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Mutations affecting somite formation and patterning in the zebrafish, *Danio rerio*

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SUMMARY

Somitogenesis is the basis of segmentation of the mesoderm in the trunk and tail of vertebrate embryos. Two groups of mutants with defects in this patterning process have been isolated in our screen for zygotic mutations affecting the embryonic development of the zebrafish (*Danio rerio*). In mutants of the first group, boundaries between individual somites are invisible early on, although the paraxial mesoderm is present. Later, irregular boundaries between somites are present. Mutations in *fused somites* (*fss*) and *beamter* (*bea*) affect all somites, whereas mutations in *deadly seven* (*des*), *after eight* (*aei*) and *white tail* (*wit*) only affect the more posterior somites. Mutants of all genes but *wit* are homozygous viable and fertile. Skeletal stainings and the expression pattern of *myoD* and *snail1* suggest that anteroposterior patterning within individual somites is abnormal. In the second group of mutants, formation of the horizontal myoseptum, which separates the dorsal and ventral part of the myotome, is reduced. Six genes have been defined in this group (*you*-type genes). *you-too* mutants show the most severe phenotype; in these the adaxial cells, muscle pioneers and the primary motoneurons are affected, in addition to the horizontal myoseptum.

The horizontal myoseptum is also missing in mutants that lack a notochord. The similarity of the somite phenotype in mutants lacking the notochord and in the *you*-type mutants suggests that the genes mutated in these two groups are involved in a signaling pathway from the notochord, important for patterning of the somites.

Key words: somitogenesis, adaxial cells, muscle pioneers, zebrafish

INTRODUCTION

Segmentation is an important strategy for patterning the anteroposterior (A/P) axis in both vertebrate and invertebrate phyla. In *Drosophila* many of the genes involved in segmentation were identified by genetic screens for mutations affecting embryogenesis (Nüsslein-Volhard and Wieschaus, 1980; Jürgens et al., 1984; Nüsslein-Volhard et al., 1984; Wieschaus et al., 1984). Investigation of these mutants and the subsequent cloning of the corresponding genes has allowed a detailed molecular analysis of this process (Martinez Arias, 1993; Pankratz and Jäckle, 1993). In vertebrates there is still little known about the segmentation process. Rotation experiments in chick indicate that metamerism in the tail depends on the somites, which are derived from mesoderm (Keynes and Stern, 1984). This differs from *Drosophila*, where the ectoderm is likely to impose segmentation onto the mesoderm (Bate, 1990).

In zebrafish (*Danio rerio*), as in most other vertebrates, somites form as epithelial spheres from the presomitic mesoderm in an anterior to posterior direction. Starting at 10 hours postfertilization (hpf), one pair of somites is formed every 20-30 minutes by formation of a new somitic furrow. Roughly 30 somite pairs form in a normal embryo; 7 above the yolk cell, 10 above the yolk extension and 13 posterior to the anus. Perpendicular to the A/P axis of the embryo the somites are subdivided into sclerotome and dermomyotome, which later gives rise to myotome and dermatome. In zebrafish, myotome is the major part of the somites, in contrast to mouse and chick, where the major part of the somites becomes sclerotome. The zebrafish sclerotome is composed of a relatively small group of cells in the ventral part of the somite (B. Morin-Kensicki, personal communication). Vertebrae are derived from the sclerotome, and are formed comparatively late in zebrafish development.

In the course of development three distinct structures are recognizable in the zebrafish myotome: adaxial cells, muscle pioneers and the horizontal myoseptum. In presomitic mesoderm, adaxial cells are distinguishable as large cuboidal cells, adjacent to the notochord. These differ from the rest of the paraxial mesoderm by morphology, behavior and gene expression (Felsenfeld et al., 1991; Hammerschmidt and Nusslein-Volhard, 1993; Thisse et al., 1993; Weinberg et al., 1996). Until the 7-somite stage, the somites have the shape of epithelial spheres. After this timepoint, the adaxial cells in the first five somites start to elongate and intercalate, until they span an entire somite. Adaxial cells from somites that form later in development elongate and intercalate shortly after somite boundary formation. The muscle pioneers, probably a subset of the adaxial cells, are the first cells to show muscle striation in the myotome (Felsenfeld et al., 1991). They lie adjacent to the notochord in the region of the horizontal myoseptum, and are labeled by anti-Engrailed antibody (4D9, Patel et al., 1989; Hatta et al., 1991) starting from the 8- to 10-somite stage and later by Zn5 monoclonal antibody (Trevarrow et al., 1990). The horizontal myoseptum is a fibrous sheet dividing the myotome into a dorsal and a ventral part, and is first visible around 28 hpf. Adaxial cells, muscle pioneers and the horizontal myoseptum are affected in mutants lacking the notochord. It has been shown by cell transplantation experiments that wild-type notochord can induce muscle pioneers and horizontal myoseptum in such mutants (Halpern et al., 1993; Odenthal et al., 1996).

Analysis of somitogenesis in other vertebrates has defined a number of properties of this process. In frogs, a short heatshock during somite formation disturbs somitogenesis. After the heatshock, 4-5 normal somite pairs form, followed by 1-2 abnormal ones (Elsdale et al., 1976). This suggests that the process of segmentation is acting in the presomitic mesoderm, before visible boundaries are formed. Similar effects have been observed in zebrafish (Kimmel et al., 1991). Somites form relatively normally from isolated presomitic mesoderm in culture (Packard and Jacobson, 1976). If newly formed somites are rotated along their A/P axis they form vertebrae with the corresponding inverse polarity (Aoyama and Asamoto, 1988). These experiments indicate that patterning of the paraxial mesoderm along its A/P axis is an intrinsic property of this tissue.

Patterning in the mediolateral (M/L) plane is dependent on induction by the notochord, neural tube and the overlying ectoderm (Brand-Saberi et al., 1993; Münsterberg and Lassar, 1995, and references therein). Transplantation and rotation experiments in chick suggest that polarity in this plane is determined shortly after somite formation (Aoyama and Asamoto, 1988).

A small number of genes are known to be involved in the process of somitogenesis. In mouse, targeted disruption of the *fibronectin* (George et al., 1993), *integrin alpha5* (Yang et al., 1993), *FGFr1* (Deng et al., 1994; Yamaguchi et al., 1994), *Wnt-3a* (Takada et al., 1994) or *Notch1* genes (Conlon et al., 1995) results in impaired somite formation.

In our screen (Haffter et al., 1996) two groups of genes were identified that will be valuable for understanding patterning of the somites in both the A/P and M/L axes. Mutations in five genes affect the formation of early somite boundaries. Two of these, *fused somites* (*fss*) and *beamter* (*bea*), affect the formation of all somite boundaries, while mutations in *deadly seven* (*des*), *after eight* (*aei*) and *white tail* (*wit*) have an effect on more posterior somite boundaries only. 11 genes are involved in the formation of the horizontal myoseptum. Six of these, *you*, *you-too*, *sonic-you*, *chameleon*, *u-boot* and *choker* are required for formation of the horizontal myoseptum but not for notochord formation. The five others, *no tail*, *floating head*, *doc*, *momo* and *dino*, also have an effect on notochord formation (Hammerschmidt et al., 1996b; Odenthal et al., 1996). This paper presents an initial characterization of mutants with defects in somite boundary or horizontal myoseptum formation, and other mutants that affect the paraxial mesoderm as a secondary effect (Table 1).

MATERIALS AND METHODS

Maintenance of fish stocks

Maintenance of fish stocks and matings were done as described by Mullins et al. (1994).

Mutant strains

All analyses were done with the strongest alleles of the respective genes, if there were detectable differences. The alleles used were *fss*^{te314a}, *bea*^{to202}, *des*^{tx201}, *aei* ^{tr233}, *yot* ^{ty119}, *syu*^{tq252}, *con*^{tm15a}, *you*^{ty97}, *ubo*^{tp39} and *cho*^{tm26}. The viability of *fss* was tested with *fss*^{ti1}.

Antibody staining and whole mount in situ hybridization

In situ hybridization and antibody staining was done as described by Hammerschmidt et al. (1996b). The probes that were used were *myoD* (Weinberg et al., 1996), *snail1* (Hammerschmidt and Nusslein-Volhard, 1993; Thisse et al., 1993), and *sonic hedgehog* (Krauss et al., 1993). The 4D9 monoclonal antibody (Patel et al., 1989), anti-Ntl rabbit polyclonal antiserum (Schulte-Merker et al., 1992), anti-Snail1 rabbit polyclonal antiserum (Hammerschmidt and Nusslein-Volhard, 1993), znp-1 monoclonal antibody (Trevarrow et al., 1990) and monoclonal anti-acetylated tubulin antibody (Sigma) were used for stainings.

Pictures

Pictures of live embryos were taken on a Zeiss Axiophot microscope using Kodak ektachrome 64T or 160T slide films; rhodamine-labeled cells were photographed using Kodak ektachrome 400T slide film. Pictures were scanned on a Nikon Coolscan slidescanner; composite pictures were made using the Adobe Photoshop software package on a Macintosh computer.

Cell transplantation

Cell transplantation was basically done as described by Ho and Kane (1990), with the following modifications. For mounting donor and host embryos, small wells were made in agar (2% in E2; Westerfield, 1989), holding single embryos. Transplantations were done using a Zeiss Stemi 2000 stereomicroscope at 20-25×, around the sphere stage (Kimmel et al., 1995).

To determine the effect of the *fss* mutation on the muscle fiber length, small numbers (5-10) of *fss* mutant cells were transplanted into *fss* mutant hosts. Embryos were allowed to develop for 48 hours. Only the length of clearly isolated muscle fibers was measured on a video screen. In addition, the A/P level of these fibers was recorded (all fibers were parallel to the A/P axis). The values obtained were divided by the length that would be expected for muscle fibers parallel to the A/P axis at that level. As a control, exactly the same experiment was done with a set of transplants from wild-type to wild-type embryos.

For the rescue of the myoseptum defect of *yot* and *ubo* mutants, slightly larger numbers of cells were transplanted (10-30 cells). Mutant chimaeric embryos were scored for rescue of the myoseptum phenotype after 2-3 days, using a Zeiss Axiophot.

Skeletal stainings

Fish were fixed in 3.7% formaldehyde/PBS for 24-72 hours. After fixation specimens were rinsed and stained with 0.1 mg/ml Alcian blue 8 GX (Sigma) in ethanol:acetic acid 4:1 (v:v). Specimens were incubated in 90%, 50% and 30% v/v ethanol in water. After a final 2 hour wash in water, specimens were digested overnight in a 50 mg/ml solution of trypsin (crude, Sigma) in a 30% saturated NaB_4O_7 solution in water. Bones were stained with 0.4 ml solution of Alizarine red S (saturated in ethanol; Sigma) in 10 ml 0.5% KOH. Destaining was done in a 1% KOH/glycerin series (3:1, 1:1, 1:3). Scales and sometimes muscles were removed manually. Specimens were stored at 4°C in glycerol. Sometimes grease was removed by another incubation in acetone and sometimes specimens were bleached in a 1% H_2O_2, 1% NH_3 solution, after Alcian blue staining.

RESULTS

Mutations affecting somitogenesis

20 mutants with defects in the formation of epithelial somites were isolated. Complementation analysis defined five complementation groups: *fused somites* (*fss*), *beamter* (*bea*), *deadly seven* (*des*), *after eight* (*aei*) and *white tail* (*wit*) (Table 1, *fss*-type genes).

The two alleles of the *fss* gene are indistinguishable in phenotypic strength. Embryos mutant for *fss* do not show any morphological evidence of boundary formation in the paraxial mesoderm at early somite stages (Fig. 1A,B). Eventually, however, irregular borders are formed in the paraxial tissue (Fig. 2A,B). The horizontal myoseptum and muscle differentiation are not obviously affected by *fss* mutations (Fig. 2C,D).

The phenotype of *bea* mutant embryos differs slightly from that of *fss* mutants. At early somite stages, before differen-

Table 1. Genes involved in somite formation

Gene	Alleles	Other phenotypes	References
Group 1: Mutations affecting epithelial somite formation			
fused somites (*fss*)	*ti1, te314a*	–	
beamter (*bea*)	*tm98, to202, tw212b*	–	
deadly seven (*des*)	*tc225, tp37, th35b, tw239, tm145, tc239c, tx201, tj247, to15e, tc322b*	–	
after eight (*aei*)	*tg22a, tg249, tr233, tm223*	–	
white tail (*wit*)	*ta52b*	Brain, spinal cord	a
Group 2: Mutations affecting the horizontal myoseptum			
you (*you*)	*ty97, tm146c, tz310c*	Circulation, motility	
you-too (*yot*)	*ty119, ty17a*	Spinal cord, circulation, RT projection, motility	b,c,d,e
sonic-you (*syu*)	*tq252*	Motility, circulation, pectoral fins	b,d,e,f
chameleon (*con*)	*tm15a, tf18b, ty60, th6*	Spinal cord, RT projection, circulation, motility, pectoral fins	b,c,d,e,f
u-boot (*ubo*)	*tp39*	Fins, motility, melanophores	f,g
choker (*cho*)	*tm26*	Pigment pattern, hindbrain	a,g
no tail (*ntl*)	*tc41b, tb244e, ts260*	Notochord	h
floating head (*flh*)	*tk241, tm229*	Notochord, floorplate, motility	h
doc (*doc*)	*tt202, tt258, tc233a*	Notochord	h
momo (*mom*)	*th211*	Notochord, midline	h
dino (*din*)	*tm84, tt250*	Ventralizing, notochord	h,i
Group 3: Mutations with denser somites, normal horizontal myoseptum, undifferentiated notochord			
sleepy (*sly*)	*ti263a, te333, ts33a te233,tf215b, ti272a, to216a, tm89, tp16*	Notochord, brain	h
grumpy (*gup*)	*ti228b, tj229a, tp42, tx221,tl17b, tm61, tg210*	Notochord, brain	h
bashful (*bal*)	*tp82, tm220, tr259, tf235, tv36, tt206, tp86, tm267a tf209, to265, tc245c, tq210, tb244f, tc248f, tr203*	Notochord, brain	h
happy (*hap*)	*tc229, te239, tr278, ty230, tk56a, tm285*	Notochord	h
sneezy (*sny*)	*td204a, tn211, tm11, tm75, tq249*	Notochord	h
dopey (*dop*)	*tr222b, tz226, tm18a*	Notochord	h
Group 4: Mutations affecting the paraxial mesoderm as a whole			
snailhouse (*snh*)	*ty68a*	Dorsalizing	j
wirligig (*wrl*)	*dta72, tc300a*	Dorsalizing	j
piggytail (*pgy*)	*dty40, tc227a, ta206a, dti216, tx223, tb241c*	Dorsalizing	j
somitabun (*sbn*)	*dtc24*	Dorsalizing	j
lost-a-fin (*laf*)	*ty130a, tc263, tf211, tt203, tv9, tm110b, tf215a*	Dorsalizing	j
spadetail (*spt*)	*tm41, tq5*	Gastrulation	i

References: a, Jiang et al. (1996); b, Brand et al. (1996); c, Karlstrom et al. (1996); d, Granato et al. (1996); e, Chen et al. (1996); f, Eeden et al. (1996); g, Kelsh et al. (1996); h, Odenthal et al. (1996); i, Hammerschmidt et al. (1996a); j, Mullins et al. (1996).

For group 5 mutations affecting muscle formation in the myotomes, see Granato et al. (1996).

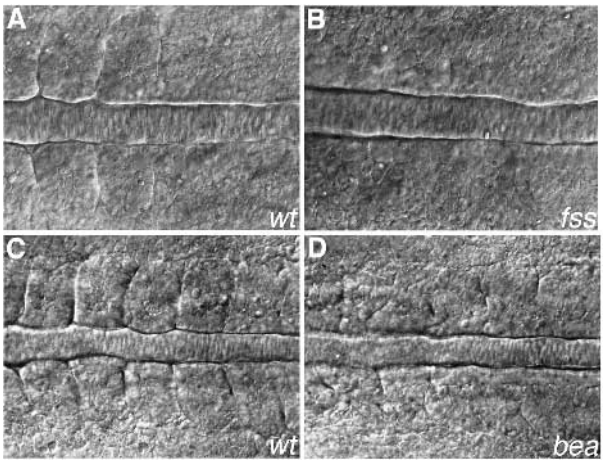

Fig. 1. Dorsal view of live embryos mutant for *fss* (B), *bea* (D) and their wild-type siblings (A and C, respectively). In A the sixth somite is just forming. (B) *fss* mutant at the same A/P level; no somite boundaries are visible. In C the seventh somite is just forming at the left of the picture. (D) *bea* mutant at the same level as C. Boundaries are present, but indistinct and irregularly placed.

tiation of the most anterior somites, segment boundaries can be seen, in contrast to *fss* mutants. These boundaries are irregularly placed and indistinct in comparison to those of wild-type siblings (Fig. 1C,D). Later in development, somitogenesis seems to be more severely disturbed, and in the tail the somite boundary defect is as severe as that of *fss* mutants (Fig. 3). As a result, at 24 hpf, *bea* mutants seem to have 1-4 relatively normal-looking anterior somite boundaries, followed by irregular boundaries more posteriorly.

In *des* and *aei* mutants the first 7±2 somites form as in wild-type siblings, but more posterior somite boundaries change gradually from a wild-type arrangement to an irregular arrangement similar to that of *fss*. The transition from normal to defective somite boundaries occurs in a similar region in all cases analyzed and no difference in allele strength has been detected among different alleles of *aei* and *des.*

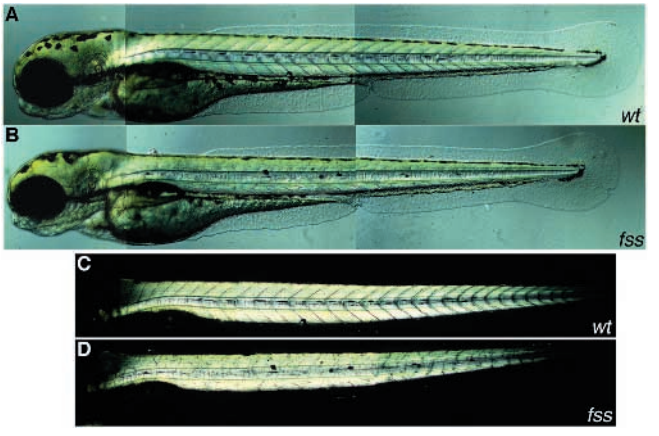

Fig. 2. An overview of the *fss* phenotype at 84 hpf. (A) Wild-type sibling, (B) homozygous mutant; no V-shaped myotomes are present. Usually mutants are slightly shorter. (C,D) Same embryos, dark-field image. Striated muscle shows a bright birefringence. Segment borders are visible as dark lines.

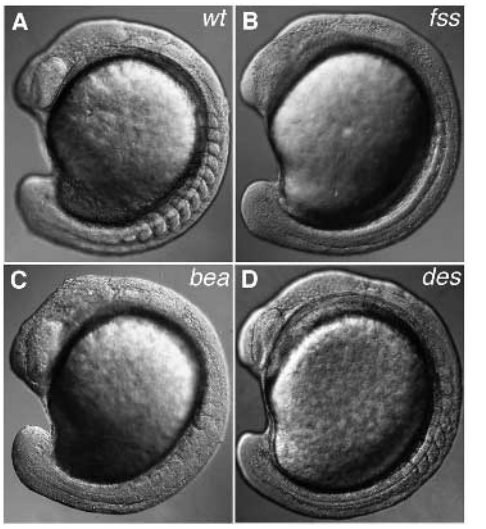

Fig. 3. Comparison of the three phenotypic groups of somite boundary mutants. All embryos are at the 11- to 13-somite stage. (A) Wild-type embryo. (B) *fss* mutant; only very few boundaries are present anteriorly. (C) *bea* mutant; in this individual the phenotype seems almost as severe as in *fss*; normally the anterior somites are more regular. (D) *des* mutant; the first eight somites look normal; no somites are formed beyond this point. The somite phenotype of *aei* and *wit* is similar to that of *des.*

The single *wit* allele exhibits a somite defect that is very similar to that of *des* and *aei*. In addition *wit* mutant embryos have an increased number of primary neurons. A detailed account of this phenotype is given elsewhere (Jiang et al., 1996).

A morphological comparison of the three different phenotypes is given in Fig. 3. For all genes except *wit*, homozygous mutants survive to adulthood. There are no obvious motility defects, but sometimes these fish are slightly kinked. For *fss* mutants, survival rates were 75% of the level of the siblings in

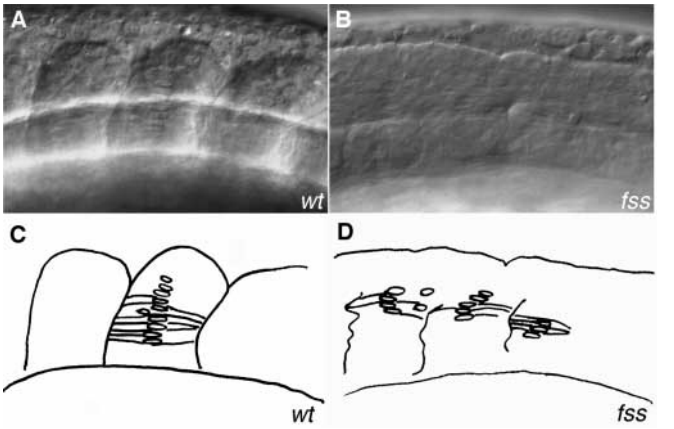

Fig. 4. Adaxial cells differentiate normally in *fss* mutants. (A) Somite 7 at the 13-somite stage, lateral view. At the middle of the somite a stack of flattened nuclei are visible, representing adaxial cells that have elongated and intercalated. (B) *fss* mutant at a similar region. Nuclear stacks can be seen, although they usually contain less cells. At the point where these cells seem to end a border is forming. (C,D) Schematic drawings of A and B, respectively.

Table 2. Effect of *fss* on muscle fiber length

Donor	Host	Measured fiber length (× expected)	Standard deviation (× expected)	Number of fibers measured
Wild type	Wild type	0.98	0.06	65
fss/*fss*	*fss*/*fss*	1.08	0.30	129

The relative length of the muscle fibers was determined as described in Materials and Methods.

an India/Tübingen hybrid background (33 versus 46 survivors out of 50).

We have raised homozygous mutants for *fss*, *bea*, *aei* and *des*. Embryos from crosses between homozygous females and heterozygous males did not have a stronger phenotype than embryos from the reciprocal cross, indicating that these genes do not have a maternal component that might be sufficient to allow somitogenesis in the anteriormost somites, or for the irregular boundaries seen in older mutant embryos.

To investigate further why the anteriormost somites are formed normally or relatively normal in mutants for *des*, *bea* and *aei*, we have made all three double mutant combinations between these three genes. No clear enhancement of the phenotype was found, indicating that these three genes do not have overlapping functions in the anterior part of the paraxial mesoderm.

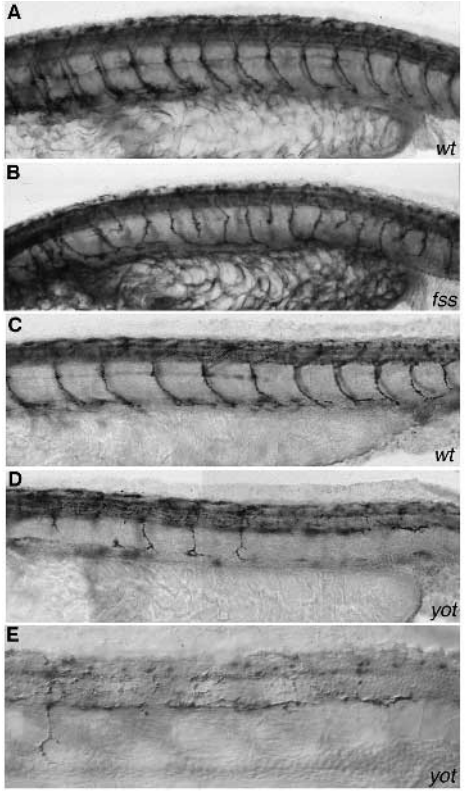

Fig. 5. Effect of *fss* and *yot* mutations on the primary motoneurons. (A,B) Znp-1 staining of wild-type sibling and *fss* mutant, respectively. The number of CaP axons is slightly increased in *fss* as compared to wild type, and some axons terminate prematurely. (C,D) Znp-1 staining of wild-type sibling and *yot* mutant, respectively. In C, CaP axons project down in every segment. In D only four CaP axons are projecting down. (E) Same *yot* mutant as in D; high power magnification of five myotomes anterior to the anus. One CaP axon projects into the muscle. In the other segments axons are running posteriorly, parallel to the neural tube.

Segmentation is irregular in *fss* mutants

At 72 hpf irregular somite boundaries are present in *fss* mutants (Fig. 2). Observation of live embryos around the 7-somite stage revealed that in *fss* mutants the elongation and intercalation of the adaxial cells proceeds normally, the only abnormality being the lack of somite boundaries. Soon after these movements have taken place, somite boundaries, although irregular, are formed (Fig. 4).

The average length of muscle fibers in homozygous *fss* embryos was estimated by transplanting fluorescently labeled mutant *fss* cells into mutant *fss* embryos before gastrulation. After 2 days of development labeled muscle fibers were measured and their lengths compared to those of muscle fibers at that particular A/P level in wild-type embryos. The length of individual fibers was variable, but on average only slightly longer in *fss* than in wild type (Table 2).

In the zebrafish, primary motoneurons are segmentally organized with each myotome having three primary motoneurons (Eisen et al., 1986), namely the Caudal, Middle and Rostral primary motoneurons (CaP, MiP and RoP, respectively). The CaP axon projects to the ventral myotome and the cell body of this neuron lies most posterior within each segment. Stainings with the znp-1 monoclonal antibody, which labels the axons of

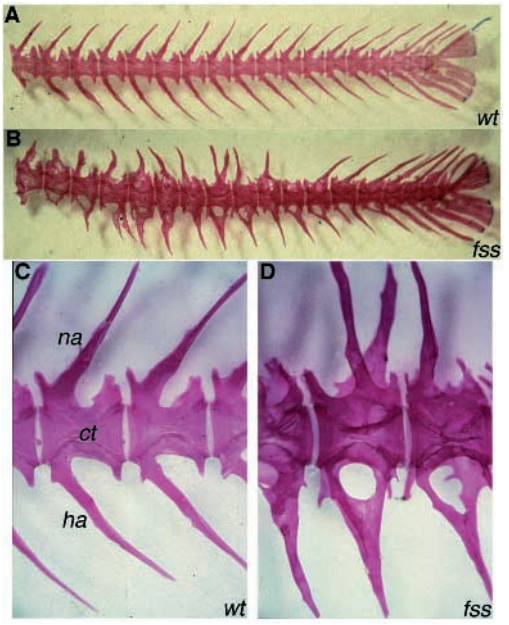

Fig. 6. Skeletal phenotype of *fss* mutants. Skeletal staining on adult wild type (A) and *fss* mutant (B) tail vertebrae. (C,D) Enlargement of wild-type and mutant vertebrae, respectively at a similar A/P level. *ct*; centrum, *na*; neural arch, *ha*; hemal arch. The centra in *fss* are almost normal in number and shape, but the length of individual centra is slightly more variable (B). In the wild type (C) every vertebra has four half arches, forming the neural arch on the dorsal side and the hemal arch on the ventral side. In *fss* mutants (B,D), arches grow out irregularly. For example, on the ventral side of the middle vertebra in D, three half arches are present, two at the left side which fuse, project to the right side, and fuse with the third half arch.

primary motoneurons (Trevarrow et al., 1990), reveals that in *fss* mutants CaP axons are present, but are more irregularly spaced, frequently truncate prematurely (10-20%, Fig. 5A,B) and sometimes bifurcate or join with a neighboring axon. MiP axons are present, and affected in a similar way. The effect on the RoP has not been analyzed so far. The motoneuron pattern seems to reflect the irregular segments in *fss* mutants at 28 hpf. Similar effects were found in *des*, *aei* and *bea* mutants.

Anteroposterior patterning within the somite might be affected

Whole-mount skeletal stainings of *fss* mutants were performed to see the effect of this mutation on the vertebral column. Tail vertebrae in the zebrafish consist of a centrum, the hemal arch ventrally, and the neural arch dorsally (Fig. 6C). In anterior vertebrae the hemal arches are replaced by ribs, the ribs and both arches being attached to the anterior of the centrum. In *fss* mutants the centra are relatively normal in number and shape but the hemal and neural arches seem to grow in an irregular fashion and from ectopic positions (Fig. 6B,D). Ribs are present, but are irregularly placed, frequently not attached to the centra, and sometimes bifurcate (data not shown).

A centrum can be divided into eight parts according to anterior/posterior, left/right and dorsal/ventral position. Thus in a wild-type fish there are four anterior positions with, and four posterior positions without a half hemal or neural arch. In the tail of two *fss* mutant fish, 15 vertebrae were analyzed for the position of these half arches. A position was scored as anterior if an outgrowth was present and posterior if not. In 35% of the positions a change in polarity was scored (82/232, 8 uncertain). If the polarity was completely randomized, a frequency of 50% would be expected, showing that some polarity is still present in the A/P axis of the centra.

Other markers for the A/P polarity within single somites are *myoD* (Fig. 7) and *snail1* (not shown). These markers are expressed in the most posterior somitic cells just after their formation, generating a clear striping pattern, and expression subsequently spreads to more anterior cells. In *fss* mutants this expression is affected such that, although there still seems to be a wave of expression that passes from anterior to posterior over the paraxial mesoderm as a whole, there are no detectable stripes of posterior somite expression (Fig. 7E,F). The expression still appears slightly patchy, suggesting that there might be some residual polarity within the somites. The adaxial cells, however, express *myoD* and *snail1* normally.

The vertebrae of the other three viable mutants, *bea*, *des* and *aei*, were analyzed in the same way as *fss*. In all three cases the frequency of positions with abnormal polarity was lower (±12%). In the tail region of *bea*, *des* and *aei*, *myoD* expression appears homogeneous, as in *fss* mutants, but more rostrally where somite formation is affected less severely (*bea*), or not at all (*des* and *aei*), a striping pattern is still visible. The transition from apparently normal to abnormal somite formation is shown by in situ hybridization with *myoD* on *des* and *bea* mutant embryos in Fig. 7A-D. We conclude that in *fss*, *bea*, *des* and *aei* mutants, A/P polarity within the individual somite is affected, although the A/P polarity in the paraxial mesoderm as a whole is still present.

Mutations affecting the horizontal myoseptum

The horizontal myoseptum is a fibrous sheet separating dorsal and ventral myotome (Fig. 8). In jawed fishes this septum is very prominent morphologically and in zebrafish it can be seen at 28 hpf. Such a separation between the dorsal and ventral axial muscles is not restricted to fish but is also found in the axial musculature of terrestrial vertebrates.

Mutants in six genes (*you*-type genes), *you* (*you*), *you-too* (*yot*), *sonic-you* (*syu*), *chameleon* (*con*), *u-boot* (*ubo*) and *choker*(*cho*) have no or a reduced horizontal myoseptum, but a morphologically normal notochord. Many of these genes were given names with 'you' to refer to the U-shape of the somites in these mutants. Both formation of vertical somite boundaries and the notochord are unaffected in *you*-type mutants. A short phenotypic comparison between *you*-type mutants is given in Table 3.

In mutants for *yot*, *syu*, *you* and *con* the floorplate is indistinct at 20 hpf, but at 72 hpf it is morphologically distinguishable. *sonic hedgehog* (*shh*) expression in the floorplate is normal in *yot*, *you* and *con* mutants, but in *syu shh* expression is reduced at 24 hpf (data not shown; Krauss et al., 1993; Brand et al., 1996). In *you*-type mutants motility in response to touch is variably reduced; *yot* and *con* mutants are severely affected,

Table 3. Phenotypic comparison of *you*-type mutants

Gene name	Number of alleles	Strongest allele	Horizontal myoseptum	Motility	Circulation	Other morphological phenotypes
you-too (*yot*)	2	*ty119*	–	–	–	Eyes closer together, retinal axons project ipsilaterally
chameleon (*con*)	4	*tm15a*	–	–	–	Eyes closer together, small pectoral fins, reduced outgrowth of retinal axons
you (*you*)	3	*ty97*	+/–	+/–	+/–	*tm146c* and *tz310b* are viable
sonic you (*syu*)	1	*tq252*	+/–	+/–	+/–	Small pectoral fins
u-boot (*ubo*)	1	*tp39*	–	+/–	+	Irregular fin edges, reduced no.of pigment cells
choker (*cho*)	1	*tm26*	+/–	+	+	Abnormal pigment pattern, dented hindbrain

For the overview of the phenotypes the strongest allele has been taken.
+ normal; +/– weakly affected; – strongly affected.

Table 4. Requirement for *yot* and *ubo*

Gene	Mutants with clone in tail	Muscle (cases)	Notochord/ floorplate (cases)	Neural tube (cases)	Rescue (cases)	Position of rescuing clone
ubo	21	16	4	8	4	Myoseptal muscle
yot	54	39	14	23	13	Myoseptal muscle

whereas *ubo*, *you*^{tm146c} and *you*^{tz310b} mutants have a very mild phenotype. Mutations in three genes also show fin defects; in *con* and *syu* the pectoral fins are variably reduced, and in *ubo* all fin edges are indented irregularly (van Eeden et al., 1996). Mutants for *you*, *yot*, *syu* and *con* have a circulation defect, probably because formation of a functional dorsal aorta is delayed or does not occur at all (Chen et al., 1996). In *ubo* and *cho* formation of the dorsal aorta is unaffected. Finally, in *yot* and *con* mutants the spacing of the eyes is variably reduced, suggesting a variable reduction in ventral brain structures. In addition, these mutants have defects in the pathfinding or outgrowth of retinal axons. Details of the neural phenotypes of *yot* and *con* are given elsewhere (Brand et al., 1996; Karlstrom et al., 1996).

All *you*-type mutations are lethal, except for *you*^{tm146c} and *you*^{tz310b}. Lethality is probably due to the circulation defect or to lack of an air-filled swimbladder. Homozygous *you*^{tm146c} or *you*^{tz310b} fish do not have a detectable adult phenotype, and do not show maternal effects. Skeletal staining of homozygous fish did not reveal obvious defects.

Mutations in another five genes, *dino* (*din*), *doc* (*doc*), *momo* (*mom*), *no tail* (*ntl*) and *floating head* (*flh*), affect notochord formation and these mutants also lack the horizontal myoseptum (Halpern et al., 1993; Talbot et al., 1995; Hammerschmidt et al., 1996a; Odenthal et al., 1996). *no tail* and *doc* mutants have an undeveloped notochord in the trunk, and form somites lacking the myoseptum. *flh* mutants lack a notochord, and notochord precursor cells are absent. Somites from the left and the right side fuse under the neural tube and these also lack the horizontal myoseptum. *momo* lacks the notochord in the trunk and *dino* lacks the notochord in the tail. In these two mutants the somite phenotype is restricted to the region where the notochord is missing, but otherwise it is similar to the *flh* phenotype. There is evidence that the lack of the horizontal myoseptum in these mutants is caused by the lack of the notochord (Halpern et al., 1993; Odenthal et al., 1996). In addition to these mutants, six more genes have been defined that are necessary for later notochord development. Somites are condensed in mutants of these genes but the horizontal myoseptum and the muscle pioneers are present (Table 1). Detailed descriptions of mutants with defects in the notochord are given elsewhere (Hammerschmidt et al., 1996a; Odenthal et al., 1996).

In *you*-type mutants adaxial cells and muscle pioneers are affected

Muscle pioneers are a subset of muscle cells in the region of the horizontal myoseptum. They can be recognized morphologically at 24 hpf, by their early formation of myofibrils. In wild-type embryos 2-6 nuclei of these cells per segment express the Engrailed antigen, which is recognized by the 4D9 monoclonal antibody (Hatta et al., 1991). Stainings with 4D9 monoclonal antibody on *yot*, the mutation with the strongest phenotype, shows that Engrailed-expressing cells are absent from the somites (Fig. 9A,B). Engrailed expression in the midbrain-hindbrain boundary is normal (Fig. 9A,B).

During somite stages, *myoD* is expressed at high levels in the adaxial cells. In situ hybridization with *myoD* on *yot* mutant embryos shows that expression is reduced at the tailbud stage (data not shown). At 15 somites no expression is detectable in the adaxial cells (Fig. 9E,G), but the expression in the posterior part of the somites, which comes up immediately after their formation, is initially normal. More anteriorly, however, *myoD* expression is reduced prematurely, and the anterior part of the somite never expresses *myoD* at normal levels (Fig. 9E,G). *yot* has a dominant effect on *myoD* expression, since an unsorted mixture of *yot* mutants and siblings revealed an approximate 1:2:1 (7:19:7) ratio in embryos with high, low or no detectable *myoD* expression in the adaxial cells, respectively (Fig. 9E,F,G). In situ hybridization with *snail1* (Hammerschmidt and Nusslein-Volhard, 1993; Thisse et al., 1993) on *yot* mutants indicates that the adaxial expression of this gene is affected in a similar way (data not shown). The morphology of adaxial cells is also affected in *yot* mutants. At the 2- to 3-somite stage, before the abnormal somite shape appears, *yot* mutants can be recognized by the morphology of the adaxial cells.

The reduction in *myoD* expression is less pronounced in *syu*, *con* and *you* mutants, especially in the tailbud where some adaxial expression is present. No difference was detected between *ubo* mutants and wild-type siblings, suggesting that this gene is not required to establish adaxial expression of *myoD*. In *syu*, *con* and mutants with the strongest *you* allele (*ty97*), segments rarely have a few Engrailed-positive cells (data not shown). Engrailed staining of a mutant with the weakest phenotype, *you*^{tm146c}, showed approximately one weakly staining nucleus per segment (Fig. 9C,D). *ubo* mutants show reduced Engrailed staining in all segments, and in *cho* mutants muscle pioneers are present, as judged by their early striation.

The somite phenotype of the five mutants that also affect notochord formation is very similar to the phenotype of *yot* mutants. Engrailed expression is not detectable in the somites and adaxial expression of *myoD* is severely reduced (Halpern et al., 1993; Weinberg et al., 1996; Odenthal et al., 1996).

In *you*-type mutants primary motoneuron patterning is abnormal

The motility defect of *you*-type mutants suggested that there might be an effect on motoneurons as well. Primary motoneurons were stained by the znp-1 monoclonal antibody in most *you*-type mutants. In *yot* mutants, CaP and MiP axons, which normally project to the ventral and dorsal myotome, respectively, are absent in many segments; instead, axons can be seen running along the neural tube (Fig. 5C,D,E). Staining with a monoclonal antibody against acetylated tubulin shows that

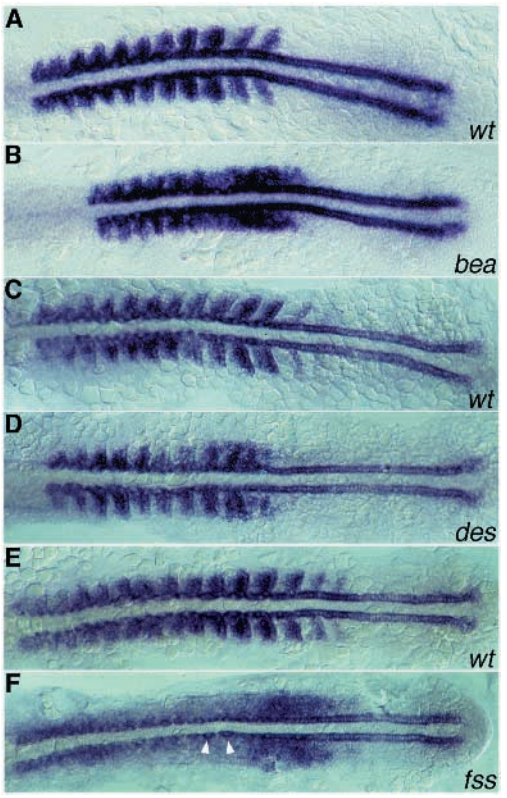

Fig. 7. *myoD* expression in *bea*, *des* and *fss* mutants. All mutants show normal adaxial expression. Wild-type sibling (A) and *bea* mutant (B), 12-somite stage. In the wild type, striped expression of *myoD* is visible in the posterior part of every somite. In *bea*, striped expression is visible in the anteriormost but not in more posterior paraxial mesoderm. (C,D) Wild-type sibling and *des* mutant, respectively, 13-somite stage. In *des*, expression in the posterior cells of the first seven somites is normal (D). More posteriorly in the paraxial mesoderm, the stripes change into irregular patches. (E,F) Wild-type sibling and *fss* mutant, respectively. In *fss* the striped pattern of expression is no longer seen. Note the elongated adaxial cells just anterior to the region where *myoD* reaches the highest level of expression (arrowheads).

early axonal tracts in the spinal cord are normal (data not shown). This indicates that the *yot* mutation does not have a general effect on the early axonal tracts, and suggests that the effect on the motoneurons is specific. At present it is not clear, however, if the primary motoneuron phenotype is due to abnormal somite patterning or to a defect in the motoneurons themselves.

The primary motoneuron phenotype is also detectable in *you*, *syu* and *con* mutants, but the frequency of abnormally projecting axons is lower. Mutations in the *flh* gene show a similar primary motoneuron defect: axons do not enter the paraxial mesoderm, but instead truncate or project along the neural tube (J.O., unpublished; Talbot et al., 1995).

you-too and *u-boot* functions are required in the paraxial mesoderm

Because there is evidence that the myoseptum is induced by the notochord (see Discussion), it was interesting to determine whether the gene products for the *you*-type genes

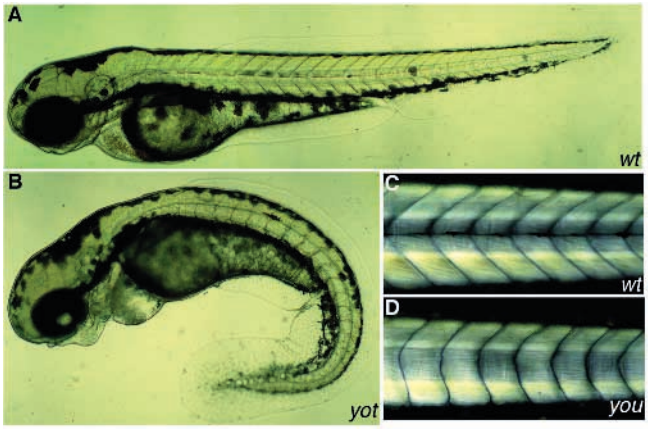

Fig. 8. Overview of the phenotype of a wild-type sibling (A) and a *yot* mutant (B) at 72 hpf. The horizontal myoseptum is visible in A as a black line running through the middle of the myotome. In B this line is absent. *yot* mutants have a variably curved tail. The swelling of the heart cavity is probably a consequence of the reduced circulation in the tail. Branchial arches are displaced ventrally in *yot*. This is observed in all mutants where the eyes lie closer together. (C,D) Dark-field image of wild-type sibling (C) and a *you* mutant (D) at 120 hpf. Myotomes in *you* are U-shaped instead of V-shaped, and lack the horizontal myoseptum.

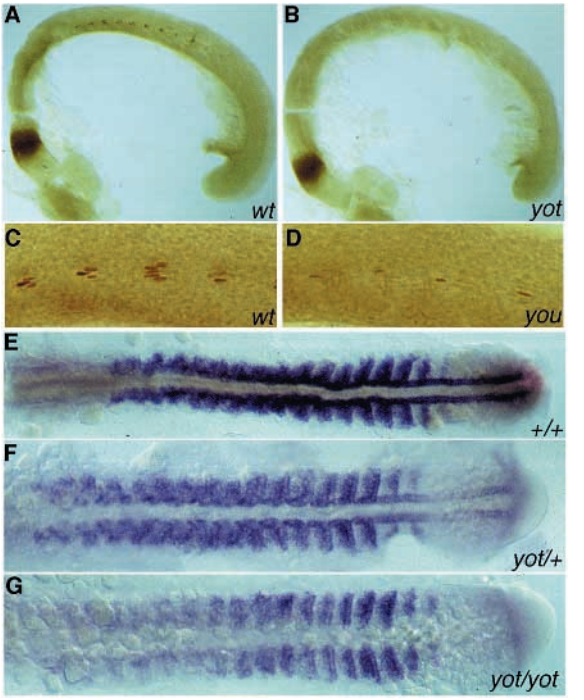

Fig. 9. (A) Engrailed (4D9) staining of a 21-somite stage wild-type embryo. Staining is visible at the midbrain-hindbrain boundary and in the somites. In every somite about 2-5 nuclei, representing the muscle pioneers, are stained. In a *yot* mutant at the same stage (B), staining of the muscle pioneers is absent. (C) 4D9 staining of a wild-type sibling and (D) the weakest *you*-type mutant, you^{tm146c}. Approximately one Engrailed-positive cell is present per segment. (E-G) *myoD*-staining of a wild type, a *yot* mutant and a putative *yot*/+ embryo at the 17-somite stage, respectively. In the homozygous *yot* mutant, adaxial staining is absent and the staining in the rest of the paraxial mesoderm is reduced (G). In the *yot* heterozygous embryo, expression in the adaxial cells is reduced, especially in the presomitic mesoderm, anterior to the tailbud (F).

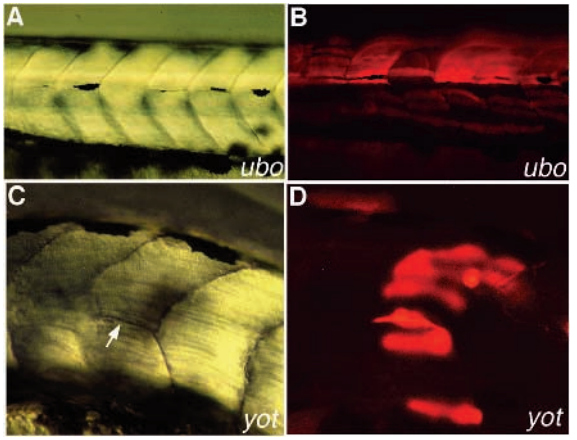

Fig. 10. Rescue of the horizontal myoseptum phenotype in *ubo* and *yot* mutants by cell transplantation. Rhodamine dextran-labeled wild-type cells were transplanted into *ubo* or *yot* mutants at mid-late blastula stage. Embryos were scored for rescue of the myoseptum at 48-72 hpf. (A) Normarski picture of five segments with a rescued myoseptum in an *ubo* mutant. Note that the segment in the middle does not show any rescue. (B) Picture of the same embryo showing the fluorescently labeled wild type cells. In all segments with rescue, labeled cells are present in the myoseptum. In the myotome that did not show rescue only muscle fibers, that are slightly above or far below the myoseptum, are labeled. (C) Normarski picture of a *yot* mutant with a single rescued horizontal myoseptum (arrow). (D) Picture of the same region showing a brightly labeled wild-type cell at the position where the myoseptum formed.

are required in the notochord or in the somites. Cell transplantations from wild-type embryos to *yot* and *ubo* mutant embryos were carried out. A small number of fluorescently labeled wild-type cells were transplanted into mutant embryos before the onset of gastrulation (Ho and Kane, 1990). The resulting genetic mosaics were analyzed morphologically. In both mutants a myoseptum is only formed if wild-type donor cells are present at the level of the horizontal myoseptum (Fig. 10). A large number of wild-type cells in the notochord, in contrast, never leads to rescue of a myoseptum (Table 4). In *syu*, *you* and *con* mutants the incomplete penetrance of the myoseptum phenotype did not allow such an analysis.

Other mutations affecting the paraxial mesoderm

A number of mutations were found to affect formation of the entire paraxial mesoderm and thus also to influence somitogenesis. Mutations in five genes result in a variable dorsalization of the embryo and show a ventral expansion of the paraxial mesoderm (Mullins et al., 1996). We have also identified two new alleles of *spadetail*, which causes a severe reduction of cells in the paraxial mesoderm (Ho and Kane, 1990; Hammerschmidt et al., 1996a). Mutations in *dino* cause partial ventralization of the embryo; in addition to the effect on posterior somite patterning as described, this mutation also leads to a reduced amount of paraxial mesoderm in the anterior region (Hammerschmidt et al., 1996b). 15 genes are required for formation of striated muscle in the myotomes and elsewhere in the embryo. A detailed description of these genes is given elsewhere (Granato et al., 1996).

DISCUSSION

Anteroposterior polarity within somites is affected in *fss*-type mutants

We have isolated mutations in five genes in our zygotic screen that are involved specifically in somite formation. Embryos mutant for these genes fail to form epithelial somites properly. Eventually, however, irregular segment boundaries become visible in the myotomes. Mutations in *fss* show this defect over the entire length of the paraxial mesoderm, while *bea* mutants have a weaker defect in the anterior paraxial mesoderm but look similar to *fss* posteriorly. Mutations in the three other genes (*aei*, *des* and *wit*) only cause this defect in the posterior region of the paraxial mesoderm.

Skeletal stainings and *myoD* expression indicate that anteroposterior polarity within individual somites is affected. The effect of *fss* is stronger than that of *bea*, *des* or *aei*, as judged by the skeletal phenotype. Although antero-posterior patterning within single somites is affected, the wave of differentiation that passes from anterior to posterior over the paraxial mesoderm as a whole seems to be relatively normal in *fss*-type mutants. The observed phenotype can be explained quite well by the ‘clock and wavefront’ model that has been proposed

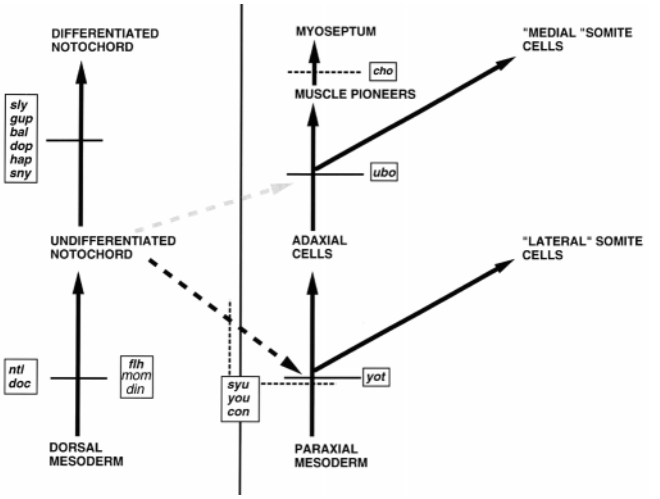

Fig. 11. Schematic representation of the main events in specification of the different structures in the myotome, and the interactions with the notochord that are thought to take place. Mutations in five genes (*ntl, doc, fln, mom, din*) affect the early specification of the notochord primordium; the notochord primordium signals at this stage to the adaxial cells to induce and maintain their adaxial character (broken black arrow). *con, you* and *syu* might be involved in the generation or the reception of this signal; *yot* is also involved in this signaling process and required in the paraxial mesoderm. Mutations in six genes (*sly, gup, bal, dop, hap, sny*) affect further differentiation of the notochord without affecting this signal. How the muscle pioneers are selected from the adaxial cells is not known. Either a high level or a prolonged exposure to the signal (gray broken arrow) might be important. *ubo* is required in the paraxial mesoderm and likely to be involved in the specification of the muscle pioneers from the adaxial cells and not in the formation of the adaxial cells themselves, since *myoD* expression is normal in this mutant. The position of *cho* is not clear since it has not been analyzed with molecular markers. It has a very weak myoseptum phenotype and muscle pioneers are present, and therefore it is likely to function downstream of the other *you*-type genes.

(Cooke and Zeeman, 1976). This model assumes that somite formation is the result of two processes, the first a differentiation 'wavefront' that moves in a smooth way over the paraxial tissue from anterior to posterior, and the second a 'clock' that periodically promotes and blocks the progression of this wavefront. The genes described above might be interpreted as essential for the clock process only.

Irregular segmentation in *fss*-type mutants

At first glance it is surprising that a mutant like *fss*, which has abnormal somite formation, survives to adulthood. The following observations might account for this. Differentiation in the mediolateral plane is only weakly affected; adaxial cells, muscle, muscle pioneers, horizontal myoseptum and vertebrae are present. Primary motoneurons have a segmental arrangement. The irregular segmentation seen later in development results in only slightly more variable muscle fiber length and an apparently normal motility.

In *fss* mutants the adaxial cells flatten and intercalate as in wild type, resulting in irregular stacks of these cells. These stacks are quite similar in length to the length of a somite. (Fig. 7F, arrowheads). Boundaries form where two stacks of adaxial cells meet (Fig. 4). Thus adaxial cells may play a role in forming the irregular boundaries between the myotomes seen at later stages. In fact, in double mutants between *fss* and *yot*, where adaxial cells are absent, most of the irregular boundaries are lost, resulting in embryos with almost unsegmented paraxial mesoderm (F.v.E, unpublished).

Somite formation appears in all vertebrates as a continuous repetition of the same process, and hence a gene required for somite formation might be expected to be required for the formation of all somites. It was therefore surprising that only *fss* shows such a requirement, mutations in *des*, *aei* and *wit* having defects only in the posterior paraxial mesoderm. The phenotype of *bea* is stronger in posterior than anterior somites. It is possible that there is a stronger requirement for these genes in the posterior versus the anterior paraxial mesoderm, and that all alleles that we isolated are weak alleles. An argument against this possibility is that all alleles we isolated for a particular gene (10 alleles for *des*) have the same strength. Furthermore, there are no further mutants in our collection that could be candidates for a null-phenotype of these genes. However, we cannot rule out that these candidates were missed in our screen.

There may be other reasons why the first few somites form normally in *bea, aei, des* and *wit.* At present we can say that there is no indication of a maternal contribution of *bea, aei,* and *des*, which might suffice for somitogenesis in the anteriormost somites. Furthermore, we have made all possible double mutant combinations between *aei, bea* and *des,* but did not detect an enhancement of the phenotype, suggesting that these three genes do not have overlapping functions; overlap with *wit* could still be possible, however. We speculate that other, maternally expressed genes may replace the function of *aei, des* and possibly *bea* in the early embryo. The products of such genes might become exhausted early in somitogenesis, their function taken over by the three zygotic genes described here.

At present there are no cloned genes that could be candidates for *fss*, *bea*, *aei* and *des*. Mice mutant for *fibronectin*, *integrin alpha5*, *FGFr1*, *Wnt-3a* and the *Notch1* gene show additional morphological defects, and always result in lethality. The neural phenotype of *wit* mutants suggests that a more general factor, involved in neurogenesis, is affected (Jiang et al., 1996).

Mutations affecting the horizontal myoseptum may define a signaling pathway

We have identified 11 genes that affect the formation of the horizontal myoseptum. Six of these, *you*, *you-too*, *chameleon*, *sonic-you, u-boot* and *choker*, have a normal notochord. The other five also show defects in the notochord, and these are discussed elsewhere (Hammerschmidt et al., 1996b; Odenthal et al., 1996). Transplantation experiments in *ntl* and *doc* mutant embryos have shown that the notochord is required for induction of the horizontal myoseptum and the muscle pioneers (Halpern et al., 1993; Odenthal et al., 1996). If the muscle pioneers are required for myoseptum formation, mutants that lack the horizontal myoseptum might lack the muscle pioneers, and thus define genes that are involved in a signaling pathway from the notochord to the somites. The *you*-type mutants might define such genes since they exhibit defects that are very similar to those observed in mutants lacking the notochord. In both groups, adaxial cells, muscle pioneers, horizontal myoseptum and motoneuron axon projection are affected.

Although *you*-type mutants have many aspects of their phenotypes in common with mutants of genes required for notochord specification, there are also differences. For example the *yot* and *con* mutations have effects on the spacing of the eyes and on retinal axon projection and outgrowth (Brand et al., 1996; Karlstrom et al., 1996), indicating that the function of these genes is also required in regions anterior to the notochord.

There are indications that Ntl expression has to be maintained in the notochord in order to induce Engrailed expression in the somites (Odenthal et al., 1996). We found that Ntl expression is normal in the notochord of *you*-type mutants, suggesting that the defect in *you*-type mutants lies downstream of Ntl. We have not yet been able to identify a *you*-type gene that might encode an inducing molecule required in the notochord instead of the paraxial mesoderm. We have shown by transplanting wild-type cells into *yot* and *ubo* mutants that these genes are required in the paraxial mesoderm for the formation of the horizontal myoseptum. This leaves three good candidate genes, *con*, *you* and *syu*, which might encode the inducer. A preliminary model of where the different *you*-type genes might act is shown in Fig. 11.

It is not clear how the identified signaling pathway can be compared to the patterning signals from the notochord, which have been defined in the chick. Only *syu* has an effect on floorplate formation, as seen by *sonic hedgehog* (*shh*, Krauss et al., 1993) expression. So far *yot* and *con* mutants have been shown to have a reduced number of secondary motoneurons (Brand et al, 1996), which can be induced by both the notochord and the floorplate. Other *you*-type mutants have not been tested yet. We have not yet analyzed the effect of the *you*-type mutants on the induction of the sclerotome. In zebrafish, the sclerotome is a rather small group of cells and the vertebral column develops long after day 6 of development. Our screening procedure did not allow isolation of mutants with defects restricted to the vertebrae.

sonic hedgehog is expressed in both floorplate and

notochord and is thought to induce structures in neighboring tissues. Injection of *shh* mRNA into zebrafish embryos causes an increase of adaxial *myoD* expression (Weinberg et al., 1996), the opposite effect to the reduction of adaxial *myoD*, seen in most *you*-type mutants. *shh* is therefore a good candidate for either *con*, *you* or *syu*. We are currently testing these mutations for linkage to this gene.

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