Differential induction of four msx homeobox genes during fin development and regeneration in zebrafish

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SUMMARY

To study the genetic regulation of growth control and pattern formation during fin development and regeneration, we have analysed the expression of four homeobox genes, msxA, msxB, msxC and msxD in zebrafish fins. The median fin fold, which gives rise to the unpaired fins, expresses these four msx genes during development. Transcripts of the genes are also present in cells of the presumptive pectoral fin buds. The most distal cells, the apical ectodermal ridge of the paired fins and the cleft and flanking cells of the median fin fold express all these msx genes with the exception of msxC. Mesenchymal cells underlying the most distal cells express all four genes. Expression of the msx genes in the fin fold and fin buds is transient and, by 3 days after fertilization, msx expression in the median fin fold falls below levels detectable by in situ hybridization. Although the fins of adult zebrafish normally have levels of msx transcripts undetectable by in situ hybridization, expression of all four genes is strongly induced during regeneration of both paired and unpaired fins. Induction of msx gene expression in regenerating caudal fins occurs as early as 30 hours postampulation. As the blastema forms, the levels of expression increase and reach a maximum between the third and fifth days. Then, msx expression progressively declines and disappears by day 12 when the caudal fin has grown back to its normal size. In the regenerating fin, the blastema cells that develop at the tip of each fin ray express msxB and msxC. Cells of the overlying epithelium express msxA and msxD, but do not express msxB or msxC. Amputations at various levels along the proximodistal axis of the fin suggest that msxB expression depends upon the position of the blastema, with cells of the rapidly proliferating proximal blastema expressing higher levels than the cells of the less rapidly proliferating distal blastema. Expression of msxC and msxD is independent of the position of the blastema cell along this axis. Our results suggest distinct roles for each of the four msx genes during fin development and regeneration and differential regulation of their expression.

Key words: blastema, Danio rerio, developmental patterning, limb regeneration

INTRODUCTION

The genetic regulation of pattern formation in the vertebrate limb is beginning to be understood (Tabin, 1991; Tickle, 1991). The molecular characterization of Drosophila developmental control genes and their vertebrate cognates, notably homeobox genes, members of a large class of transcriptional regulators, permitted the identification of genes that may control limb outgrowth and patterning. Of the homeodomain-containing proteins expressed at various times and positions during limb development, the Hox and Msx families have been most extensively studied. The Hox genes, related in sequence to the Drosophila homeotic genes, are organized into the HoxA, HoxB, HoxC and HoxD complexes, each containing about ten genes (Duboule and Dollé, 1989; Graham et al., 1989; Kappen et al., 1989; Kessel and Gruss, 1990). During limb development, mesenchymal cells coordinately express some members of the HoxA and HoxD complexes in a spatial pattern within the limb bud that corresponds to their chromosomal positions within their respective gene complex (Dollé et al., 1989; Izpisua-Belmonte et al., 1991; Oliver et al., 1989). Experimental manipulation of Hox gene expression in the limbs suggests that these genes provide positional information during limb development (Dollé et al., 1993; Izpisua-Belmonte et al., 1991; Morgan et al., 1992; Nohno et al., 1991).

Vertebrate genes with homeoboxes related in sequence to that of the Drosophila muscle segment homeobox (msh) gene are also thought to play an important role in limb development. The two best studied vertebrate members of this gene family (termed Msx) are the mouse Msxl and Msx2 genes (Hill et al., 1989; Monaghan et al., 1991; Robert et al., 1989) and their cognates in chicken (Coelho et al., 1991a, b; Robert et al., 1991). In contrast to the Hox genes, Msx genes are not organized in clusters (Monaghan et al., 1991). Msxl and Msx2 could be involved in epithelial-mesenchymal interactions that link growth of a tissue to its patterning (Muneoka and Sassoon, 1992). In the developing limb of 11.5 day mouse embryos, Msxl expression correlates spatially with the progress zone...
whereas cells of the apical ectodermal ridge (AER) express Msx2. The functions of Msx1 and Msx2 in epithelial-mesenchymal interactions were investigated with chicken-mouse interspecies grafts (Davidson et al., 1991) and in chicken by removal of the AER (Ros et al., 1992), by grafting a second AER (Robert et al., 1991) and by analysis of Msx1 expression in limbless mutants (Coelho et al., 1991b; Robert et al., 1991).

Results of these studies suggest that Msx1 expression in the progress zone may depend upon a signal provided by the AER, whereas Msx2 could be involved in some aspect of AER function. Msx1 may regulate the proliferative and undifferentiated properties of cells of the progress zone, as suggested by the differentiation-defective phenotype of myogenic cells in which Msx1 expression has been ectopically induced (Song et al., 1992).

The role of homeobox-containing genes in the development or regeneration of fish fins, the evolutionary relatives of tetrapod limbs, has not yet been reported. Analysis of regulatory gene functions in fish fins and comparisons with cognate genes in tetrapod limbs should provide a basis for understanding the evolution of patterning during limb development. To analyse fin growth, we have studied the expression of zebrafish msx genes. The zebrafish genome contains at least five msx genes (Akimenko et al., 1991; Ekker et al., 1992a; Holland 1991; E. Weinberg, personal communication). We have previously reported our characterization of two of these genes, msxC and msxD (previously called mshC and mshD, respectively), including their expression in the developing inner ear (Ekker et al., 1992a). We now report the isolation of the zebrafish msxA and msxB genes and the expression patterns of msxA, msxB, msxC and msxD during fin development and regeneration. Preliminary analysis of the fifth zebrafish msx gene, msxE (from E. Weinberg), indicates that cells of the pectoral fins express this gene only weakly; its transcripts are undetectable in the median fin fold and in any of the fins during regeneration. For this reason, msxE is omitted from the present report.

Our results demonstrate that various cell types express distinct combinations of msx genes in a specific time course during development and that, although msx expression is normally undetectable in the adult fin by in situ hybridization, the four msx genes are reinduced during regeneration in a pattern that differs only slightly from their initial expression during embryonic development. The differential regulation of these four msx genes during fin development and regeneration argues for distinct roles for each of the genes in fin growth.

MATERIALS AND METHODS

Animals
Embryos were obtained from the Oregon AB line and were staged according to hours (h) and days postfertilization. Adults and embryos were maintained at 28.5°C using standard methods (Westerfield, 1993). Some of the embryos were raised in 0.2 mM phenylthiocarbamideto inhibit pigment formation (Hyatt et al., 1992).

Fin amputation
Adult zebrafish of at least 10 weeks of age were anesthetized by immersion in water containing 0.17 mg/ml tricaine (ethyl-m-amino benzoate; Westerfield, 1993). The fins were then amputated using a scalpel. Fish were returned to their tanks and, after various times of regeneration, the fins were cut off again, following the same procedure and immediately fixed in a phosphate buffer saline solution containing 4% paraformaldehyde (Westerfield, 1993).

In all regeneration experiments, we cut the fins in a consistent manner using branch points of the lepidotrichia as references. For the temporal analysis of the expression of the msx genes, the fins were cut proximal to the first branch point. In experiments where the fins were cut at three different levels, the ‘proximal’ cut was proximal to the first branch point, the intermediate cut was localized between the first and the second branch points and the ‘distal’ cut was a few lepidotrichia segments after the second branch point.

Library screening
A λ EMBL-4 genomic library and a λgt-10 cDNA library prepared from 2 day zebrafish embryo mRNA (kindly provided by A. Fjose) were screened under reduced stringency conditions (55°C; 3.5x SSC) with the 200 bp Spht-PsrI fragment of the mouse Hox-7/b12 plasmid (Robert et al., 1989). This probe includes the entire Msx1 homeobox sequence together with 20 bp 5' of the homeobox. Four recombinant genomic clones and eight recombinant cDNA clones were isolated. Three of the four genomic clones contained the msxA gene and five cDNA clones contained the msxB gene.

DNA sequencing
Restriction fragments of the cDNA inserts or of the genomic clones were isolated and subcloned into Bluescript phagemids (Stratagene, San Diego, CA). Single strand templates were prepared from these phagemids according to the manufacturer’s instructions. DNA sequencing was performed either on single-stranded or double-stranded templates by the dideoxy-termination method (Sanger et al., 1977) using Sequenase (USB, Inc.), according to the manufacturer’s directions.

Gene expression analysis
In situ hybridization on whole-mount embryos or on whole-mount fins was performed as previously described (Püschel et al., 1992) with minor modifications (Akimenko et al., 1994). The probes consisted of antisense RNA corresponding to the longest available cDNA for msxB, msxC and msxD (Fig. 2; Ekker et al., 1992a; probe sizes: 1234 nucleotides (nt), 2024 nt and 1145 nt, respectively). For msxA, the probe consisted in a 641 bp PCR fragment amplified from cDNA prepared from a 20-28 h library (see below and Fig. 1).

RESULTS

Isolation of the zebrafish msxA and msxB genes
We first isolated the zebrafish msxA gene from a genomic library as three recombinant phage using a probe corresponding to the homebox of the mouse Msx1 gene (Akimenko et al., 1991). Analysis of the genomic clones (not shown) indicates that the zebrafish msxA gene contains two exons separated by an intron of approximately 500 bp. The second exon contains the homeobox (Fig. 1). To localize the first exon of msxA in the genomic clones, we used a probe derived from the 5’ terminal coding region of msxD (Ekker et al., 1992a) that includes sequences coding for a decapeptide conserved among all vertebrate Msx genes (Ekker et al., 1992a) We sequenced restriction fragments of the genomic clone hybridizing to the msxD probe and found an open reading frame encoding the conserved decapeptide. We used this sequence information to design PCR primers and subsequently amplified a cDNA for the msxA gene by PCR using DNA prepared from a cDNA library of 20-28 h embryos (kindly provided by David Grunwald). The two oligonucleotide primers were derived
genes and zebrafish fin regeneration

In the long open reading frame of 266 amino acid residues (aa) that includes the home-odomain, two possible initiation codons are located upstream of the conserved decapeptide. It is difficult to determine which one serves as a translation start site because neither matches the eukaryotic initiation codon consensus sequence (Kozak, 1987). The predicted sizes of the proteins would be either 245 or 257 aa.

We isolated the \textit{msxB} gene as five cDNA clones, the longest corresponding to a transcript of 1234 nucleotides (Fig. 2). It encodes a homeodomain that shares 55 out of 60 aa with that of the mouse Msx1 (Akimenko et al., 1991). There is only one ATG codon upstream of the conserved decapeptide (at nucleotide position 75). This start site would produce a predicted protein of 229 aa. We cannot be sure that this ATG serves as the initiation codon, because there is no stop codon upstream. However, northern blot analysis with a probe specific for \textit{msxB} detects a transcript in RNA from 2 day embryos with an approximate size of 1400 nucleotides (not shown). This suggests that the longest cDNA clone is nearly complete.

\textbf{Msx expression during development of paired fins}

Zebrafish pectoral fin buds form on both sides of the trunk at about 28 h. However, expression of \textit{msxA}, \textit{msxB}, \textit{msxC} and \textit{msxD} genes was first detected at this stage. The \textit{msxA} probe used for in situ hybridization consisted of the 641 bp PCR fragment delineated by the two oligonucleotide primers used for PCR amplification (underlined). The sequence accession number for \textit{msxA} is U16310.
msxD in the presumptive pectoral fin buds starts earlier and, by 24 h, two ovoid patches of cells on the surface of the yolk lateral to the anterior somites express each of the four genes (Fig. 3A). As the pectoral fins develop, cells of the apical ectodermal ridge (AER) of the pectoral fins (Wood, 1982) express msxA, msxB, and msxD (Fig. 3B,C,E) but not msxC, whereas cells that occupy an immediately more proximal position, closer to the base of the fin, express all four msx genes. msxA, msxB, and msxD are uniformly expressed in these cells (Fig. 3B,C,E), whereas the msxC hybridization signal is stronger along the anterior edge of the fin bud (Fig. 3D). We also observe weak hybridization signals for msxA and msxD, but not for msxB and msxC throughout the surface of the fin bud (Fig. 3B,E). We previously reported differential expression of three members of the dlx homeobox gene family in the anterior and posterior regions of the AER (Akimenko et al., 1994). Comparisons of the in situ hybridization patterns of msx and dlx genes (not shown) suggest that regions of msx-expression include the regions that express dlx genes, as well as cells closer to the base of the fin buds that do not express dlx.

Pelvic fins form much later in zebrafish development. The first observable pelvic fin buds about 3 to 4 weeks after fertilization and, as in the pectoral fin buds, cells on the edges of the developing pelvic fin buds express at least msxB, msxC and msxD (not shown).

**Msx expression during development of the median fin fold**

The unpaired fins, dorsal, caudal and anal, originate from a single embryonic median fin fold, which extends along the dorsal and ventral midlines of the trunk and the tail. The median fin fold is visible by about 20 h. Msx transcripts appear in the tail region before formation of the fin fold beginning with msxD. Cells at the caudal tip of the embryo are the first to express msx genes in the tail with msxD transcripts appearing as early as 12 h (not shown). By 16 h, a few cells on the median dorsal and ventral surfaces of the tail bud express msxB (Fig. 4A) and msxD (not shown), whereas deep cells of the tail bud express msxC (data not shown). By 24 h, cells in the median fin fold express msxB uniformly (Fig. 4B). We observe similar patterns with the other three msx genes (not shown) although msxC expression is weaker and more difficult to see in the dorsal aspect of the fold due to strong expression of this gene in the dorsal neuroectoderm (Ekker et al., 1992a).

By 30 h, differences in the expression patterns of the four genes in the median fin fold are more prominent and reflect those observed in the pectoral fin buds. Thus, the most distal cells of the fold, which include cleft cells and flanking cells (Dane and Tucker, 1985), express msxA (Fig. 4E), msxB (Fig. 4F) and msxD (Fig. 4D,H), but not msxC (Fig. 4C,G) whereas transcripts of all four msx genes are found in mesenchymal cells in the space underlying the cleft and flanking cells. Early fin bud mesenchyme has been recently shown to be of neural crest origin in the zebrafish (Smith et al., 1994). Interestingly, transcripts of two of the msx genes expressed in mesenchymal cells of the median fin fold, msxB (Fig. 4F) and msxC (Fig. 4C,G) are earlier found in a position corresponding to that of premigratory neural crest cells (Ekker et al., 1992a and unpublished results).

**Fig. 3.** Expression of four msx genes in pectoral fin buds and their primordia. (A) Cells on the surface of the yolk sac in the primordia of the pectoral fin buds (arrows) express msxB at 24 h. The hybridization signal indicated by the arrowhead corresponds to msxB expression by cells of the dorsal hindbrain and spinal cord (Ekker et al., unpublished observations). (B-E) Dissected pectoral fin buds from 48 h zebrafish embryos. Anterior is to the left, distal to the top. Cells in the AER as well as cells that occupy a more proximal position next to the AER express msxA, msxB and msxD (B, C and E, respectively). There are also weak hybridization signals for both msxA and msxD throughout the fin bud. (D) Expression of msxC at 48 h is restricted to cells next to the AER and is more intense along the anterior edge. Scale bar: 20 µm in (B,C); 40 µm in (D,E).
Reinduction of msx gene expression during fin regeneration

Zebrafish, like other fish, regenerate their fins after injury and reestablish normal patterning although branch points in the lepidotrichia, the bony component of the fin rays, may vary (Géraudie and Singer, 1990). Thus, if the msx genes play a role in patterning during development, we anticipate that they should be reexpressed during regeneration. To examine this possibility, we amputated caudal fins of adult zebrafish by removing 60% of the distal part of the fin (Fig. 5A) and analysed msx gene expression 3 days after the amputation.

As shown in Fig. 5, strong hybridization signals corresponding to the transcripts of the msxA, msxB, msxC and msxD genes were detected in the regenerating fins. The expression patterns were similar to those observed during development. This suggests that the msx genes play a role in the regeneration process.
genes appear in the distal part of the fin during regeneration, and the expression patterns of the four msx genes are distinct. Cells in the most distal layers of the fin, corresponding to the wound epidermis that covers the blastema, express high levels of msxA (Fig. 5A, 6A) and msxD (Fig. 5D, 6D), but undetectable levels of msxB (Fig. 6B) and msxC (Fig. 6C). Expression of msxB (Fig. 5B, 6B) and msxC (Fig. 5C, 6C) is confined to the dense mesenchymal blastema formed at the distal part of each fin ray. There is no detectable expression of the four zebrafish msx genes in the regenerated part of the fin that has already differentiated.

We observed similar expression patterns in regenerating pectoral and anal fins (data not shown). These results demonstrate that cells of both the paired and the unpaired fins strongly induce reexpression of members of the msx gene family during regeneration.

**Temporal expression of the msx genes during regeneration**

We examined the time course of msx gene expression during regeneration of the caudal fin. To compare the expression of the various msx genes, we divided regenerating caudal fins, after fixation, into two pieces corresponding to the ventral and dorsal lobes, and hybridized each half with a different msx probe. The skeleton of the zebrafish fin is formed by segmented rays, the lepidotrichia, each of which is composed of two parallel hemisegments, that resemble a pair of parentheses in cross section. As early as 30 hours postamputation, we detect a very faint hybridization signal for msxB in each fin ray immediately distal to the end of the truncated lepidotrichia, in cells located between the two hemisegments (data not shown). As the blastema forms, the levels of msxB expression rapidly increase and remain high in blastema cells between day 2 and day 5 after amputation. During the same time period, distal blastema cells of the caudal fin also express the zebrafish retinoic acid receptor-γ gene (White et al., 1994). Later, msxB expression progressively decreases and disappears by 12 days of regeneration. At this time, amputated caudal fins have grown back to their normal size, as determined by cutting half the fin as above and measuring the time necessary for the amputated half to grow back to the same size as the uncut half. msxC and msxD transcripts appear and disappear in regenerating caudal fins with a time course similar to that of msxB (data not shown) whereas the time course of msxA induction in the regenerating caudal fin has not yet been determined.

**Correlation of msxB expression with the rate of growth of fin regenerates**

The temporal change in msx gene expression during regeneration may indicate that msx function is only transiently required during the process of regeneration. Because regeneration proceeds from proximal to distal, an alternative, although not mutually exclusive interpretation, is that the level of expression of each gene depends upon the position of the regenerating cells along the proximodistal axis of the fin. To examine these two possibilities, we amputated individual caudal fins at three different levels along the proximodistal axis (Fig. 7A) and analyzed the expression of the msx genes 4.5 days after the amputation. During regeneration, the growth rate of the fin is faster in its proximal part than in its distal part. Thus, after

![Fig. 5. Induction of msx gene expression during regeneration of the caudal fin. Blastema cells at the tip of each fin ray express msxB (B) and msxC (C). Cells overlying the blastema express msxA and msxD (A,D). Gene expression was determined 3 d after amputation. The plane of amputation is indicated by an arrow in C-D. The inset in A is the schematic representation of the skeleton of a zebrafish caudal fin. The standard plane of amputation is indicated by a dashed line and corresponds to a level proximal to the first branch point of the lepidotrichia (arrow). Scale bar: 40 µm.](image)
similar periods of regeneration, the distance separating the tip of the regenerate from the plane of amputation, which is an indicator of the amount of tissue that has regenerated, is longer for the proximal amputation than for the more distal amputations (Fig. 7B). A similar observation has been made for the pelvic fins of the opaline gourami, *Trichogaster sp* (Tassava and Goss, 1966).

We found that the level of expression of *msx*B correlates with the growth rate of the fin (Fig. 7B-E); there is a progressive decrease in the expression of *msx*B from the proximal cut to the distal cut. Examination of the regenerates suggests that the number of cells that express *msx*B is roughly equal in all three regenerates (not shown) but that the signal is stronger in individual cells of the proximal regenerate. This observation suggests that the expression of *msx*B reflects the growth rate of the blastema, which may be regulated by cues along the proximodistal axis. Alternatively, *msx*B expression may be regulated by positional cues along the proximodistal axis and higher levels of *msx*B expression may be required for the higher growth rate of proximal blastema cells.

In contrast to *msx*B, there is little if any variation in the levels of *msx*A, *msx*C and *msx*D (not shown) expression after amputation at different positions.

**DISCUSSION**

**Reinduction of zebrafish msx genes in regenerating fins**

Cells in the primordia of zebrafish fins transiently express four homeobox genes of the *msx* family and re-express the same genes during fin regeneration. Expression of homeobox genes that belong to other families has previously been reported in the regenerating limbs of newts, including *HoxC6* (Savard et al., 1988), *HoxD11* (Brown and Brockes, 1991), *HoxC10* and *HoxD10* (Simon and Tabin, 1993). The blastema of regenerating newt limbs also expresses two newt homeobox genes related to *distal-less* (Beauchemin and Savard, 1992). These previous studies measured gene expression either by northern blot or RNase protection assays of dissected tissues, which precludes visualization of individual cells. Analyses of dissected hindlimb blastema tissues suggested that *HoxC10* and *HoxD10* transcripts are present only in the mesenchymal tissue. Transcripts of the *HoxC6*, *HoxC10* and of the two *distal-less* genes are not restricted to regenerating tissue, but are constitutively present in the normal adult limb. Our results demonstrate that, in contrast, expression of *msxA*, *msxB*, *msxC* and *msxD* genes in adult zebrafish is regeneration-specific, like the newt *HoxD10* and *HoxD11* genes, and that both the paired and unpaired fins express the four *msx* genes.

Proximal blastema cells express higher levels of *msx*B transcripts than do cells in the distal blastema. Transcripts of two of the newt genes, *HoxD10* and *HoxD11* are also present at higher levels in proximal blastema (Brown and Brockes, 1991; Simon and Tabin, 1993). Therefore, genes like *msx*B and/or *HoxD10-11* could act to provide positional information along the proximodistal axis of the regenerating limb or fin, because the mesenchymal cells of the blastema express them and because, at least in amphibians, transplantation experiments have shown that information for patterning resides in these mesenchymal cells rather than in the wound epidermis that covers them (Stocum and Dearlove, 1972). Another possibility is that *msx*B is a target gene of homeodomain proteins such as *HoxD10* and *HoxD11*; its role could be to convert the positional information coded by the *Hox* genes into various rates.
of cell proliferation, such that proximal fin blastemas grow faster than distal blastemas, as can be seen in Fig. 7.

Relationships between limb/fin expression and the structures of *Msx* genes in different species

In regenerating fins, mesenchymal cells of the blastema express *msxB* and *msxC*, whereas *msxA* and *msxD* transcripts are present in epidermal cells overlying the blastema (Figs 5,6). This difference may be related to the differences in expression between *Msx1* and *Msx2* in the developing limbs of mouse and chicken. Mesenchymal cells of the progress zone underlying the AER express the *Msx1* gene, although low levels of *Msx1* transcripts are also detected in the AER. Cells in the AER predominantly express *Msx2* (Coelho et al., 1991a; Davidson et al., 1991; Hill et al., 1989; Roberts et al., 1989, 1991; Suzuki et al., 1991; Yokouchi et al., 1991). Therefore, there may be a functional similarity between *Msx1* and the zebrafish *msxB* and *msxC* and between *Msx2* and *Msx1* and *Msx2*.

Cognate relationships based upon DNA or protein sequence analyses are difficult to establish between the zebrafish *msx* genes and the *Msx* genes of other vertebrates whereas similar relationships between mouse and chicken *Msx* genes or between mouse and *Xenopus* *Msx* genes are more obvious (Ekker et al., 1992a and unpublished observations). Therefore, it is not presently possible to establish, only on the basis of sequence analyses, which one of the zebrafish *msx* genes is more closely related to *Msx1* and which one is more closely related to *Msx2*. We cannot exclude that the zebrafish *msx* genes are related to additional, as yet unidentified, *Msx* genes in mouse, birds, or amphibians. Holland has described the homeobox sequence of a third mouse *Msx* gene (Holland, 1991), but expression of this gene has not yet been reported. Mice missing *Msx1* function, produced by homologous recombination in embryonic stem cells, show no apparent limb defect (Satokata and Maas, 1994). Although, it was suggested that *Msx2* may substitute for *Msx1* in these mice, the absence of a limb phenotype in *Msx1−*mice might also be explained by the presence of another, as yet unidentified, *Msx* gene with patterns of expression more closely related to those of *Msx1*, a situation that would thus resemble what we observed for *msxB* and *msxC* in zebrafish fins.

We have previously shown that zebrafish have at least three
**Table 1. Expression of msx genes during development of pectoral fin buds, the median fin fold and their primordia was determined between 16 h and 72 h**

<table>
<thead>
<tr>
<th>Development</th>
<th>Pectoral fin buds</th>
<th>Median fin fold</th>
<th>Regeneration</th>
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<tr>
<td>AER* to AER†</td>
<td><strong>Epidermal cells</strong> (Cleft and flanking cells‡)</td>
<td><strong>Mesenchymal cells</strong></td>
<td><strong>Wound Epidermis</strong></td>
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<tr>
<td>msxA</td>
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The results shown are for 40 h zebrafish embryos (see text). For fin regeneration, in situ hybridization was performed between 3.5 and 5 days after amputation.

†Cells proximal to the AER are defined as the two or three rows of cells that occupy a position in the fin bud immediately proximal to AER cells.
‡Dane and Tucker, 1985.

**engrailed (eng)** genes (Ekker et al., 1992b) whereas only two **eng** genes have been identified in other vertebrates. The presence of a larger number of **msx** and **eng** genes in zebrafish may result from the continued expression and divergence of genes duplicated by rounds of polyploidization during evolution, a hypothesis that could also explain the presence of multiple unlinked isozymes in fish species (Morizot, 1990; Ohno, 1970).

Our sequence comparisons also show that the zebrafish **msxB** and **msxC** genes are closely related (unpublished observations). This relationship may explain the similarities in patterns observed for the two genes in regenerating fins. Nevertheless, the expression patterns of **msxB** and **msxC** are clearly distinct in the developing embryo, notably in the sensory cells of the inner ear that express **msxC** but not **msxB** (Ekker et al., 1992a).

**Relationship between msx expression in paired and unpaired fins**

The expression patterns of the four **msx** genes in paired fins are comparable to the patterns that we observed in unpaired fins. During development of paired fin buds, cells of the AER express **msxA**, **msxB** and **msxD**, but not **msxC**; whereas cells underlying the AER express all four **msx** genes (Fig. 3; Table 1). Similarly, during development of the median fin fold, the cleft cells and flanking cells (Dane and Tucker, 1985) express **msxA**, **msxB** and **msxD**, but not **msxC**; whereas cells underlying the cleft and flanking cells express all four **msx** genes (Fig. 4; Table 1). This observation may suggest that, at least in some aspects, the cleft cells of the median fin fold correspond to the AER cells of the paired fin buds and that each **msx** gene exerts the same function in the early stages of development of the paired fins as it does during development of the unpaired fins. This function may include patterning along the proximodistal axis of the fin primordium as suggested by the restricted expression of **msx** genes to distal cells in the AER of the pectoral fin buds and of the median fin fold. **msx** genes may also be involved in or regulated by interactions between the epidermal cells and underlying mesenchymal cells. This putative role of **msx** genes in patterning is further supported by the differential distributions of **msxC** expression in the fin buds (Figs 3, 4; Table 1). The patterns of induction of individual **msx** genes during regeneration of the paired fins are also identical to the patterns in the unpaired fins (Table 1). Therefore, the mechanisms of fin development and regeneration that involve the **msx** genes seem to be the same in paired and unpaired fins.

**Relationships between fin development and regeneration**

Although there are many similarities in the patterns of **msx** gene expression between developing and regenerating fins, we also noted clear differences. For example, the cells that occupy the most distal position in the fins, the AER and cleft cells during development and the wound epidermis during regeneration, express **msxA** and **msxD** (Table 1). However, cells underlying the AER/epidermis express these two genes only during development; during regeneration, cells in the blastema, the cells immediately proximal to the wound epidermis, express **msxA** and **msxD** only very weakly if at all (Fig. 6A,D). Thus, expression of **msxA** and **msxD** is restricted to more distal regions of the fin during regeneration than during development (Table 1). Similarly, the distribution of **msxB**-expressing cells also differs between developing and regenerating fins. AER and cleft cells, as well as underlying cells express **msxB**, in addition to **msxA** and **msxD**; but, in this case, **msxB** transcripts appear in the blastema and not in the wound epidermis of the regenerating fin (Table 1). Thus, in contrast to **msxA** and **msxD**, expression of **msxB** is restricted to more proximal regions of the fin during regeneration than during development.

Our results also suggest that expression of each of the **msx** genes is differentially regulated during fin development and regeneration. Of the four **msx** genes, only **msxC** is apparently not expressed in the most distal cells, the AER and cleft cells of the developing fins and the wound epidermis of the regenerating fin. Instead, **msxC** transcription is restricted to the cells immediately proximal to the AER of the pectoral fin buds, to mesenchymal cells underlying the epidermis in the median fin fold and to the blastema cells that underly the wound epidermis during regeneration. This result indicates that the **msxC** gene may play a similar role in these three cell types during various types of fin growth.

In the pectoral fin buds, the levels of **msxC** but not **msxB** expression vary along the anteroposterior axis whereas, in the regenerating fin, levels of **msxB**, but not **msxC** expression vary along the proximodistal axis of the blastema. This result suggests distinct mechanisms for the regulation of these two genes during development and regeneration.

Expression of vertebrate Mst genes appears in tissues where
epithelial-mesenchymal interactions take place, not only in the limbs but also in the pharyngeal arches (Hill et al., 1989; Robert et al., 1989; Takahashi et al., 1991), the teeth (MacKenzie et al., 1992) and the urogenital tract (Lyons et al., 1992). At least some of these interactions have been shown to be essential for the maintenance of Msx gene expression (Coelho et al., 1991b; Davidson et al., 1991; Robert et al., 1991). Our results suggest that epithelial-mesenchymal interactions, involving various members of the zebrafish msx gene family, may also occur during growth of regenerating fins and that the genetic regulation of patterning is similar in fins and tetrapod limbs. Additionally, our observations of the distributions of msx transcripts during the early stages of fin development suggest a role for the msx genes in the establishment of proximodistal polarity.

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