments are patterned differently. Analysis of the expression pattern of *her1* has shown that the first somites are specified during gastrulation. In fact, the first stripe of *her1* expression, which corresponds to the fifth somite, is visible at 70% epiboly (Muller et al., 1996). This suggests that *mesp-a* does not function during the AP patterning of the first somites to form, since it is not segmentally expressed until around 80% epiboly. This again suggests that the first few somites could be patterned differently along their AP axis to the more posterior ones. It will be of great interest to isolate and clone further mutants that help resolve the conserved and divergent mechanisms regulating somite formation along the AP axis.

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