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Structure of the zebrafish *snail1* gene and its expression in wild-type, *spadetail* and *no tail* mutant embryos

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SUMMARY

Mesoderm formation is critical for the establishment of the animal body plan and in *Drosophila* requires the *snail* gene. This report concerns the cloning and expression pattern of the structurally similar gene *snail1* from zebrafish. In situ hybridization shows that the quantity of *snail1* RNA increases at the margin of the blastoderm in cells that involute during gastrulation. As gastrulation begins, *snail1* RNA disappears from the dorsal axial mesoderm and becomes restricted to the paraxial mesoderm and the tail bud. *snail1* RNA increases in cells that define the posterior border of each somite and then disappears when somitic cells differentiate. Later in development, expression appears in cephalic neural crest derivatives. Many *snail1*-expressing cells were missing from mutant *spadetail* embryos and the quantity of *snail1* RNA was greatly reduced in mutant *no tail* embryos. The work presented here suggests that *snail1* is involved in morphogenetic events during gastrulation, somitogenesis and development of the cephalic neural crest, and that *no tail* may act as a positive regulator of *snail1*.

Key words: branchial arches, *Brachydanio rerio*, epiboly, gastrulation, mesoderm, neural crest, *no tail*, paraxial mesoderm, *snail1*, somitogenesis, *spadetail*, zebrafish, zinc finger

INTRODUCTION

Gastrulation in a variety of organisms is a critical event that leads to the formation of the three embryonic layers - endoderm, mesoderm and ectoderm. In vertebrates, much work has focussed on mesoderm since it plays a pivotal role in organizing the body axis. Although the morphogenetic events resulting in mesoderm formation vary among different species, a number of genes and gene products involved in mesoderm formation have been conserved during evolution. Studies of *Xenopus* embryos have shown, for example, that molecules related to peptide growth factors (for review see Hopwood, 1990; Jessell and Melton, 1992) and activin (for review see Moon and Christian, 1992) play a role in initiating mesoderm formation in vivo. One of their target genes is the frog homologue of the mouse *Brachyury* or *T* gene (Smith et al., 1991), which is crucial for mesoderm development (Herrmann et al., 1990).

Another way to identify genes that might be involved in mesoderm formation in vertebrates is to search for genes similar to those that program a comparable process in *Drosophila melanogaster*. Genetic and molecular analyses have identified a regulatory cascade of maternally expressed genes that leads to a gradient of the transcription factor *dorsal* in cell nuclei and thus define pattern along the dorsoventral axis (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). The *dorsal* protein then initiates the expression of at least two genes, *twist* (Thisse et al., 1991; Jiang et al., 1991; Pan et al., 1991; Thisse and Thisse, 1992) and *snail* (Ip et al., 1991) that are required for mesoderm formation (Thisse et al., 1988; Alberga et al., 1991). Both *twist* and *snail* genes have been shown to be conserved in vertebrates: a gene homologous to *twist* has been identified in *Xenopus* (*Xtwi*, Hopwood et al., 1989) and in mouse (*M-twist*, Wolf et al., 1991), and *snail* homologues have been identified in *Xenopus* (*Xsna*, Sargent and Bennett, 1990) and mouse (*Sna*, Nieto et al., 1992; Smith et al., 1992).

The zebrafish (*Brachydanio rerio*) has advantages for studying vertebrate development because it combines the benefits of amphibians as an embryological system and mice as a genetic system (Streisinger et al., 1981, 1986; Kimmel, 1989). Here, we report the cloning and structural analysis of a zebrafish *snail* gene (called *snail1*). Our results show that *snail1* encodes a zinc finger protein that is structurally more related to the mouse *Sna* than the *Xenopus Xsna*. We then describe the distribution of *snail1* RNA in more detail than has been possible in other vertebrates because of the transparency of zebrafish embryos and improvements in the method of in situ hybridization. We show that *snail1* in zebrafish is expressed in paraxial mesoderm during gastrulation and then in somites. Later on, it is detected in head mesoderm and neural crest cell derivatives.

We also examined the expression pattern of *snail1* in two mutants affecting mesoderm formation. We show a shift of *snail1*-expressing cells to the tail bud in *spadetail* (Kimmel et al., 1989; Ho and Kane, 1990), consistent with a failure in the convergence of paraxial mesoderm precursors in this mutant. We also studied the expression pattern of *snail1* in

mutant *no tail (ntl)* embryos, which are defective in axial mesoderm development (Schulte-Merker et al., 1992, 1994a; Halpern et al., 1993) similar to *Brachyury* in mice (Chesley, 1935; Gluecksohn-Schoenheimer, 1944; Yanagisawa et al., 1981). We show that *no tail*, the zebrafish homologue of the mice *T* gene (Halpern et al., 1993; Schulte-Merker et al., 1994b), may act as a positive regulator of *snail1*.

The results suggest the hypothesis that *snail1* is involved in morphogenetic movements during epiboly and gastrulation, cell condensation during formation of somites and the formation of cranial cartilages.

MATERIALS AND METHODS

Cloning and sequencing of snail1 cDNA

To clone the full-length *snail1* cDNA, a gastrula cDNA library (gift from B. Riggleman) was screened at low stringency as published in Wolf et al. (1991). The probe used was the entire cDNA of the *Drosophila snail* gene (kindly provided by A. Alberga, described in Boulay et al., 1987). Deletions were made according to Lin et al. (1985) and sequencing was performed from single-strand templates by the dideoxy-termination method using Sequenase (USB Inc.) following the manufacturer's directions.

In situ hybridization on whole-mount embryos

The procedure described by Harland (1991) was used with the following modifications. The digoxigenin RNA probes were synthesized and subjected to alkaline hydrolysis according to Boehringer Mannheim Biochemica recommendations (Cat 1175041) to provide of an average length of 100-200 nucleotides. Embryos staged as described by Kimmel et al. (1993) were fixed 24 hours in 4% paraformaldehyde 1× PBS (phosphate-buffered saline), hand dechorionated and dehydrated overnight in methanol at −20°C. Then the embryos were rehydrated stepwise in methanol/PBS and finally put back in 100% PBT (1× PBS 0.1% Tween 20). Embryos older than the beginning of somitogenesis were treated 10 minutes with proteinase K (10 μg/ml in PBT). The reaction was stopped by rinsing in glycine (2 mg/ml in PBT). Embryos were postfixed in 4% paraformaldehyde 1× PBS for 20 minutes and then rinsed in PBT 5 times for 5 minutes each. The embryos were prehybridized at least 1 hour at 70°C in hybridization buffer [50% formamide, 5× SSC, 50 μg/ml heparin, 500 μg/ml tRNA, 0.1% Tween 20, 9 mM citric acid]. The hybridization was done in the same buffer containing 50 ng to 100 ng of probe overnight at 70°C. Then the embryos were washed at 70°C for 10 minutes in [75% hybridization buffer, 25% 2× SSC], 10 minutes in [50% hybridization buffer, 50% 2× SSC], 10 minutes in [25% hybridization mix, 75% 2× SSC], 10 minutes in 2× SSC, 2 times 30 minutes in 0.2× SSC. Further washes were performed at room temperature for 5 minutes in [75% 0.2× SSC, 25% PBT], 5 minutes in [50% 0.2× SSC, 50% PBT], 5 minutes in [25% 0.2× SSC, 75% PBT], 5 minutes in PBT, and then 1 hour in [PBT with 2 mg/ml BSA (bovine serum albumin), 2% sheep serum]. Then the embryos were incubated overnight at 4°C with the preabsorbed alkaline-phosphatase-coupled anti-digoxigenin antiserum (described in Boehringer instruction manual) at a 1/5000 dilution in a PBT buffer containing 2 mg/ml BSA, 2% sheep serum. Finally the embryos were washed 6 times for 15 minutes each in PBT at room temperature. Detection was performed in alkaline phosphatase reaction buffer described in the Boehringer instruction manual. When the color was developed, the reaction was stopped in 1× PBS. The embryos were then dehydrated, clarified in methylsalicylate and mounted in Permount.

RESULTS

Isolation and sequence analysis of zebrafish *snail1* cDNA

To isolate zebrafish *snail* cDNA, a gastrula cDNA library (kindly provided by B. Riggleman) was screened at low stringency using as probe the entire *Drosophila snail* cDNA (a gift from A. Alberga). Sequence data showed that the initial clone encoded a zinc finger protein, similar to the *Drosophila snail* gene. Since this 1.4 kb (kilobase) clone did not include the entire coding region, it was used to rescreen the cDNA library. Several clones were isolated and sequenced and one of them contains the entire 3′ untranslated region, a complete protein coding region and a portion of the 5′ untranslated leader sequence of a gene that we call *snail1*.

The *snail1* cDNA is 1950 base pairs long and contains an open reading frame of 789 nucleotides encoding a protein of 263 amino-acids (Fig. 1). 65 base pairs 5′ to the ATG translation initiation codon is an open reading frame 42 nucleotides long; this short open reading frame might play a role in the regulation of *snail1* expression at the level of translation. *snail1* encodes four zinc fingers. Fingers 1 to 3 share the classical structure CXXC(12X)HXXXH found in diverse nucleic acid binding proteins (reviewed in El Baradi and Pieler, 1991). Finger 4 is a variant on the zinc finger motif with the structure CXXC(12X)HXXXXC.

The amino-acid sequences of different members of the Snail family were aligned and compared (Fig. 2). The Snail family currently includes two *Drosophila* genes, *snail* and *escargot* (Whiteley et al., 1992), and three vertebrate genes. The fly and frog genes each contain five zinc fingers; in contrast, the mouse and fish genes both lack the first finger. The carboxy-termini of all five Snail family proteins are at about the same position, and the last four fingers are very similar. In all known members of the Snail family, the sequence of the last finger differs from the classical zinc finger motif. A search of the Swissprot. databank for the CXXC(12X)HXXXXC zinc finger structure uncovered only the Snail sequences discussed above. This motif can therefore be considered to be 'Snail' specific.

Developmental pattern of *snail1* RNA

Transcripts of *snail1* were localized during development by whole-mount in situ hybridization on zebrafish embryos. Digoxigenin-labelled sense and antisense RNA probes were hybridized to embryos of various stages. The pattern of *snail1* expression described in the following paragraphs was obtained using a *snail1*-specific RNA probe corresponding to the 5′ part of the *snail1* cDNA (a fragment containing the 5′ untranslated leader sequences and coding sequences up to the *Acc*I restriction site before the first zinc finger).

(1) Zygote, cleavage stage and blastula

snail1 RNA was first detected as a maternal RNA in the zygote (0-0.7 hours postfertilization at 28.5°C) and during the cleavage period (0.7-2.2 hours). Blastomeres were homogeneously labelled with the antisense RNA probe but no signal was detected in the yolk cell (Fig. 3A). No labelling was observed with the sense RNA probe (data not shown).

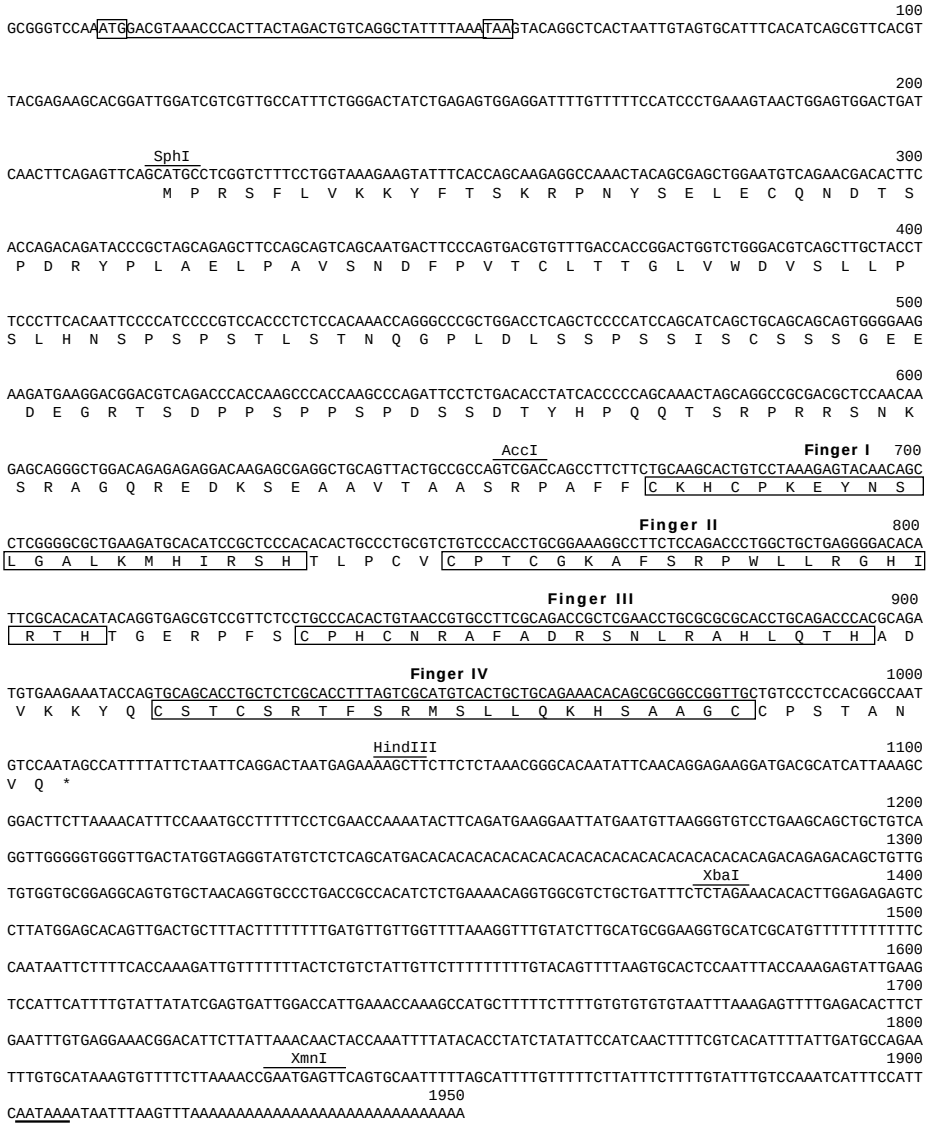

Fig. 1. Nucleotide sequence of *snail1* and deduced amino-acid sequence of the Snail1 protein. The cDNA is 1950 base pairs long and codes for a 263 amino acid residue protein. A short 5′ open reading frame 42 nucleotides long is underlined. Each zinc finger is boxed. Some useful restriction sites are indicated: *Sph*I, *Acc*I, *Hin*dIII, *Xba*I and *Xmn*I. Nucleotide numbering is shown to the right. The presumed polyadenylation signal at the 3′ end of the transcript is underlined. The stop codon is indicated with a star.

During the early blastula period, maternal RNA persisted in all of the blastomeres. However, at the end of the sphere stage and beginning of dome stage (see Kimmel et al., 1993), strong labelling appeared at the margin in a restricted area corresponding to about 60° of arc on the circumference (Fig. 3B,C). In cells immediately surrounding this region, the amount of maternal RNA was clearly reduced (Fig. 3B). Then during the dome stage, zygotic expression spread progressively and *snail1* RNA became localized all around the margin (Fig. 3D) in the deep cell layer (DEL).

(2) Gastrula

At the beginning of gastrulation, *snail1* RNA was detected as a homogeneous ring around the margin of the embryo. At 60% epiboly, when the embryonic shield begins to elongate along the anteroposterior axis by convergence and extension movements (Kimmel et al., 1993), *snail1* RNA disappears from the central region of the embryonic shield. This region corresponds to the axial hypoblast (Fig. 3E), including the presumptive notochord (chordamesoderm) caudally, and the prechordal plate rostrally. Paraxial hypoblast gives rise to muscles of the eye, jaw and gill, anteriorly and the segmental plate, which forms somites, posteriorly. Throughout gastrulation, *snail1* transcripts continued to be detected in the paraxial hypoblast of the segmental plate and around the margin except for the axial hypoblast (Fig. 3F).

(3) Segmentation (10-24 hours)

During segmentation, the embryo elongates along the anteroposterior axis and somites appear sequentially from anterior to posterior. At the beginning of somitogenesis, labelling was very strong around the blastopore, except in the axial hypoblast. Anteriorly, *snail1* RNA staining was intense in a cell sheet immediately adjacent to the axial hypoblast (Fig. 4A). We call these special cells adaxial cells. Lateral to the adaxial cells, *snail1* RNA was more diffuse. *snail1* RNA was not observed in the hypoblast rostral to the first somite.

As cells intercalate and converge toward the dorsal midline, the embryo elongates and narrows. During these events, the quantity of *snail1* RNA increased in the prospective tail bud and in the paraxial hypoblast, which showed

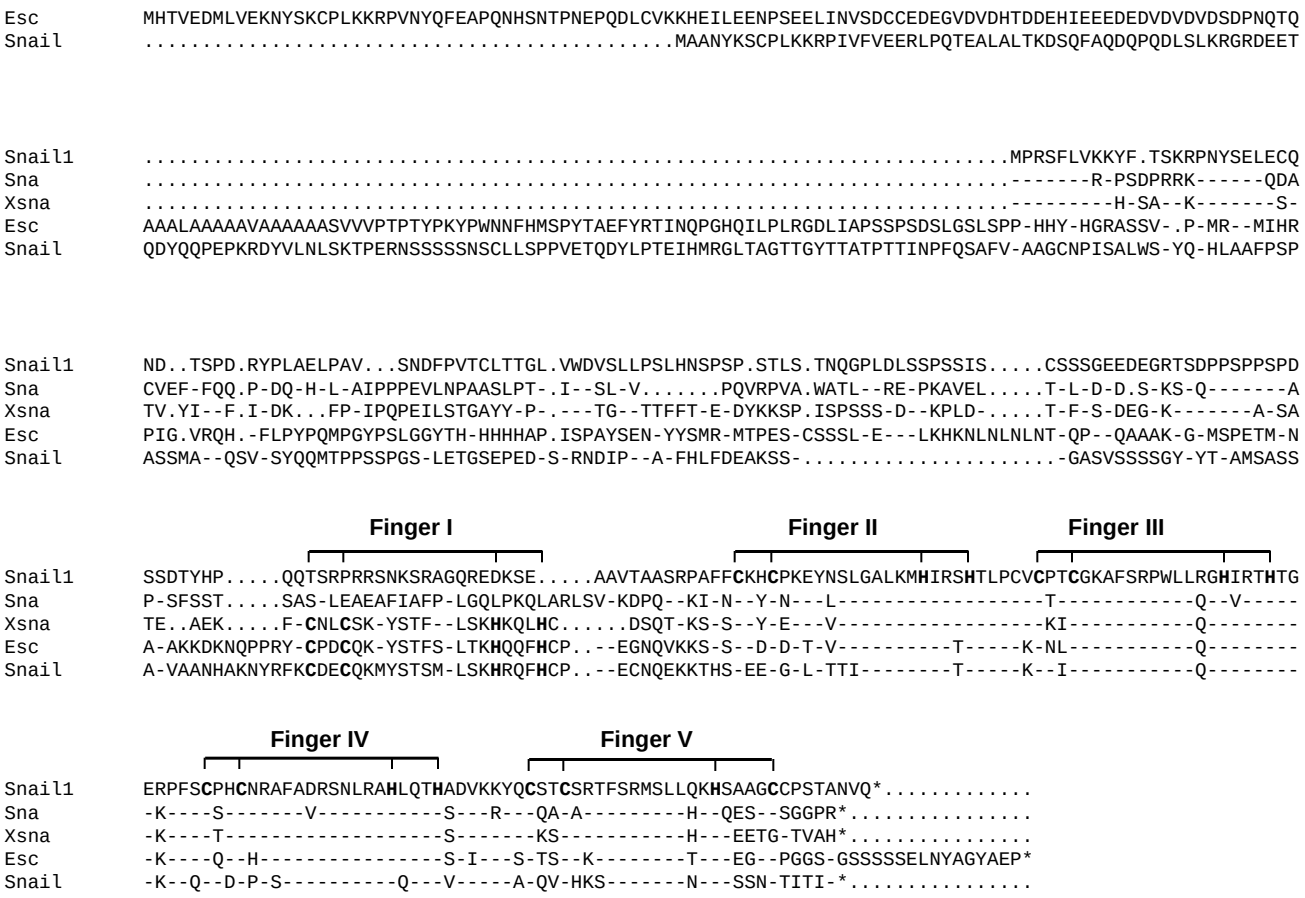

Fig. 2. Comparison of the predicted protein sequences of the *snail1* gene family. The zebrafish *snail1* protein is compared to the mouse Sna protein (Nieto et al., 1992; Smith et al., 1992), *Xenopus* Xsna (Sargent and Bennett, 1990), and Snail and Escargot from *Drosophila* (Whiteley et al., 1992; Boulay et al., 1987). Zinc fingers are indicated. A dash indicates identity to the *snail1* sequence. Dots indicate gaps inserted in the sequences to maximize homology. Cysteine (C) and histidine (H) in the zinc fingers are bold faced. A star signifies the translation stop signal at the carboxy terminus of the proteins.

well-demarcated lateral limits of expression (Fig. 4B-D). As segmentation progresses, the territory of *snail1* RNA in the tail bud and caudal paraxial mesoderm continues to narrow until the end of tail formation (Fig. 4).

In 6- to 9-somite embryos, the quantity of *snail1* transcripts increased transiently in the most lateral part of the segmented paraxial hypoblast in a row of cells parallel to the adaxial cells in the three anterior somites (arrow Fig. 4C). Expression in this territory decreased from posterior to anterior and completely disappeared by the 12-somite stage. The future identity of these cells is unknown.

Development of *snail1* RNA pattern in adaxial cells

At the beginning of somitogenesis, adaxial cells were intensely labelled up to the level of the first somite. The signal in this sheet of cells was homogeneous along the anteroposterior axis (Fig. 4A). At about the 4-somite stage, the signal started to fade posterior to the furrow separating somites 3 and 4 (Fig. 4B). Expression in these cells decreased progressively for the three anterior somites and was no longer observed in this territory after the 7- or 8-somite stage (Fig. 4C). More caudally, *snail1* RNA in adaxial cells disappeared progressively following the advancing wave of somite formation. The anterior extent of adaxial cell labelling reached the level of the most recently formed somitic furrow (Fig. 4C-F).

In the segmental plate, adaxial cells narrowed to a sheet 1-cell wide by the 6-somite stage. Each cell's nucleus occupied an asymmetric position near the notochord. This pattern remained unchanged until the last somite had been added (Fig. 4D-G).

Since adaxial cells include muscle pioneer precursors (Felsenfeld et al., 1991), we tested whether the adaxial cells accumulated transcripts from a zebrafish myotome marker: the α-*tropomyosin* gene (probe kindly provided by B. Riggleman, see also Ohara et al., 1989; Riggleman et al., 1993). Along the anterior-posterior axis, α-*tropomyosin* RNA was detected in adaxial cells, in particular in cells that form muscle pioneer precursors (Fig. 4H). In newly formed posterior-most somites, α-*tropomyosin* RNA was maintained in muscle pioneer precursors. In contrast, *snail1* RNA disappeared from these muscle pioneer precursors at this time (Fig. 4G). A comparison of the expression of these two genes shows that *snail1* RNA and α-*tropomyosin* RNA are both found in adaxial cells before the formation of the somite, at which time there is a specific extinction of *snail1* expression in adaxial cells.

snail1 RNA along the somitic furrows

When the first few somitic furrows form, *snail1* RNA increases in cells just anterior to the furrow (Fig. 4B,C). Later, as each subsequent furrow forms, *snail1* RNA increases in the lateral part of the paraxial mesoderm in cells just anterior to the furrow (Fig. 4D,E). Until the 14-somite stage, the level of *snail1* transcripts increases nearly simultaneously in all somites. As each somite matures, *snail1* RNA spreads anteriorly from a single row of cells at the posterior border of the somite (Fig. 4G). Transcripts of the *snail1* gene were not detected in the anterior quarter of the somite.

Posterior to the most-recent somitic furrow, *snail1* labelling was not homogeneously distributed. In the unsegmented segmental plate, transcripts of *snail1* RNA appeared more strongly in two stripes spaced at segmental intervals posterior to the most recent somite (arrows in Fig. 4D,E). This pattern persisted until the end of somitogenesis.

From the 14-somite stage, as the tail elongates, *snail1* RNA accumulates in posterior newly formed somites and

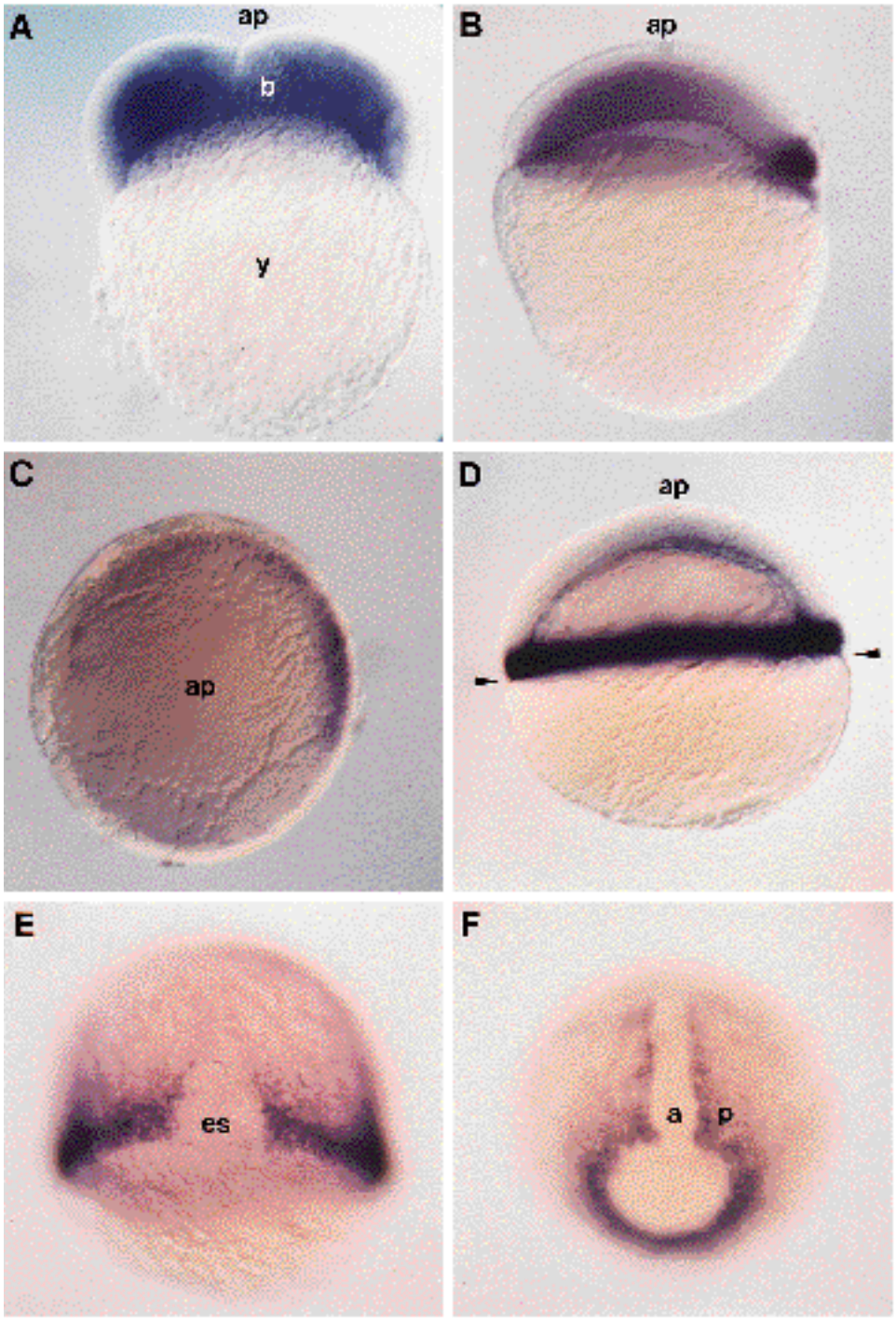

Fig. 3. Distribution of *snail1* RNA during cleavage, blastula and gastrulation. Transcripts from the *snail1* gene were revealed by whole-mount in situ hybridization. Lateral views of embryos are oriented with their animal pole up and dorsal side to the right. Animal pole views also have dorsal sides to the right. (A) 2-cell stage, lateral view. Blastomeres are labelled with *snail1* antisense probe. ap, animal pole; b, blastomeres; y, yolk cell. (B) Beginning of dome stage, lateral view. The zygotic expression of *snail1* has begun on one side of the embryo. Double labelling with the zebrafish *goosecoid* RNA shows that this is the future dorsal side of the embryo (our unpublished data). (C) Same stage as B, animal pole view. The zygotic RNA occupies an arc on one side of the embryo. The rest of the embryo is labelled more faintly with maternal RNA. (D) 40% epiboly, lateral view. *snail1* RNA is localized all around the margin and maternal RNA has mostly disappeared. The two arrows show the position of the margin. (E) 70% epiboly, dorsal view. *snail1* transcript disappears from the central part of the embryonic shield (es). (F) 90% epiboly, posterior or vegetal pole view. *snail1* RNA is excluded from the axial mesoderm (a) and restricted to the paraxial mesoderm (p) and marginal region.

disappears progressively in anterior differentiated somites (data not shown).

Disappearance of *snail1* RNA

RNA from the *snail1* gene was present until 36 hours in the extremity of the tail, but disappeared when the tail was completely formed. Transcripts of *snail1* disappeared from somites dynamically. Cells close to the notochord stained in the most posterior somites, but as muscles differentiated in a wave from medial to lateral in each somite, *snail1* labelling decreased in a similar fashion. Differentiated muscle cells never contained detectable amounts of *snail1* transcript. The latest cells to

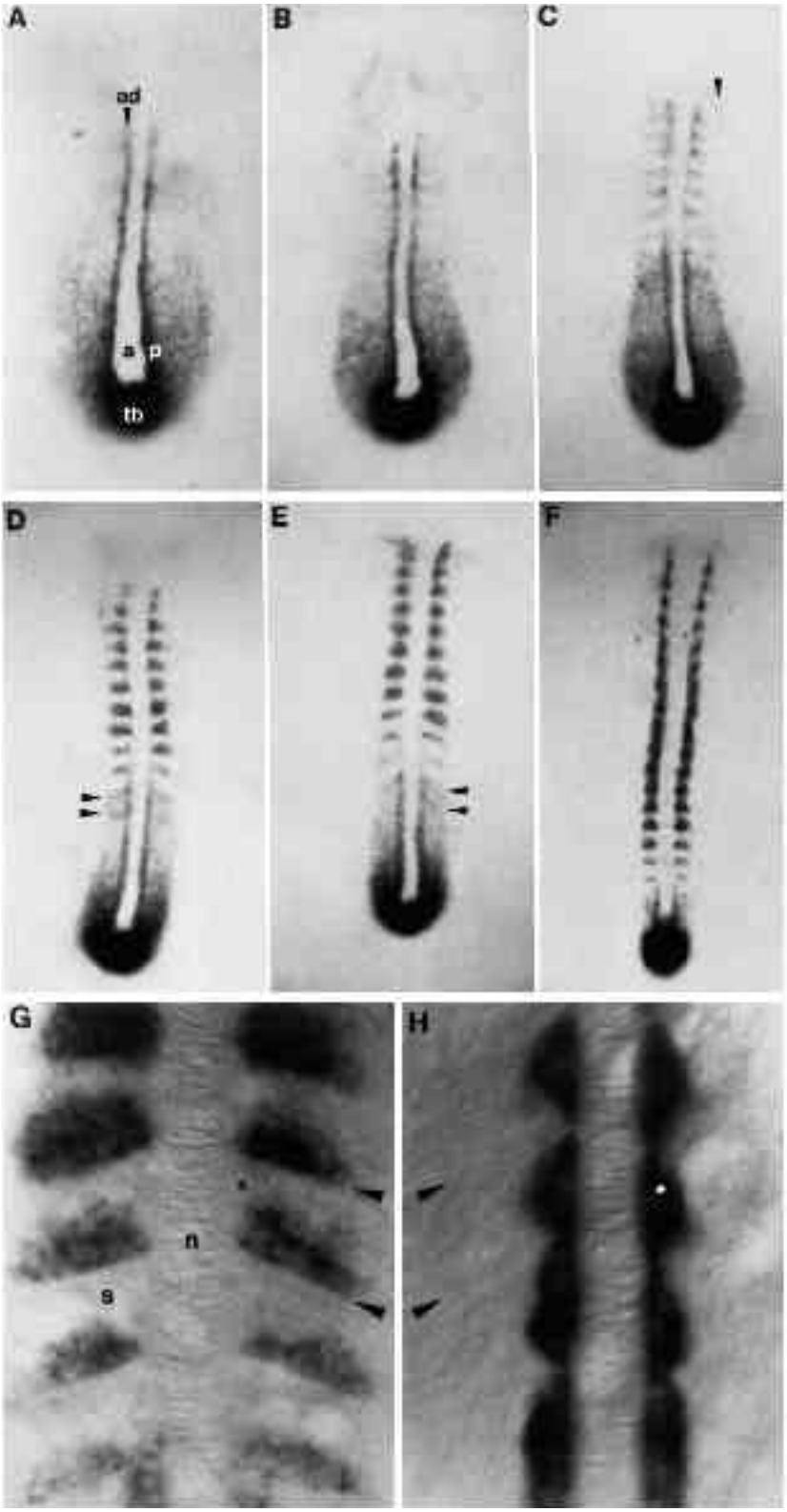

Fig. 4. Change in the *snail1* expression pattern during somitogenesis. (A) 3-somite stage. *snail1* RNA is intensively stained in adaxial cells (ad), a single row of paraxial hypoblast cells (p) adjacent to axial mesoderm (a). Lateral cells are also labelled but in a diffuse manner. The tail bud (tb) is strongly stained. (B) 4-somite stage. Labelling adjacent to the notochord starts to fade posterior to the furrow separating the third and fourth somite. Staining increases just anterior to each newly formed somitic furrow. (C) 7-somite stage. In the segmented mesoderm, signal disappears from adaxial cells. Note the epithelial character of adaxial cells more posteriorly in the unsegmented mesoderm. *snail1* RNA is detected anterior to the somitic furrow in a single row of cells. See arrow showing *snail1* RNA transiently expressed in the most lateral part of the segmental paraxial hypoblast. (D,E) 10- and 12-somite stages, respectively. In the unsegmented paraxial mesoderm (the segmental plate), there are two stripes of *snail1* RNA at segment periodicity posterior to the most recently formed somite (see arrows). As each somite matures, the territory of *snail1* expression spreads anteriorly within the somite. (F) 17-somite stage. In the most recently formed somite, *snail1* RNA accumulates in a unique line of cells just anterior to the newly formed furrow. (G) Details of *snail1* expression pattern in the somite (s) at 17-somite stage, dorsal view. Note the position of the notochord (n) in the middle and the location of the somitic furrow (arrows). *snail1* RNA is detected in the posterior compartment of the somite. In the most recently formed somite, a single sheet of cells accumulates *snail1* transcript. In older somites, the labelling occupies more of the posterior portion of the somite. The star indicates the position of muscle pioneer precursors which are not labelled with *snail1* RNA. (H) 17-somite stage. Dorsal view of an embryo probed with α-*tropomyosin*. The arrow indicates the position of the somitic furrow. αtropomyosin is detected in adaxial cells and in particular, in muscle pioneer precursors (indicated by a star). Note morphogenetic changes in α−*tropomyosin*-expressing cells as somites mature.

continue expressing *snail1* in each somite were localized superficially next to the body wall in a position expected for dermatome cells. In addition, some cells labelled with the *snail1* RNA probe were also observed along the notochord at a position expected for sclerotome cells (data not shown).

(4) Pattern of snail1 RNA in the head

Transcript from the *snail1* gene was first detected in the head at the 16-somite (17 hour) stage in a region posterior and ventral to the eye. These cells give rise to the primordia of the most anterior pharyngeal arches. Labelled cells were also observed dorsal to the eyes in the space between eyes and forebrain. These territories of expression gradually increased in size and intensity until 24 hours of development.

At 24 hours, a zebrafish embryo has well-developed eyes, several distinct brain regions, a prominent otic vesicle and six pharyngeal arch primordia. Cells located in mesenchyme surrounding and within some of these structures contained *snail1* RNA (Fig. 5A, B). Labelled cells encapsulated the eyes and appeared to fill the region between the eyes and the neighboring midbrain and forebrain. Similarly, stained mesenchymal cells were found in spaces between the olfactory placodes and the forebrain (Fig. 5B), and at the midbrain-hindbrain junction. The otic vesicle was also surrounded with label, except along its dorsal and medial borders.

By 24 hours, *snail1* was expressed in at least the first four pharyngeal arch primordia. These primordia appeared as a reiterated series of darkly stained cells that extended anteriorly and ventrally below the developing hindbrain and otic capsule. From rostral to caudal, they constitute the precursors of the mandibular, hyoid and five gill segments (Schilling, 1993). The mesenchyme of each arch is composed predominantly of neural crest cells as well as some paraxial mesoderm. Most if not all of the mesenchymal cells within an arch expressed *snail1* (Fig. 5A). Epithelium of the endoderm that forms the boundaries between arches showed no expression. Mandibular and hyoid segments appeared as larger labelled cell groups. The first two gill segments are smaller beneath the otic capsule and the most caudal segments appeared as a fused mass of staining. A bilateral string of labelled cells extended from the caudal boundary of the otic capsule (Fig. 5A,B) caudally along the notochord into the rostral trunk.

By 36 hours, the head has shortened and eyes and body pigmentation are well developed. At this time, *snail1* RNA is located in a few cells surrounding the developing lens and the eye capsule. The labelled regions in the pharyngeal arches have enlarged and become more distinct (Fig. 5C,D). Staining in the mandibular arch extends across the ventral midline but, in more caudal arches, the two sides remain widely separated.

After 2 days of development, *snail1* expression is largely confined to the pharyngeal region (Fig. 5E,F). By 3 days of development, pharyngeal cartilages and muscles have begun to differentiate. *snail1* RNA fades from newly differentiated chondrocytes (Fig. 5E,F) and becomes restricted to cells that surround the differentiated cartilages. Labelling is stronger at the dorsal and ventral extremities of the arch, and weaker in the center where the first cells appear to differentiate. By 96 hours, cells throughout the embryo stop accumulating *snail1* transcript.

In the pectoral fin buds, after a uniform signal at 24 hours, staining became localized in two distinct regions: one in the mesenchyme beneath the apical ectodermal ridge and another located in dorsal and ventral cells in the center of the fin. At 48 hours, *snail1* RNA was not observed in differentiated chondrocytes of the fin (data not shown).

snail1, *spadetail* and the process of convergence.

The in situ hybridization experiments showed that *snail1* expression occurs in hypoblast cells undergoing convergence and forming paraxial mesoderm. This result suggests the hypothesis that *snail1* might be involved in the process of convergence. This process is disrupted in homozygous *spadetail* embryos (Kimmel et al., 1989; Ho and Kane, 1990). Mutant *spadetail (spt)* embryos develop apparently normally until gastrulation, but then paraxial mesoderm fails to converge normally towards the dorsal axis of the embryo. Instead, paraxial mesoderm congregates in the tail bud, giving embryos deficient in derivatives of paraxial mesoderm. The notochord is morphologically normal in *spadetail* embryos (Kimmel et al., 1989; Ho and Kane, 1990). Interestingly, cells that express *snail1* in wild-type gastrulas develop abnormally in *spadetail* embryos. Does *spadetail* inhibit convergence because it blocks the action of *snail1*? Or do *snail1*-expressing cells simply fail to converge in *spadetail* embryos?

To investigate the relationship of *snail1* to the process of convergence, we examined the pattern of *snail1* RNA in *spadetail* mutant embryos. Expression of *snail1* RNA in *spadetail* embryos appears normal before gastrulation. Then, in the early gastrula (after 60% epiboly), *snail1* RNA fails to occupy the lateral edges of the embryonic shield in presumed *spadetail* embryos (i.e., in a quarter of the offspring of a mating of two *spadetail* heterozygotes) in contrast to wild-type embryos (Fig. 6A,B). This is long before the mutant phenotype becomes morphologically visible otherwise. During somitogenesis in mutant embryos, *snail1* RNA was concentrated in the tail bud, where non-converging cells accumulate (Fig. 6C-F). While somites fail to form in *spadetail* embryos, a few *snail1*-positive cells were found scattered along the notochord (Fig. 6C-F). These results show that *snail1*-expressing cells are mislocalized in *spadetail* mutant gastrulas.

snail1 expression in *no tail* embryos

The expression pattern of *snail1* just before gastrulation is similar to that of the *no tail* gene, which is homologous to *Brachyury* in mouse (Chesley et al., 1935; Schulte-Merker et al., 1992; Wilkinson et al., 1990). RNA from both genes accumulates in a ring of cells completely encircling the margin of the late blastula stage embryo. As gastrulation begins, the situation suddenly changes: axial cells continue to accumulate *no tail* message, but no longer contain *snail1* transcript, while, reciprocally, the rest of the margin continues to contain *snail1* RNA, but loses *no tail* RNA. Thus, in the anterior part of the embryo, *snail1* RNA becomes restricted to segmental paraxial mesoderm and *no tail* RNA becomes limited to the axial mesoderm. In contrast, in the posterior, cells of the presumptive tail bud

express both genes throughout somitogenesis. To investigate further the interactions of these two genes, we studied the localization of *snail1* transcripts in mutant *no tail* embryos, which contain axial mesoderm but do not form differentiated notochord cells (Halpern et al., 1993).

During early gastrulation, when the *no tail* phenotype can not be recognized morphologically, all offspring of matings between two *no tail* heterozygotes had a normal spatial distribution of *snail1* RNA. While the spatial distribution was normal, the quantity of RNA in paraxial mesoderm cells

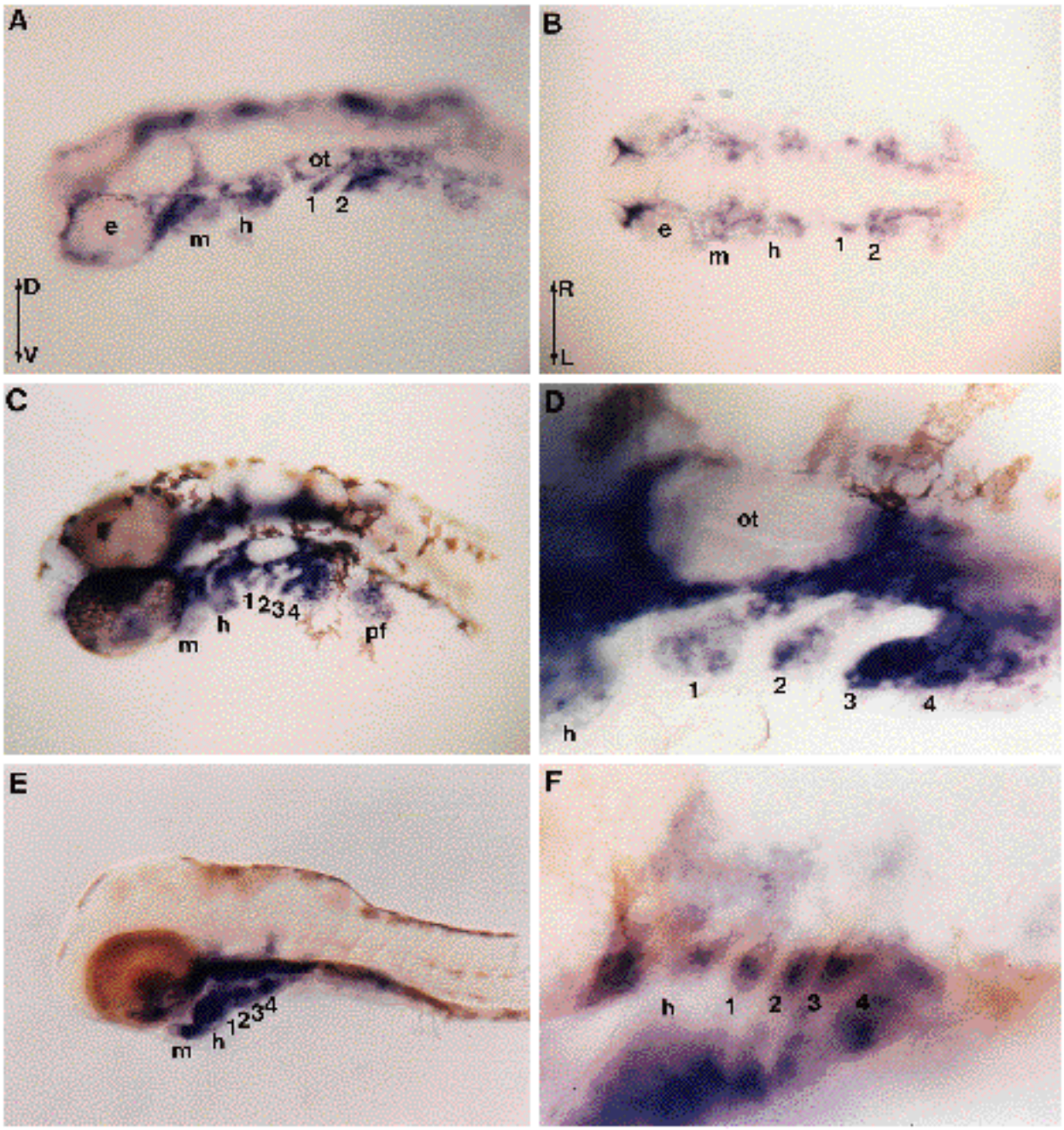

Fig. 5. Late expression pattern of *snail1* in the head. Embryos were dissected and the yolk was removed. (A) 24 hour embryo, dorsolateral view and (B) 24 hour embryo, dorsal view. *snail1* RNA is localized in various cells around the eyes (e) and otic capsule (ot), and in pharyngeal arch primordia (mandibular arch (m), hyoid arch (h), and the first (1) and second (2) gill segments). D, Dorsal; V, ventral; R, right; L, left. (C) 36 hour embryo, lateral view. The extent of labelling has expanded in the branchial arches and is easily distinguishable in the mandibular (m) arch and the hyoid (h) arch, and in the first four (1, 2, 3 and 4) gill arches. Pectoral fin buds (pf) also accumulate *snail1* transcript. (D) High magnification of the ear region (ot, otic capsule) and caudal branchial arches focusing on *snail1* RNA expression in gill arches. The mesenchyme of each arch is composed of neural crest cells and paraxial mesodermal cells. (E) 60 hour embryo, lateral view. *snail1* RNA disappears from the middle of the arches where chondrocytes are beginning to differentiate. (F) Details of the embryo shown in E, focusing on branchial arches.

was considerably reduced in about a quarter of the embryos. When the mutant phenotype could be distinguished at the beginning of somitogenesis, *snail1* RNA was observed in the paraxial mesoderm of *no tail* mutants but the amount of labelling was much less than in phenotypically wild-type sibling embryos stained for the same amount of time. Adaxial cells were more strongly labelled in the anterior part than in the posterior region of *no tail* embryos. Tail bud cells in *no tail* embryos failed to show the intense staining that they display in wild-type embryos; in Fig. 7A,B, wild-type embryos were stained for 2 hours and mutant embryos were stained for 6 hours in order to fully visualize the weak *snail1* staining in *no tail* embryos. The lateral limit of *snail1* expression in *no tail* paraxial mesoderm was less well defined than in wild type (Fig. 7A,B). The spatial distribution of *snail1* RNA in somites resembled that of wild-type embryos, but the amount of RNA was substantially reduced.

These studies show that in the absence of *no tail* gene function, the spatial distribution of *snail1* RNA is similar to wild type in segmented paraxial mesoderm, but the quantity of *snail1* RNA is drastically reduced. Likewise, the strong accumulation of *snail1* RNA observed in adaxial and tail bud cells of wild-type embryos disappears in mutant embryos. These results suggest that *no tail* gene function is required for a high level of expression of *snail1*.

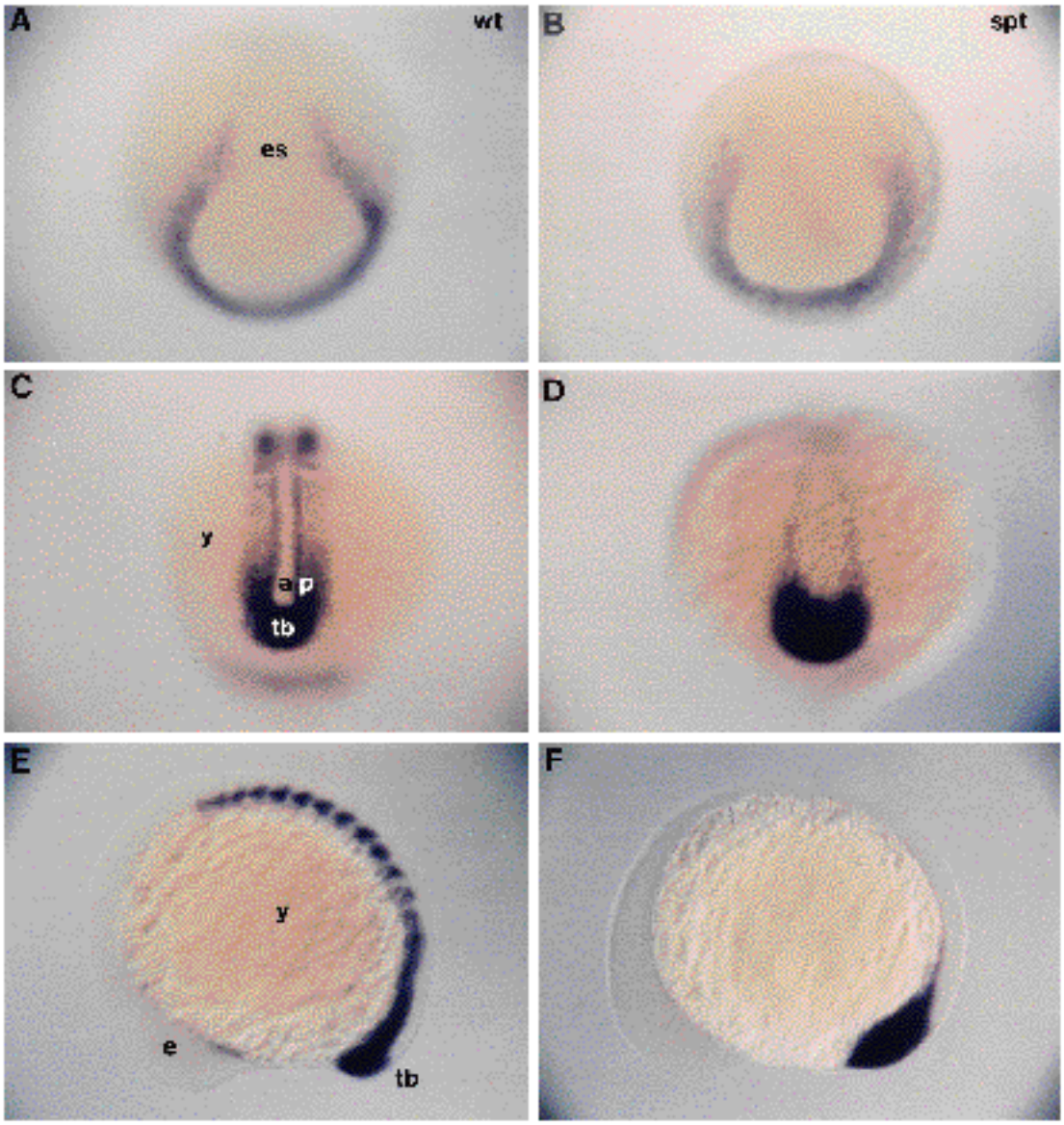

Fig. 6. Expression pattern of *snail1* in *spadetail* embryos. (A-D) Posterior views; (E,F) lateral views. (A,B) 80% epiboly; (C,D) 10-somite stage; (E,F) 12-somite stage. (A,C,E) Wild-type embryos; (B,D,F) *spadetail* embryos. While *snail1* RNA lines the lateral border of the embryonic shield in wild-type (A), it is missing in *spadetail* embryos (B). The axial mesoderm is broader in a *spadetail* embryo (C) than in a wild-type embryo (D). Wild-type embryos have extensive areas containing *snail1* transcripts in the paraxial mesoderm and somites (E), while *spadetail* embryos have much less *snail1* transcript in the paraxial region (F). In the mutant, *snail1* transcript is present only in the tail bud and in a few cells scattered along the axial mesoderm.

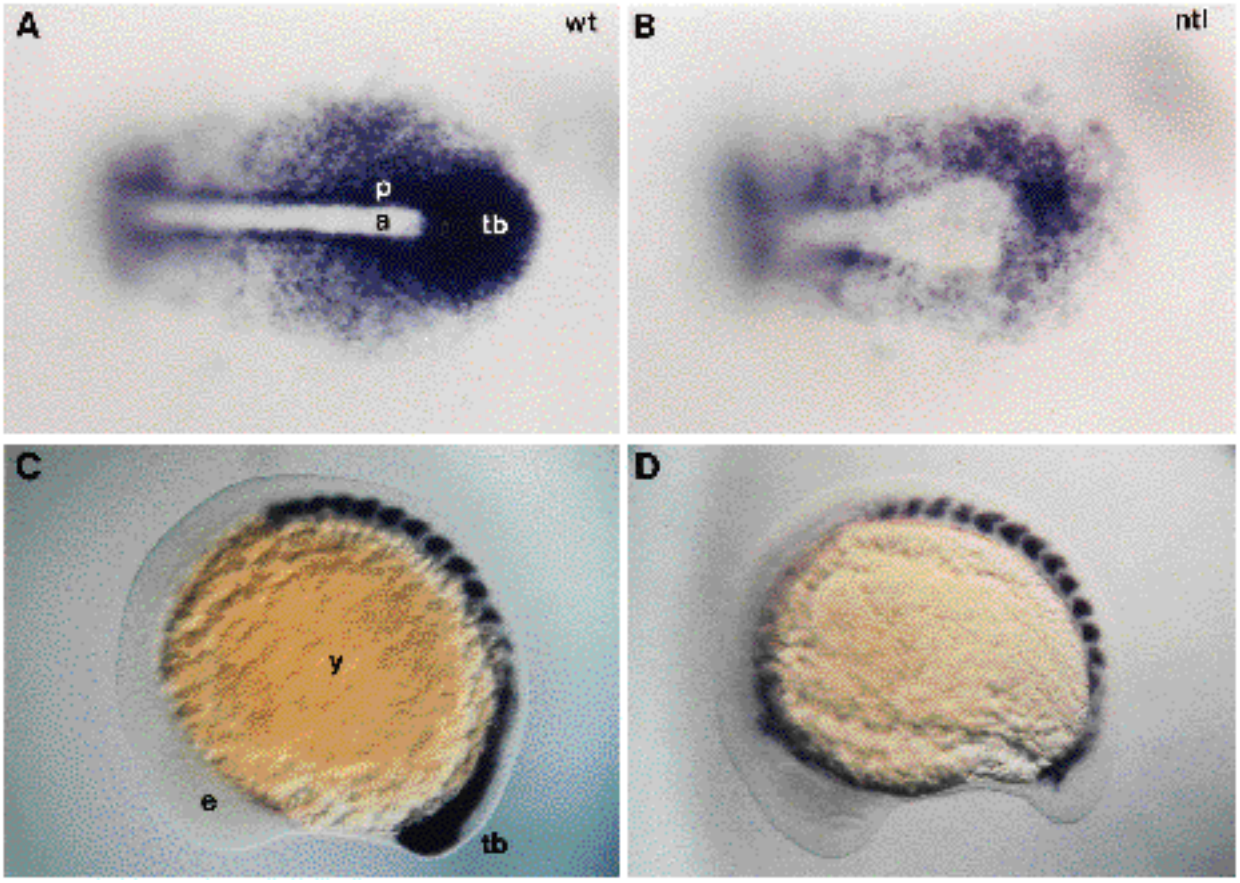

Fig. 7. Expression pattern of *snail1* in *no tail* embryos. (A,B) Posterior views of 5-somite stage; (C,D) lateral views of 12-somite stage; (A,C) wild-type embryos; (B,D) *no tail* embryos. Compared to a wild-type embryo (A), note the reduction of *snail1* signal in *no tail*, especially in the tail bud, even though this *no tail* embryo was stained for 6 hours to be able to see a clear signal, and the wild-type control was stained for just 2 hours. The axial mesoderm territory is broader in *no tail* embryos. In contrast to wild type (C), an overstained *no tail* embryo shows an absence of *snail1* staining in the tail. Since the mutant embryo was stained for a long time, background staining started to become visible.

DISCUSSION

The *snail* gene family

Sequence analysis suggests that there are two sub-types of *snail*-family genes: some with five fingers (described in *Drosophila* and *Xenopus*), and some with four (described in zebrafish and mouse). We have recently found (in progress) that zebrafish has a second *snail* gene (*snail2*) with five fingers like the *Xenopus* protein. This raises the possibility that mouse and *Xenopus* may also have two *snail*-family genes, but that the four-finger gene is the only one yet described in mouse and the five-finger gene is the only one yet described in *Xenopus*. For the zebrafish experiments reported here, a *snail1*-specific RNA probe was generated from the 5′ untranslated leader and the coding portion of the gene excluding the zinc finger region.

The expression pattern of *Sna* in mouse embryos appears to be a combination of the *snail1* and *snail2* pattern in zebrafish; *Sna* is expressed not only in mesoderm as is *snail1*, but also in presumptive pre-migratory neural crest cells (Nieto et al., 1992; Smith et al., 1992) like *snail2* (our unpublished data). Two hypotheses might explain these observations: either the expression pattern of *snail1* and *Sna* are different in these two species or the expression of *Sna* in presumptive migratory neural crest cells is a result of cross hybridization to an undescribed *snail2*-like gene in mouse.

Expression and role of *snail1*

The optical clarity and ease of obtaining zebrafish embryos permits an analysis of *snail* gene expression in greater detail than was possible for mouse or *Xenopus*. In addition, we have substantially improved the in situ hybridization protocol to preserve morphology and increase specificity. Improvements include fixing embryos for prolonged times, deleting the proteinase K step for young embryos, hybridizing at very high stringency, dissecting embryos from the yolk and flattening them between coverslips, which allows single cell resolution.

(1) Early snail1 RNA pattern

Expression of *snail1* is one of the earliest known asymmetries in the zebrafish embryo. Just before epiboly (4 hours), at the end of the sphere stage, zygotic expression of *snail1*

RNA begins in a small crescent on one side of the embryo margin. Only a few zebrafish genes are known to be expressed this early, including *no tail* (Schulte-Merker et al., 1992) and *goosecoid* (our own observations). In situ hybridization using *snail1* and *goosecoid* RNA probes in the same embryos demonstrated that both genes are expressed in the same cells (our unpublished data). In *Xenopus*, *goosecoid* expression marks the dorsal side of the embryo (Cho et al., 1991; Blumberg et al., 1991). This suggests that zygotic expression of *snail1* initiates on the dorsal side of the embryo shortly after the mid-blastula transition.

The initial crescent of *snail1* expression spreads to form a ring encompassing the entire blastoderm margin at the beginning of epiboly. These cells also express the *no tail* gene (Schulte-Merker et al., 1992), and become lateral, paraxial and axial mesoderm, as well as heart and blood (Kimmel and Warga, 1987; Kimmel et al., 1990). The ring of *snail1* RNA becomes broken at the beginning of gastrulation, as staining disappears from the central part of the embryonic shield. This suggests that *snail1* is repressed initially in the axial, presumptive notochordal and prechordal territories, and thus becomes restricted to the paraxial hypoblast. At the same time, *no tail* transcripts disappear from hypoblast cells except in the axial territory and continue to be detected at high levels in presumptive notochord (Schulte-Merker et al., 1992). Thus, *snail1* and *no tail* are initially expressed in the same set of cells, then as the shield forms, *snail1* turns off in one subset of cells and *no tail* turns off in the complementary subset of cells. Factors regulating this abrupt, complementary shift in gene expression have yet to be identified.

After gastrulation, *snail1* is no longer expressed in the lateral and ventral mesoderm. It is possible that lateral and ventral mesoderm might not get a dorsalizing signal emanating from axial cells that is needed to specify the paraxial hypoblast (Jessell and Melton, 1992; Moon and Christian, 1992).

(2) snail1 and somitogenesis

Snail1 RNA in adaxial cells

At the end of gastrulation, adaxial cells express *snail1* intensively while the adjacent paraxial cells contain reduced quantities of *snail1* transcript. The mechanism that controls intense *snail1* expression in adaxial cells is unknown. One reasonable hypothesis, to be discussed below, is that the axial mesoderm, which is in contact with adaxial cells, stimulates *snail1* expression in adjacent cells at this time. Extinction of *snail1* expression in adaxial cells is related to cell maturation: except for the most anterior somites, for which a delay is observed, the loss of expression of *snail1* in adaxial cells follows the anterior-posterior sequence of somite maturation. Adaxial cells form the muscle pioneer precursors, the first myotome cells to differentiate into muscle (Felsenfeld et al., 1991). Since *snail1* expression is only observed in undifferentiated cells, the repression of *snail1* expression in muscle pioneer precursors might reflect an obligatory step in muscle differentiation. This hypothesis predicts that ectopic expression of *snail1* in these cells might suppress muscle differentiation.

snail1 and segmentation

The segmentally repeated pattern of *snail1* expression appears to represent an up-regulation of *snail1* in cells of unsegmented paraxial mesoderm just anterior to the new somitic furrow, and a down-regulation of *snail1* in the anterior of the newly formed somite. Since this change in expression has already begun in the unsegmented segmental plate, *snail1* might be involved in the definition of the posterior border of newly forming somites. While *snail1* expression identifies the presumptive somite border two segments before the segmental furrow appears, a heat-shock sensitive process necessary for proper furrow formation occurs four segments before the furrow becomes visible (Kimmel et al., 1988). Since changes in *snail1* expression occur about an hour later than the heat-shock-sensitive step, *snail1* may not trigger segment specification; instead, it may be involved in an early but subsequent step in this process, perhaps the regulation of genes that cause morphogenetic events.

snail1 defines a posterior domain in the somite

Since *snail1* is expressed in the posterior three quarters of the somite and most of the somite is myotome (Kimmel et al., 1993), *snail1* is clearly expressed in at least a portion of the myotome. In more mature somites, *snail1* RNA is detected in a group of superficial cells assuming a position expected for dermis, and in a second group of cells along the notochord that are probably sclerotome (data not shown). Thus, *snail1* seems to be expressed in a portion of all three somite compartments.

Rather than distinguishing myotome, dermatome and sclerotome, *snail1* seems to define a posterior domain in the somite. This suggests that mesoderm segmentation in vertebrate embryos may be analogous in some ways to segmentation in *Drosophila* embryos, where gene expression patterns define anterior and posterior compartments in each segmental unit, the parasegment (Martinez-Ariaz and Lawrence, 1985; Lawrence, 1989). The proposed analogy is that *snail1* defines the posterior domain of each somite and a different gene or genes define the complementary anterior domain. Such genes have not yet been identified. We predict that anterior domain genes would repress *snail1* in anterior cells and perhaps stimulate *snail1* in adjacent cells. Other genes would define segment identity (i.e., Wilkinson and Krumlauf, 1990).

Expression of *snail1* in mutant embryos

The *spadetail* phenotype involves an inability of paraxial mesoderm (i.e., *snail1*-expressing cells) to converge dorsally during gastrulation (Kimmel et al., 1989; Ho and Kane, 1990). This raises the hypothesis that *spadetail* is defective in *snail1* function. The earliest detected phenotype in *spadetail* embryos is in the pattern of *snail1* expression. Work is in progress to see if *spadetail* embryos lack normal *snail1* function; if so, then injecting *snail1* RNA into *spadetail* embryos could rescue the mutant phenotype.

Analysis of *snail1* expression in *no tail* embryos suggests that mesoderm formation in zebrafish has an anterior and a posterior phase as it does in *Xenopus* (Cunliffe and Smith, 1992). The anterior (head and thorax) distribution of *snail1*

RNA is normal in *no tail* embryos, although its quantity is reduced. That result suggests that a *no tail*-dependent *snail*-stimulating signal may come from notochordal cells or, alternatively, that a *no tail*-*snail1* interaction occurs earlier in the tail bud at the time when both genes are expressed in the same cells. In contrast, the posterior (tail) expression of *snail1* is abolished in *no tail* embryos. These genetic results suggest that *no tail* may act upstream of *snail1* as a positive regulator. This conclusion supports the finding that injecting *Xenopus* embryos with RNA from the *Xenopus no tail* homologue *Xbra* stimulates the frog's *snail* family homologue (Cunliffe and Smith, 1992).

snail1 function

Analysis of wild-type and mutant embryos suggests that *snail1* is involved in several morphogenetic events, including involution and convergence during gastrulation, invagination of the somitic furrow and the condensation of pharyngeal cartilages. *snail1* may regulate the expression of genes needed for cell mobility or adhesivity during these morphogenetic processes. Alberga et al. (1991) have proposed that *snail* is involved in morphogenetic movements during *Drosophila* embryogenesis. Ectopic expression of *snail1* is being used to test this hypothesis.

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