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The expression of a zebrafish gene homologous to *Drosophila snail* suggests a conserved function in invertebrate and vertebrate gastrulation

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SUMMARY

Snail, a zinc finger protein, is required for the formation of the ventral furrow and the mesoderm during gastrulation of the *Drosophila* embryo. *snail* homologues have been cloned from *Xenopus* and mouse. We have isolated a zebrafish homologue of *snail*, designated *sna-1*. Like its *Drosophila* counterpart, Sna-1 protein is nuclear. Maternal and zygotic *sna-1* transcripts are ubiquitously distributed in zebrafish embryos of cleavage and blastula stages. In gastrulating embryos, *sna-1* is expressed in involuting cells of the germ ring, but not in those at the dorsal midline, the presumptive notochordal region. After involution, the expression is maintained in the paraxial mesoderm and becomes prominent in the muscle pioneer precursors, followed by expression at the posterior somite boundaries. Later, *sna-1* is expressed in neural crest and mesodermal derivatives of the head region. Sna-1 expression is induced in animal cap cells by activin A. The early *sna-1* expression pattern in gastrulating zebrafish *no tail* (*ntl*) mutant embryos is normal except a reduction in the level of *sna-1* transcription, suggesting that Ntl protein is not the key activator of *sna-1* transcription in vivo, but might be involved in the enhancement or maintenance of *sna-1* transcription. Data obtained in studies with ectopic *ntl* expression support this model.

Key words: zebrafish, gastrulation, mesoderm, somitogenesis, *snail, no tail, Brachyury,T*

INTRODUCTION

During gastrulation, the embryonic tissue of the blastula becomes subdivided into three distinct germ layers, the ectoderm, endoderm and mesoderm. Germ layer formation and cell specification during gastrulation is coupled to specific cell movements. In zebrafish, three morphogenetic movements can be distinguished: epiboly, involution and dorsal convergent extension (Warga and Kimmel, 1990). During epiboly, the blastoderm thins and spreads over the yolk towards the vegetal pole. During involution, cells migrate around the blastodermal margin, causing the blastoderm to fold under itself and to become bilayered. The outer layer, the epiblast, gives rise to ectodermal derivatives, the inner layer, the hypoblast, forms endodermal and mesodermal derivatives (Warga and Kimmel, 1990). Dorsally involuting cells give rise to anterior/dorsal structures, while laterally and ventrally involuting cells form posterior/ventral structures (Kimmel et al., 1990). Dorsal convergence brings the cells of the epiblast and the hypoblast to the dorsal side of the embryo, resulting in the formation and the elongation of the embryonic axis (Warga and Kimmel, 1990).

Two zebrafish mutants have been described that show defects in gastrulation and mesoderm formation. In *spadetail* (*spt*) mutant embryos, laterally involuting cells fail to converge to the dorsal side, causing a lack of trunk somites (Kimmel et al., 1989; Ho and Kane, 1990). *no tail* (*ntl*) mutant embryos fail to form sufficient mesoderm and lack the notochord and all posterior structures. Furthermore, they show defects in the muscle pioneer cells of the trunk somites, indicating that the differentiation of these cells depends on signalling properties of the differentiated notochord (Halpern et al., 1993). The phenotype of *ntl* is very similar to that of mouse embryos mutant in the *brachyury* (*T*) gene (Chesley, 1935; Yanagisawa et al., 1981; Herrmann et al., 1990). The zebrafish homologue of the *T* gene was cloned and shown to be identical with the *ntl* gene (Schulte-Merker et al., 1992, 1994b).

The control of gastrulation and mesoderm induction in vertebrates have been most extensively studied in *Xenopus laevis*. The initial mesoderm-inducing signals are probably maternally supplied and appear to come from vegetal cells which themselves do not contribute to mesodermal structures (Nieuwkoop, 1969). There seem to exist at least two signals, one inducing dorsal, the other inducing ventral mesoderm. Factors have been found that induce presumptive ectodermal cells to form mesoderm in the animal cap assay. Activin A, a transforming growth factor β (TGF β), preferentially induces anterior/dorsal mesoderm; the basic fibroblast growth factor (bFGF) induces posterior/ventral mesoderm (for reviews, see Smith, 1989). Studies with dominant-negative FGF- and activin-receptors (Amaya et al., 1991; Hemmati-Brivanlou and Melton, 1992) provide evidence that the growth factors are involved in mesoderm induction in vivo.

In *Drosophila*, the control of gastrulation and mesoderm

formation has been elucidated based on mutational analysis (for review see Anderson, 1987; St. Johnston and Nüsslein-Volhard, 1992). Zygotically expressed genes have been identified that control the invagination of the presumptive mesoderm in the ventral furrow of the embryo. Embryos mutant for the genes *twist* (Thisse et al., 1988) or *snail* (Boulay et al., 1987) fail to form a ventral furrow and completely lack mesoderm, suggesting that the genes regulate downstream target genes that are involved in the determination of mesodermal fates as well as in the morphogenetic movements of invagination (Leptin and Grunewald, 1990).

After the finding that many developmental genes are conserved among invertebrates and vertebrates (see Discussion), *snail* homologues have been cloned and characterized from *Xenopus* (*xsna*, Sargent and Bennett, 1990), mouse (*msna*, Nieto et al., 1992; Smith et al., 1992) and zebrafish. Similar to *snail* in *Drosophila*, the vertebrate *snail* homologues are expressed in involuting cells, as they become mesodermal, suggesting a role in mesoderm formation and the morphogenetic movements of gastrulation similar to the function of *snail* in *Drosophila*.

Animal cap assays in *Xenopus* revealed that *xsna* and *xbra*, the *Xenopus T*-homologue, can be induced by activin A and bFGF, suggesting an in vivo regulation of the transcription factors by the peptide growth factors. *xbra* is induced directly as an immediate-early response (Smith et al., 1991), while it is not known whether the induction of *xsna* is mediated by another gene product (Sargent and Bennett, 1989). Ectopic expression of *xbra* leads to ectopic induction of *xsna* (Cunliffe and Smith, 1992), suggesting a regulatory cascade with activin inducing *xbra* (*T*), which in turn induces *xsna*. We show that *sna-1* expression is induced by activin A in animal caps. However, this induction, as well as the early *sna-1* expression in vivo, is independent of *ntl*. The early coexpression of zebrafish *ntl* and *sna-1* in a simple pattern in the same population of cells suggests that these genes might instead respond to the same inducing signals in a parallel pathway.

MATERIALS AND METHODS

Isolation and characterization of cDNA clones

A zebrafish gastrula cDNA library in Lambda Zap (Stratagene) was screened with a ^{32}P-labelled, randomly primed probe derived from a *Xenopus xsna* cDNA clone. Replica filter lifts were taken using positively charged nylon membranes (Pall Biodyne B). Hybridisation was performed overnight at 55°C in 5× SSPE, 1% SDS, 5× Denhardt's solution, filters were washed at 58°C in 4× SSPE, 1% SDS. 13 phage clones were isolated and recombinant Bluescript SK plasmids were recovered by in vivo excision according to suppliers' instructions (Stratagene). Eight different clones were identified by restriction analysis. About 300 nucleotides at both ends of the inserts were sequenced using vector specific primer. Clones with an open reading frame were completely sequenced by primer walking. Both strands of the 1.8 kb *sna-1* cDNA of the plasmid pBS-Sn3 were sequenced. Sequencing was performed with an ALF sequencer (Pharmacia). Specific primers were synthesized with a Pharmacia Gene assembler.

Fish strains

All studies, unless noted otherwise, were performed with embryos from wild-type strains that have been kept and inbred several times in the Tübingen laboratory. The *spadetail* mutant line *spt^{b104}* and the *no tail* mutant lines *ntl^{b160}* and *ntl^{b195}* were kindly provided by the laboratory of C. B. Kimmel (Eugene, Oregon) and were outcrossed several times to the Tübingen wild-type strains. *spt* mutant embryos and *ntl* mutant embryos of postgastrula stages used for in situ hybridisation were identified by their mutant phenotype, *ntl* mutant embryos of gastrula stages were diagnosed with anti-Ntl antibody.

Preparation of RNA and northern analysis

Total RNA of 300 or more embryos and of adult males was prepared by the hot phenol method (Schulte-Merker et al., 1992). For northern analysis, 5-10 μg of total RNA were loaded on 1.4% agarose (BRL) formaldehyde gels and run under denaturing conditions. After high-salt transfer onto Hybond N membranes (Amersham), filters were baked at 80°C for 20 minutes, exposed to UV light for 1 minute and stained with methylene blue in order to visualize the rRNAs for loading control. Hybridisation was performed overnight at 68°C in 7% SDS, 0.3 M sodium phosphate buffer pH 7.0, 10 mM EDTA and filters were washed at 68°C in 1% SDS, 40 mM sodium phosphate buffer pH 7.0. ^{32}P-labelled probe was obtained by randomly primed DNA polymerisation using the *Bam*HI-*Hin*dIII fragment of pBS-Sn3 (nucleotides 1-933) as template. Unincorporated nucleotides were removed by gel filtration (Sepharose CL 6B).

Production and purification of antibodies

The His-tag vector system (Quiagen) was used to express the full-length Sna-1 protein (pQE12-sn) and a fusion protein of dihydrofolate reductase and the N-terminal part of Sna-1 without the zinc finger domain (pQE16-snΔ114), respectively. Using the appropriate primers for oligonucleotide site-directed mutagenesis, the required restriction sites were introduced into the cDNA by polymerase chain reaction. Recombinant plasmids were transformed into *E. coli* M15 cells (Quiagen). Cells were harvested 5 hours after induction of protein expression. Recombinant Sna-1 protein was purified by affinity chromatography with Ni-NTA-Agarose under denaturing conditions according to suppliers' instructions (Quiagen). For immunization of rabbits, Ni-purified protein was further purified by preparative SDS-PAGE.

For affinity purification of the antisera, 10 mg of Ni-purified recombinant full-length Sna-1 protein were coupled to CNBr-Sepharose (Sigma) in the presence of 100 mM $NaHCO_3$, 500 mM NaCl, 6 M guanidine hydrochloride pH 8.3. Antisera were loaded onto the affinity column in the presence of 0.5 M NaCl, 0.1% Tween 20, and the antibodies were eluted with 0.1 M glycine pH 2.5. Collected fractions were neutralized with Tris-HCl pH 8.0, and BSA, glycerol and azide were added.

Western blot analysis

12.5% acrylamide gels were run at constant current (30 mA) using the buffer system described by Laemmli (1970). Blotting was performed for 45 minutes at constant current (400 mA) using immobilon-P membranes (Millipore) essentially as described by Towbin et al. (1979). After transfer, membranes were blocked at least 2 hours in blocking buffer (BB: 100 mM Tris-HCl pH 9.2, 300 mM NaCl, 0.1% Tween 20) + 10% milk powder, followed by incubation with 10^3-fold diluted antibody in BB + 5% milk powder. After four washes in BB + 5% milk powder, signal detection was carried out with peroxidase-coupled goat anti-rabbit antibodies and the ECL system (Amersham) according to suppliers' instructions.

Whole-mount staining of embryos and sectioning

Whole-mount in situ hybridisation with digoxigenin-labeled RNA probes and whole-mount antibody stainings were performed as previously described (Schulte-Merker et al., 1992). For *sna-1*/Ntl

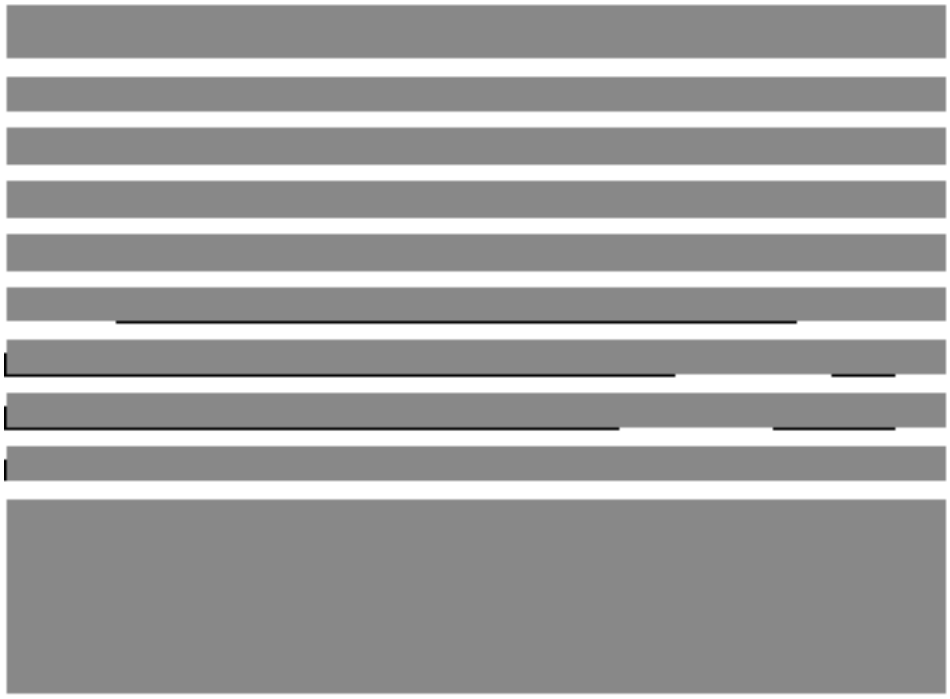


Fig. 1. Sequence of zebrafish *sna-1*. The DNA sequence of the coding strand of the cDNA of pBS-Sn3 and the predicted amino acid sequence of the coding region using the one letter amino acid code are shown. An in frame stop codon upstream the translation initiation codon and a polyadenylation signal in the 3′ untranslated region are underlined. The four zinc fingers are boxed.

double stainings, embryos first underwent complete in situ hybridisation, followed by complete antibody staining.

To show that *sna-1* expression starts at the dorsal side, dome-stage embryos were divided into three fractions. One fraction was hybridised with a *sna-1* probe, the second with a *gsc* probe (Schulte-Merker et al., 1994a) and the third with both the *sna-1* and the *gsc* probe. The fact that, in all three fractions, the in situ signal came up at one side of the embryos was taken as indication for an expression of *sna-1* and *gsc* at the same side of the embryo.

Embryos were orientated with the help of bridged coverslips or spread after manual removal of the yolk. Sections of embryos were taken after whole-mount staining. In situ hybridized embryos first were incubated for 2 hours at room temperature in 4% paraformaldehyde/PBS in order to fix the stain of the alkaline phosphatase colour reaction. Embryos were embedded in Technovit 7100 (Kulzer, FRG) and 5-8 mm sections were cut. Sections were mounted in DPX mountant (Fluka). Photographs were taken at an Axiophot photomicroscope (Zeiss).

RNA injection

Plasmid pBSCT-ZFc1 (Schulte-Merker et al., 1992) was linearized with *Bam*HI, pBS-Sn3 with *Kpn*I. Following the protocol of Driever et al. (1990), capped sense RNAs were obtained using T3 RNA polymerase. The transcripts thus obtained could be translated in vitro. Embryos were injected as described by E. S. Weinberg in Westerfield (1993). Stability of the injected RNA was controlled on northern blots and its in vivo translation was tested on western blots and by whole-mount antibody stainings.

Animal cap assay

The animal-most quarter of 3.5-hour-old embryos was cut off using an eyelash and transferred to agarose-coated Petri dishes containing incubation medium with or without 50 u/ml human activin A. The incubation medium used was LDF supplemented with 1% FCS (Gibco), 0.4% trout serum and 25 mg/ml trout embryonic extract (Collodi et al., 1992). After incubation for 4-5 hours, animal caps were subjected to immunostaining as described for whole embryos (Schulte-Merker et al., 1992).

RESULTS

Isolation and characterization of sna-1 cDNA clones

The *xsna* cDNA was used to screen a zebrafish gastrula cDNA library at low stringency. Of 3×10^5 phage clones screened, two code for a *snail* homologue, designated *sna-1*. The longer 1.8 kb clone (pBS-Sn3, Fig. 1) contains 115 nucleotides of 5′ untranslated region, a 3′ poly(A)-tail and an open reading frame encoding a putative protein of 260 amino acids with a calculated relative molecular mass of 28.6×10^3 and four zinc fingers in its C-terminal part. There is an in frame stop codon 7 triplets upstream of the first Met codon, indicating that this ATG triplet is the initiation codon for translation of zebrafish *sna-1*. The N-terminal part of Sna-1 protein contains small regions shared with mouse Msna and *Xenopus* Xsna (Fig. 2), but bears no significant homology to the *Drosophila* Snail and Escargot proteins (Whiteley et al., 1992). However, all proteins show significant homology in the zinc finger region, although the first zinc finger is missing in the mouse and the zebrafish gene (Fig. 2). Amino acid sequence comparison of zinc fingers 2-5 reveals 88% identity to *xsna*, 83% to *msna*, 71% identity to *Drosophila snail* and 82% to *Drosophila escargot*.

Temporal expression of *sna-1*

Northern blot analysis of RNA (Fig. 3) reveals a single *sna-1* transcript of about 1.9 kb in length. *Sna-1* message can be detected in cleavage and early blastula stages before zygotic transcription starts (Kane and Kimmel, 1993), suggesting that the transcripts are supplied maternally. In the sphere stage, slightly after midblastula transition, the abundance of *sna-1* mRNA is significantly higher, suggesting that zygotic *sna-1* transcription starts in blastula stages.

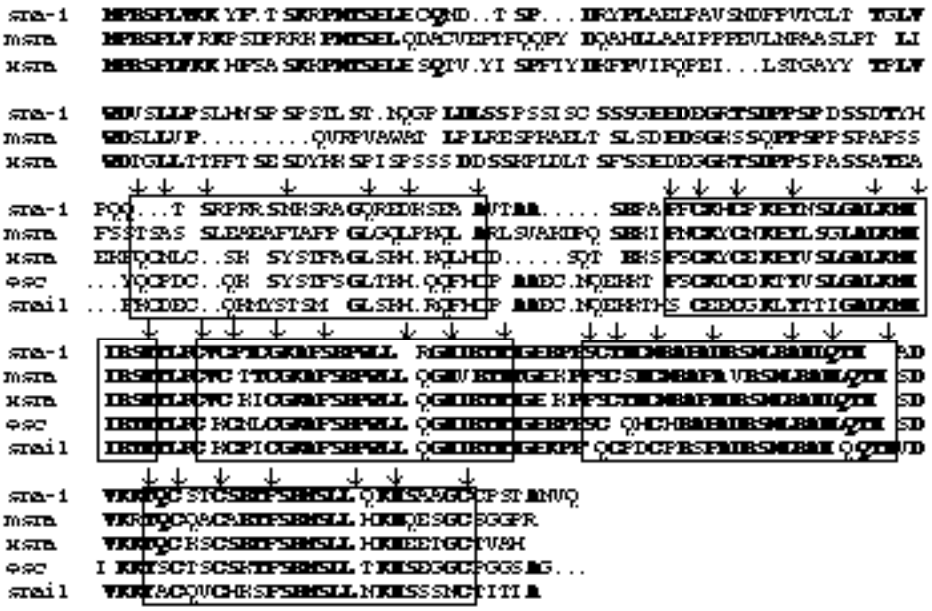


Fig. 2. Sequence comparison between the predicted amino acid sequences of *snail*-related proteins. Sequences of the full-length vertebrate proteins zebrafish *sna-1*, mouse *msna* and *Xenopus xsna* and the sequences of the zinc finger domains of *Drosophila escargot* and *Drosophila snail* are aligned. Bold letters indicate amino acid identity compared with *sna-1*. Zinc fingers 1-5 are marked by open boxes. Arrows indicate amino acid residues conserved in many zinc fingers. *sna-1* and *msna* lack the key residues of the first zinc finger found in *xsna*, *snail* and *escargot.*

The abundance of *sna-1* mRNA continuously increases until the onset of gastrulation, is maximal in stages of midgastrula and drops continuously during later gastrula, neurula and somite stages. *sna-1* mRNA can still be detected in 1-, 2- and 3-day-old zebrafish larvae, but cannot be found in older embryos and adult males.

Affinity-purified polyclonal antibodies raised against full-length Sna-1 protein expressed in *E. coli* specifically detect recombinant Sna-1 protein on western blots (Fig. 4A) and specifically immunoprecipitate Sna-1 protein expressed in reticulocyte lysate (not shown). Western blot analysis with zebrafish embryonic extracts (Fig. 4B) shows that the temporal distribution of the Sna-1 protein reflects that of the mRNA.

Further analysis of temporal and spatial *sna-1* expression was performed by whole-mount in situ hybridisation and whole-mount antibody stainings.

sna-1 is ubiquitiously expressed in blastula stages

In cleavage and blastula stages, before and after the onset of zygotic *sna-1* transcription, the *sna-1* mRNA is ubiquitously distributed in the embryo (Fig. 5A). In apparently all cells of the blastula embryos, Sna-1 protein is localized in the nucleus. Differences in the nuclear Sna-1 staining among different cells of later blastula stages are due to the asynchrony of the cell cycle: in noncleaving cells the nuclear DAPI fluorescence is quenched by the Sna-1 antibody staining, while cells lacking Sna-1-positive nuclei show unquenched DAPI-positive mitotic figures (Fig. 5B). Shortly before gastrulation (dome stage), the ubiquitous expression of *sna-1* mRNA decreases, until it is completely gone by the onset of gastrulation.

sna-1 expression during gastrulation

Patterned *sna-1* expression begins in the dorsal marginal zone, before rapidly spreading circumferentially along the whole margin of the embryo. In the dome stage, *sna-1* mRNA is restricted to the dorsal margin of the embryos (Fig. 5C). The dorsal side was identified by simultaneous staining for *goosecoid* (*gsc*) mRNA, which is restricted to the dorsal side (Schulte-Merker et al., 1994a) (see Material and methods, not shown). By 30% epiboly, *sna-1* is expressed as a ring around the whole marginal zone of the embryo with highest expression at the dorsal side. There, marginal cells express *sna-1* prior to their involution (Fig. 5D). After onset of involution at 50% epiboly, *sna-1* is uniformly expressed around the whole ring (Fig. 5E) in the cells of the hypoblast, during and after their involution (Fig. 5F).

By 60% epiboly (Fig. 5G), *sna-1* mRNA can no longer be detected in involuting cells at the dorsal midline. However, *sna-1* continues to be expressed in involuting cells in more lateral and ventral positions. Among the cells that expressed *sna-1* during their involution, *sna-1* expression is maintained in cells forming the paraxial trunk mesoderm, while *sna-1* mRNA can no longer be detected in all other cells shortly after their involution (Fig. 5H). In somitogenesis stages, one row of cells within the paraxial trunk mesoderm adjacent to each side of the presumptive notochord becomes more prominently positive for *sna-1* (Fig. 6A,C,E,F). From this cell population, the muscle pioneer cells are drawn (Felsenfeld et al., 1991). Additionally, *sna-1* is highly expressed in the tailbud, which develops after closure of the germ ring and where the tail mesoderm is formed (Fig. 6A,E,F,J).

sna-1 expression during somitogenesis and cephalic muscle and cartilage formation

During somite formation, the paraxial *sna-1* expression in the hypoblast is organized into a segmented pattern (Fig. 6E). In an intermediate stage, no *sna-1* expression can be detected in the loosely organized cells inside the forming somites, while the epithelial cells at the anterior and the posterior boundary of the somites and the muscle pioneer precursors express it at low level (Fig. 6E, small photo). Shortly after formation of the somite, the expression in the muscle pioneer precursors and in the cells of the anterior

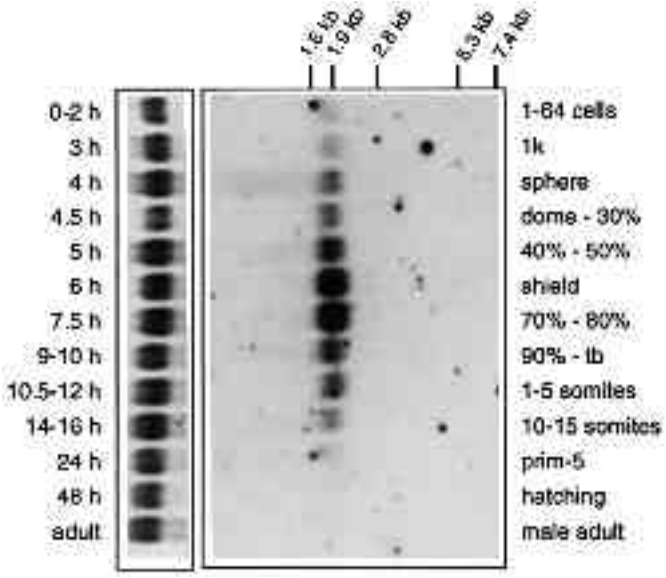


Fig. 3. Temporal expression of *sna-1* mRNA. 10 μg of total RNA of the indicated stages were blotted. Staging was done according to Westerfield (1993). A single transcript of about 1.9 kb was detected upon probing with the 933 bp *Bam*HI-*Hin*dIII fragment of pBS-Sn3. The left panel shows methylene blue staining of ribosomal RNA of the same blot.

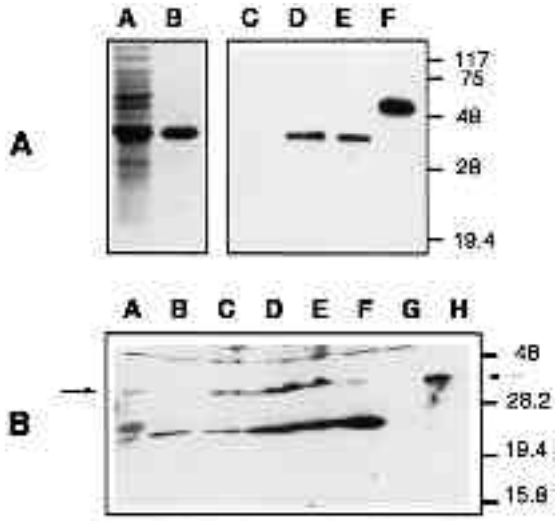


Fig. 4. (A) Specificity of anti-Sna-1 antibodies. Coomassie-blue-stained SDS-PAGE gel (lanes A, B) and Western blot (lanes C-F) showing total protein from induced (lanes A,D) and uninduced bacteria (lane C) carrying expression vector pQE12-sn, and Ni-NBT purified protein from pQE12-sn containing (lanes B,E) and pQE16-snΔ114 containing bacteria (lane F). The band with an apparent M_r of 39×10^3 was used to raise the antiserum.
(B) Temporal expression pattern of Sna-1 protein. Western blot showing Ni-NBT-purified protein of pQE12-sn carrying bacteria (lane H) and total protein equivalent to 2-3 zebrafish embryos of following stages: 1-64 cells (lane A), sphere (lane B), shield (lane D), tailbud (lane E), 1 day (lane F) and 6 days (lane G). The zebrafish Sna-1 band was identified by comparison of the protein pattern of sphere-stage embryos, which were either untreated (lane B) or injected with pBS-sn3 encoded *sna-1* sense RNA in the 1-cell stage (lane C). The injection leads to the exclusive increase of one band of an apparent M_r of 39×10^3 which comigrates with the pQE12-sn expressed recombinant protein.

somite epithelium decreases, whereas the *sna-1* expression in the posterior region of the somites becomes significantly stronger (Fig. 6F).

In later forming somites from the 7th somite onwards, the complete rearrangement of the *sna-1* expression pattern is accomplished 40 minutes after somite formation, the time required to form two new somites. In contrast, the rearrangement of the first 5-6 somites arrests in the intermediate stage described above (Fig. 6E, small photo). These somites continue the rearrangement synchronously at the 7- to 8-somite stage (Fig. 6E). This is in line with the synchronous onset of the expression of an *engrailed*-like gene in the muscle pioneer cells of the first 7-8 somites in 10- to 11-somite-stage embryos (Hatta et al., 1991; Ekker et al., 1992).

Somitic *sna-1* mRNA disappears in a rostrocaudal wave beginning at 18 hours after fertilization. In 1-day-old embryos, *sna-1* is expressed in ventral lateral positions of the head region except the region of the trigeminal ganglion and the otic placode (Fig. 7A). From lineage analysis studies, it is known that the cells of this region derive from the neural crest and the paraxial head mesoderm (Schilling, 1993). In hatched larvae (2 days after egg deposition), *sna-1* mRNA is found in the lateral and medial parts of the mandibular and the hyoid (1. and 2. pharyngeal arch), while the central regions of the arches are devoid of *sna-1* mRNA (Fig. 7B). *sna-1* mRNA is also detected in medial regions of the gill arches (Fig. 7C). The *sna-1* expression reflects the course of cartilage differentiation, which starts in central regions of the arches and proceeds laterally and medially (Schilling, 1993), suggesting that *sna-1* is expressed in presumptive cartilage cells of the arches and turned off as the cells differentiate. Furthermore, *sna-1* is expressed in two parallel bands of muscle precursor cells in the pectoral fin buds of larvae 2.5 days after egg deposition (Fig. 7D). The myoblasts of the fin buds are supposed to derive from invading myotomal cells as in chick (Solursh et al., 1987).

sna-1 expression is affected in *spt* mutant embryos

As shown above, cells of the paraxial trunk mesoderm express *sna-1* from the time that they involute in lateral positions. In *spt* mutant embryos, laterally involuting cells fail to converge during gastrulation. They finally end up in an enlarged tailbud so that somites are missing in the trunk, but are present in the tail (Kimmel et al., 1989; Ho and Kane, 1990). In *spt* mutant embryos of the 4-somite stage, the *sna-1* staining in paraxial positions and the *sna-1*-positive lines adjacent to the notochord are absent (Fig. 6H). Only in the posterior-most region of the hypoblast can two faint lateral *sna-1*-positive stripes be detected. These might correspond to paraxial cells that have reached their position after closure of the germ ring independent of convergent movements and which give rise to the tail somites. The tailbud of mutant embryos is much more strongly stained than in wild-type siblings (Fig. 6H,M versus E,J). Thus, the alteration of the *sna-1* pattern in gastrulating *spt* mutant embryos exactly reflects the described defects of the mutants in the morphogenetic movements of gastrulation.

After early coexpression, *ntl* and *sna-1* develop complementary expression domains

A number of genes have been identified that are expressed in the presumptive mesodermal region during early gastrulation. *ntl*, the zebrafish homologue of the mouse *T* (*brachyury*) gene, is expressed in the germ ring in the same pattern as *sna-1*. While descendants of lateral regions of the germ ring abolish the *ntl* expression after their involution, its expression is maintained in the presumptive notochordal cells at the dorsal midline (Schulte-Merker et al., 1992). At that time, this region is devoid of *sna-1* expression. We analyzed the relationship of *ntl* and *sna-1* expression more thoroughly by staining for *sna-1* mRNA and Ntl protein in the same embryos.

Ntl protein can be detected in marginal cells on the dorsal side of late blastulas before the onset of the margin-specific *sna-1* transcription. In dome-stage embryos, when the first *sna-1* transcripts can be detected in dorsal marginal cells, the *ntl* expression has spread around the whole margin of the embryo. In germ ring embryos (Fig. 8D), *sna-1* and *ntl* are expressed in all involuting cells of the germ ring. Cross sections through the germ ring (Fig. 8C) reveal that cells in the marginal epiblast before their involution express *ntl*, but not yet *sna-1*, the expression of which is restricted to the cells in the hypoblast. This indicates that also in these later involuting cells *sna-1* expression is preceded by expression of *ntl*.

Dorsal cells that involute in later stages of gastrulation and lack *sna-1* mRNA maintain *ntl* expression after their involution (Fig. 8E,F). However, in paraxial cells, which coexpressed *ntl* and *sna-1* during involution, only *sna-1* expression can be detected later on. This leads to a clear separation of *ntl* and *sna-1* expression in the developing mesoderm. *Ntl* is expressed in the axial mesoderm and *sna-1* in the paraxial trunk mesoderm (Fig. 8F,G,H).

sna-1 expression is progressively reduced in *ntl* mutant embryos

Ntl mutant embryos lack the tail and a differentiated notochord. Both isolated *ntl* alleles, ntl^{b160} and ntl^{b195}, have *ntl* mRNA, but lack functional Ntl protein (Schulte-Merker et al., 1992, 1994b). The observation that the onset of *sna-1* transcription in involuting cells is slightly preceded by the onset of *ntl* expression (Fig. 8C), as well as the induction of ectopic *xsna* transcription after ectopic *xbra* expression in *Xenopus* (Cunliffe and Smith, 1992), suggested that *ntl* might activate the *sna-1* transcription. However, in *ntl* mutant gastrulas, the expression pattern of *sna-1* is very similar to that in wild-type siblings, although the *sna-1* signals appear to be fainter (Fig. 8A,B versus D,E). The weaker expression of *sna-1* in *ntl* mutant embryos is significant: in gastrulating embryos of the shield stage, when mutant and wild-type embryos cannot yet be distinguished

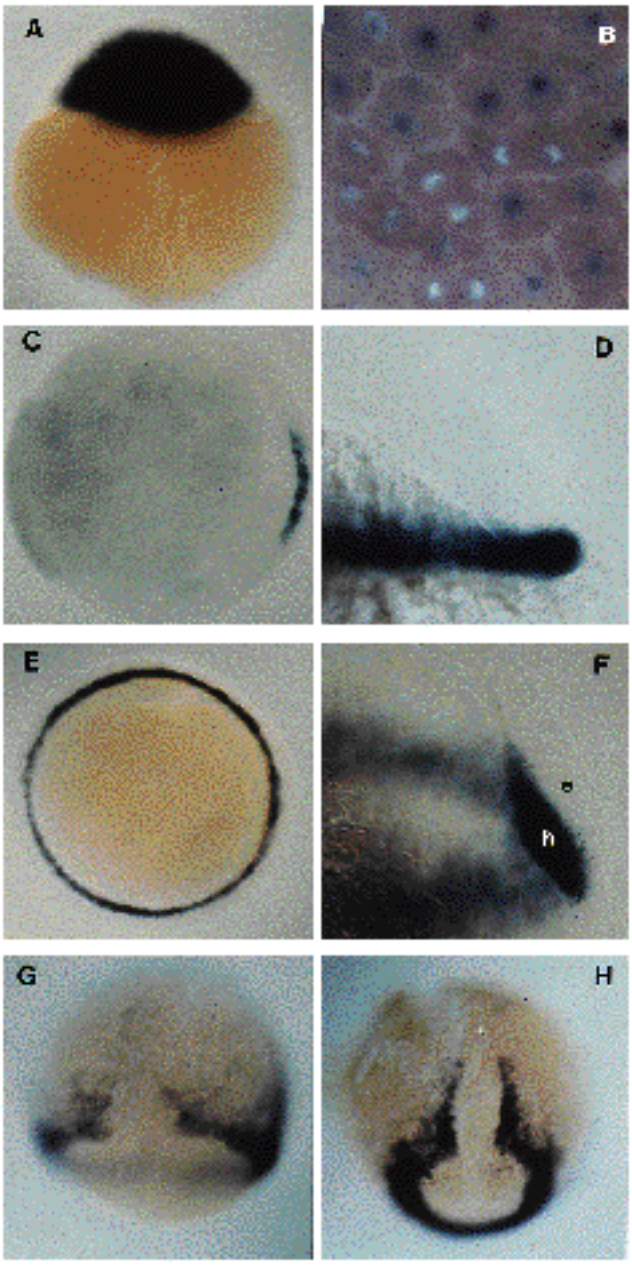
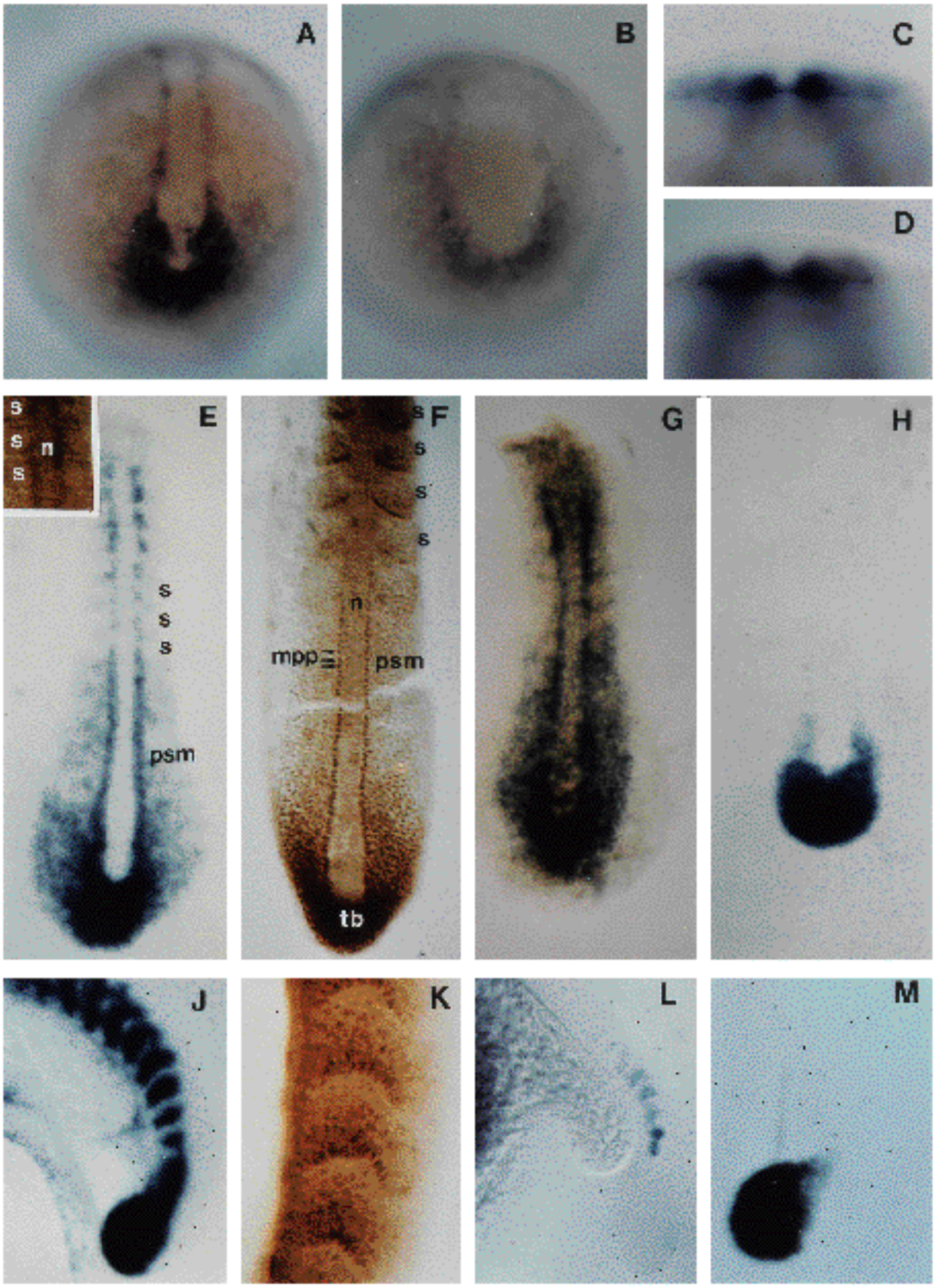


Fig. 5. Expression of *sna-1* in wild-type embryos before and during gastrulation, investigated by whole-mount in situ hybridisation (A,C,D,E,F,G,H) and anti-Sna-1 antibody staining (B). (A) Sphere stage, lateral view; (B) sphere stage, animal view, counterstained with DAPI; (C) dome stage, animal view, dorsal right; (D) 30% epiboly, lateral view, dorsal right; (E) germ ring stage, animal view, dorsal right; (F) germ ring stage; lateral view; (G) 60% epiboly, dorsal view; (H) 80% epiboly, dorsal view. Abbreviations: epiblast (e), hypoblast (h). For details see text.

Fig. 6. Expression of *sna-1* in wild-type, *spt* and *ntl* mutant postgastrula embryos, investigated by whole-mount in situ hybridisation (A,B,C,D,E,G,H,J,L,M) and anti-Sna-1 antibody staining (E, small photo, F,K). (A,B) 95% epiboly, dorsal view: (A) wild-type, (B) *ntl*. (C,D) 4-somite stage, optical cross section at level of presomitic mesoderm: (C) wild-type, (D) *ntl*. (E, small photo) 5-somite stage, dorsal view on somites. (F) 15-somite stage, spread wild-type embryo, dorsal view on posterior part of the embryo up to somite 12. (E, big photo, G,H) 8-somite stage, dorsal view, spread embryo: (E) wild type; (G) *ntl* ; (H) *spt*. (K) 15-somite stage, wild type, lateral view on somites 10-13. (J,L,M) 22 hour embryos, lateral view of the tail and the posterior part of the trunk; borders of embryo outlined by dots: (J) wild-type; (L) *ntl* ; (M) *spt*. Abbreviations: muscle pioneer precursor (mpp), notochord (n), somite (s), tailbud (tb). For details see text.

by morphological criteria, we could enrich for *ntl* mutants by sorting embryos with weaker *sna-1* staining after in situ hybridisation (60% *ntl* in the sorted fraction versus 27% total, Table 1). As an unambiguous identification, however, was not possible, the influence of *ntl* on the level of *sna-1* expression is low. From late stages of gastrulation onwards,



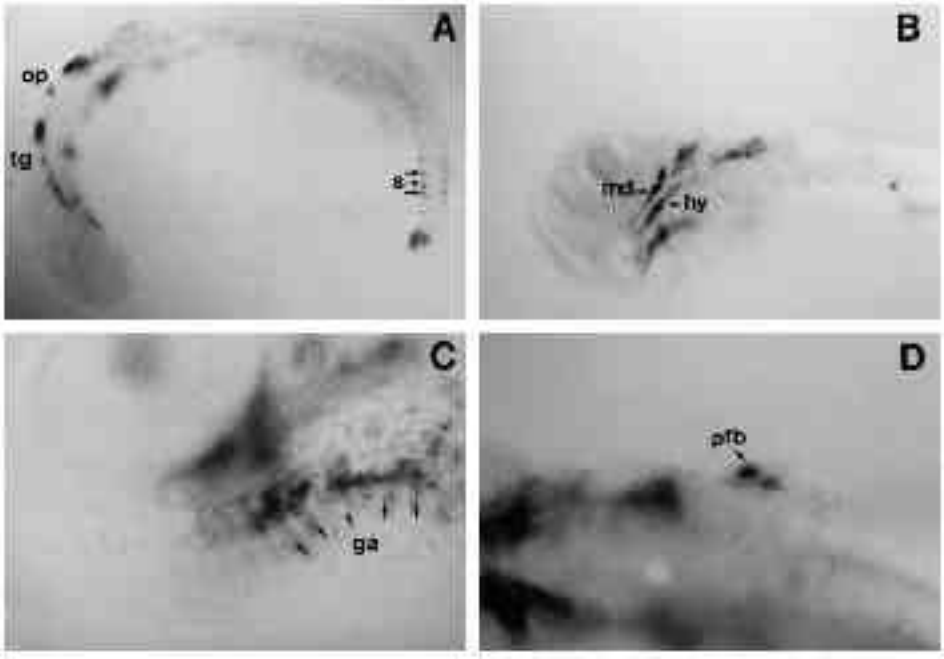


Fig. 7. Expression of *sna-1* in the head region and the pectoral fin buds investigated by whole-mount in situ hybridisation. (A) 1 day, lateral view; *sna-1* is expressed in the tip of the tail, the posterior somites and in ventral lateral regions of the head sparing the trigeminal ganglion and the otic placode. (B,C) 2 days, lateral view; *sna-1* is predominantly expressed in lateral regions of mandibular and hyoid (B), and in medial parts of all arches including the 5 gill arches (C). (D) 2.5 days, dorsal view; *sna-1* expression in the two presumptive muscle bundles of the pectoral fin bud. Abbreviations: trigeminal ganglion (tg), otic placode (op), somites (s), mandibular (md), hyoid (hy), gill arches (ga), pectoral fin bud (pfb).

ntl and wild-type embryos are morphologically distinguishable. In these stages, the *sna-1* expression in *ntl* embryos is significantly lower than in wild-type siblings (Fig. 6B versus A). The expression pattern, however, is relatively normal: as in the wild type, two rows of cells adjacent to the dorsal midline predominantly express *sna-1* (Fig. 6D,G versus C,E), followed by *sna-1* expression in the posterior part of differentiated somites (Fig. 6L). Striking differences in the *sna-1* expression pattern are visible in older embryos: while the tailbud is less prominently stained in *ntl* mutant embryos after closure of the germ ring, no *sna-1* staining at all is left in the tip of the 'tail' of 22h *ntl* embryos (Fig. 6L versus J). However, this does not necessarily mean that in these late stages *ntl* is required for *sna-1* transcription. More likely, the absence of *sna-1* mRNA is due to a cessation of mesoderm formation in the tail tip of *ntl* mutant embryos, which also is indicated by the absence of *ntl* mRNA as a marker for newly forming mesoderm (Schulte-Merker et al., 1994b; not shown). Thus, although the level of *sna-1* expression is progressively reduced in *ntl* mutant embryos, the *sna-1* expression pattern itself appears to be virtually independent of *ntl*.

Table 1. Level of *sna-1* expression in wild-type and *ntl* mutant shield-stage embryos

	Ntl antibody staining		
sna-1 in situ	No. of embryos	No. of wild type	No. of *ntl*
Faint signal	30	12 (40%)	18 (60%)
Normal signal	87	73 (84%)	14 (16%)
∑	117	85 (73%)	32 (27%)

Offspring of two *ntl* heterozygous fish were fixed in shield stage and in situ stained for *sna-1* mRNA. The quarter of the embryos with the faintest *sna-1* signal was sorted. All embryos were counterstained with anti-Ntl antibodies to identify their genotype. If the level of *sna-1* expression were unambiguously lower in *ntl* mutants, 100% of the embryos in the fraction with the fainter signal should be *ntl*. If the expression were indistinguishable between wild-type and mutant embryos, 25% of this fraction should be *ntl*. Here, we get 60% *ntl* embryos. Similar results were obtained in further experiments.

Ectopically expressed *ntl* is not sufficient to induce, but might enhance *sna-1* expression

In order to test whether the lower degree of *sna-1* expression in *ntl* mutant embryos might be due to a maintenance or enhancement of *sna-1* transcription by Ntl protein, Ntl protein was ectopically expressed by the injection of *ntl* sense RNA into one cell of 1- to 4-cell-stage wild-type embryos. Injected embryos were fixed at different stages and double stained for Ntl protein and *sna-1* mRNA. In embryos of the germ ring stage, the intensity of the Ntl antibody staining was similar in nuclei ectopically expressing Ntl and in marginal nuclei with endogenous Ntl expression, suggesting that ectopic Ntl was expressed at physiological levels. Ntl and *sna-1* are coexpressed in the germ ring as in uninjected embryos. In more animal regions, ectopic Ntl expression does not lead to the production of *sna-1* mRNA (Fig. 9A). However, an effect of *ntl* overexpression on *sna-1* mRNA levels can be demonstrated in embryos of pregastrula stages, when the marginal expression of *ntl* and *sna-1* is still low: in the injected region, the *sna-1* staining is significantly stronger than in the uninjected region. The stronger expression is restricted to the margin, the expression domain of endogenous *sna-1* (Fig. 9B). Thus, at least in early stages of gastrulation, *ntl* is not sufficient to initiate, but appears to enhance the *sna-1* transcription.

sna-1 expression is induced by activin A

It has been previously shown that *ntl* is induced by activin in animal caps (Schulte-Merker et al., 1992). After our finding that *sna-1* activation is independent of Ntl protein in vivo, we studied whether *sna-1* and *ntl* might be induced in a parallel pathway responding to the same signals. Animal

caps incubated in activin A-containing medium express Ntl- and Sna-1 protein (Fig. 9C), while animal caps incubated in medium lacking the activin do not (Fig. 9D). Expression of Sna-1 protein is also induced in animal caps derived from *ntl* mutant embryos, which were identified by their failure to express Ntl protein upon treatment with activin (not shown).

DISCUSSION

We have described a zebrafish gene, *sna-1*, that encodes a zinc finger protein with striking similarity to the zinc finger domains of *Xenopus xsna*, mouse *msna* and the *Drosophila* genes *snail* and *escargot*. The zebrafish and mouse proteins lack a counterpart to the first zinc finger of *xsna*, *snail* and *escargot*. While to date only one *snail*-related gene was described in mouse and *Xenopus*, a second zebrafish *snail*-related gene has been isolated in addition to the gene described here (B. and C. Thisse and J. Postlethwait, personal communication), suggesting that, as in *Drosophila*, *sna-1* might be a member of a family of *snail*-related genes. However, the conservation of some regions of the *msna*, *xsna* and *sna-1* genes outside of the zinc finger domain might support the notion that *sna-1* is the homologue of the *Xenopus* and the mouse gene. Despite the higher structural similarity of *sna-1*, *xsna* and *msna* to *Drosophila escargot*, we assume that they are functionally more closely related to *Drosophila snail*, because all of them are expressed in the mesoderm, whereas *escargot* shows a complex and highly dynamic expression pattern primarily in ectodermal derivatives (Whiteley et al., 1992).

Comparison of the expression pattern of *sna-1* in zebrafish and *snail* in *Drosophila*

sna-1 expression in early zebrafish gastrulas and *snail* expression in early gastrulating *Drosophila* embryos both are restricted to involuting/invaginating cells, which give rise to mesodermal and endodermal derivatives. In gastrula stages of zebrafish embryos, all presumptive endodermal and mesodermal cells express *sna-1* during their involution, except dorsally involuting cells of stages from 60% epiboly onwards, which give rise to the notochord (Kimmel et al., 1990). Similarly, in gastrulating *Drosophila* embryos, *snail* is expressed in the invaginating presumptive mesodermal cells of the ventral furrow and in cells of the anterior midgut invagination, which give rise to anterior endodermal derivatives. Thus, the *snail*/*sna-1* expression in cells with similar fates in zebrafish and *Drosophila* suggests that, despite the different temporal and spatial organisation of gastrulation, the genes might have a conserved function in mesoderm and endoderm formation and in the principle morphogenetic movements of gastrulation.

In the recent years, many other developmental genes have been found to be conserved between *Drosophila* and other organisms. Most strikingly, the system of the homeotic genes, which specifies positional identities along the anteroposterior axis in *Drosophila* embryos, is conserved in vertebrates, where it apparently serves a similar function. Furthermore, other homeobox genes like *caudal/Cdx* and *even-skipped*/*Evx* as well as members of the *MyoD* family, the *achaete scute*/*MASH* family and the *forkhead*/*HNF* family are found both in *Drosophila* and vertebrates and show some similarities in the expression patterns with respect to cell types or positions within the embryo of both species (reviewed in McGinnis and Krumlauf, 1992).

However, other structurally conserved genes like the *Drosophila* segmentation genes *Krüppel*, *engrailed*, *hairy* and *wingless* and their vertebrate counterparts have unrelated expression patterns in *Drosophila* and vertebrates, making a functional conservation of the genes unlikely. The zebrafish *twist*-related gene *twi*, like *twist* homologues in *Xenopus* (Hopwood et al., 1989) and mouse (Wolf et al., 1991), is expressed in the presumptive notochordal cells in the axial hypoblast after the cells have involuted (R. Riggleman and M. H., unpublished results). This suggests that *twi* in zebrafish is involved in the regional specification of parts of the mesoderm but, in contrast to *twist* in *Drosophila*, not in the initial mesoderm formation.

Ntl is neither necessary nor sufficient for activation of *sna-1* transcription

In late blastula stages, *ntl* (Schulte-Merker et al., 1992) and *sna-1* are coexpressed in marginal cells, suggesting that they respond to the same inducing signals. Both *ntl* and *sna-1* are induced by activin A in the animal cap assay (Schulte-Merker et al., 1992 and this paper). We have shown that the induction of *sna-1* expression in animal cap cells by activin A is independent of *ntl*. Furthermore, *ntl* is not necessary to activate *sna-1* transcription in vivo, as in *ntl* mutant embryos the pattern of *sna-1* expression is normal except for a reduction in the level of expression. Additionally, our studies of ectopic *ntl* expression, in contrast to the results of Cunliffe and Smith (1992), indicate that in gastrulating embryos *ntl* is not sufficient to activate *sna-1* transcription. However, in line with the reduction of *sna-1* expression in *ntl* mutant gastrulating embryos, *ntl* does appear to enhance *sna-1* expression. The discrepancy between our results and those of Cunliffe and Smith could be due to experimental differences: they determined the *sna-1* expression in animal caps corresponding to stage 12 embryos (mid- to late gastrula), whereas we used intact embryos of early gastrula stages. Thus, the data might suggest that the nature of *ntl* control on *sna-1* expression changes during the course of gastrulation and that *ntl* becomes sufficient to activate *sna-1* transcription in later gastrula stages. However, from our results in *ntl* mutant embryos, we at least know that also in these late gastrula stages *ntl* is not necessary for *sna-1* transcription.

sna-1 expression in somites and the head region

In late gastrula and somite stages, *sna-1* is expressed in the presomitic mesoderm with prominent expression in the muscle pioneer precursor (MPP) cells (Felsenfeld et al., 1991). The MPP cells stop *sna-1* expression shortly after somite formation and shortly before their differentiation during which they change their size, shape and relative position. At the same time, *sna-1* becomes more strongly expressed in the posterior part of the newly formed somites. A rostrocaudal polarity of the expression pattern was described for a few other genes in chicken somites. These genes are probably involved in guiding innervation and

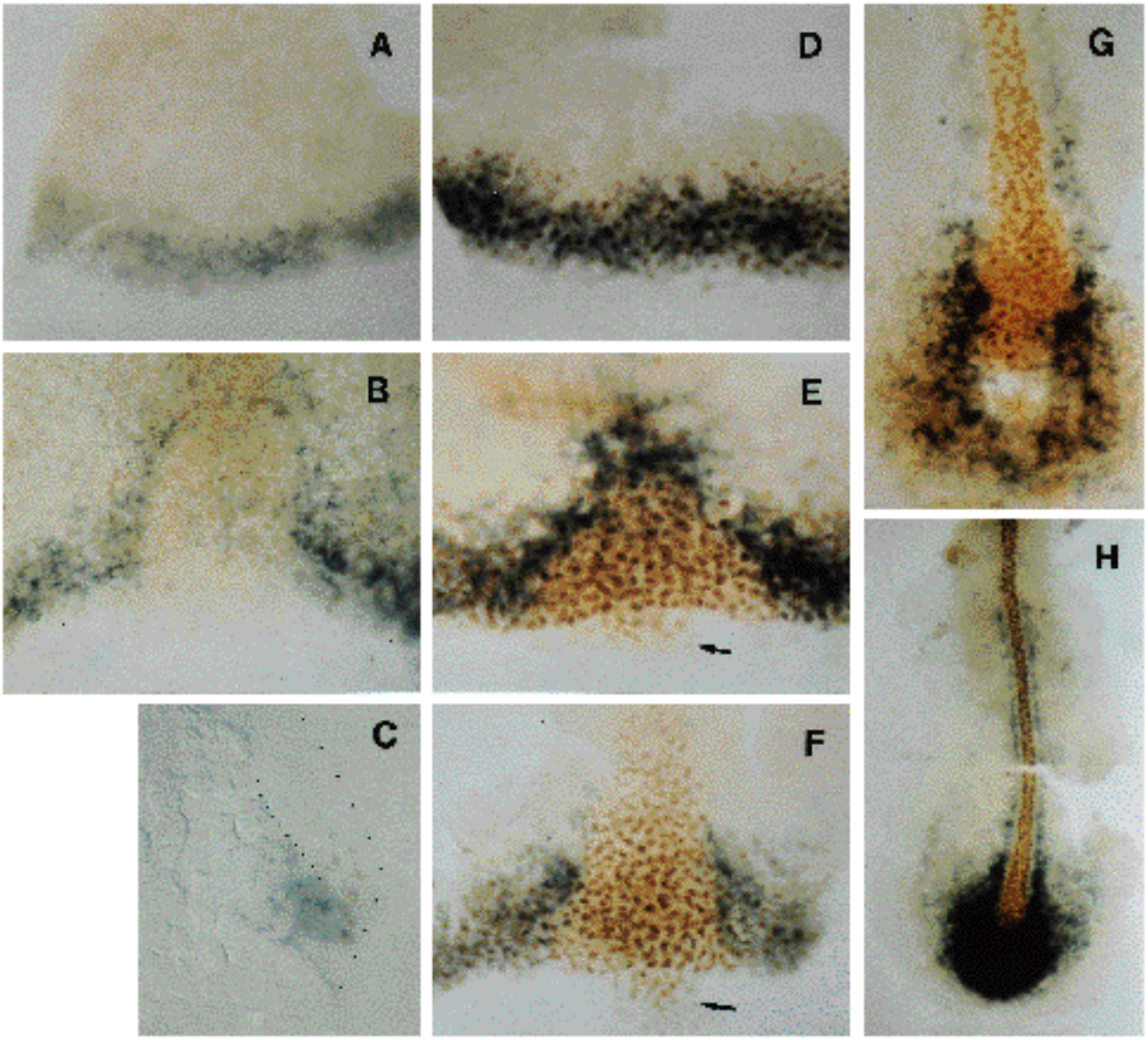


Fig. 8. Relationship of *sna-1* and *ntl* expression in wild-type (C-H) and *ntl* mutant (A,B) embryos investigated by *sna-1* in situ (blue) and anti-Ntl antibody (brown) double stainings. All embryos were spread. (A,D) Germ ring stage, dorsal view: (A) *ntl*, (D) wild-type. (C) Section through germ ring of germ ring-stage embryo, lateral position. The border of the embryo and the separation of epiblast and hypoblast are outlined by dots. (B,E) 60% epiboly, dorsal view: (B) *ntl*, (E) wild type. (F) 80% epiboly, dorsal view.(G) 95% epiboly, dorsal view. (H) 4-somite stage, dorsal view. For details see text. Arrows in E and F indicate the 'forerunning' cells, a group of *sna-1*-negative and *ntl*-positive cells located on the yolk posterior to the germ ring at the dorsal midline of the embryo.

neural crest migration (e.g. Layer et al., 1988; Bronner-Fraser et al., 1992). However, in contrast to chick (Rickmann et al., 1985; Keynes and Stern, 1984), these processes in zebrafish occur in a nonpolar fashion in the middle of the somites (Myers et al., 1986; Raible et al., 1992). So far, it is not known whether the polar distribution of *sna-1* is necessary for and how it might contribute to normal somite development.

In *ntl* mutant embryos of late gastrula and somite stages, the two *sna-1*-positive lines of muscle pioneer precursor cells are present, although they are not as well organized as in wild-type siblings. This suggests that the determination of muscle pioneer precursors, at least at the level of *sna-1* expression, does not depend on *ntl* and a differentiated notochord. It might either be totally independent of the notochord or be induced by notochord precursor cells, which can be detected in *ntl* mutant embryos and which are supposed to induce other tissues like the floor plate independent of Ntl protein (Halpern et al., 1993). However, the differentiation of the precursors to muscle pioneers does depend on *ntl* or a differentiated notochord (Halpern et al., 1993).

Xsna in *Xenopus* and *msna* in mouse in addition to their mesodermal expression are expressed in neural crest cells before and during their migration (Nieto et al., 1992; Smith et al., 1992; Essex et al., 1993). In contrast to *xsna* and *msna*, the earliest neural crest-specific expression of zebrafish *sna-1* can be detected in cranial non-neurogenic cells only

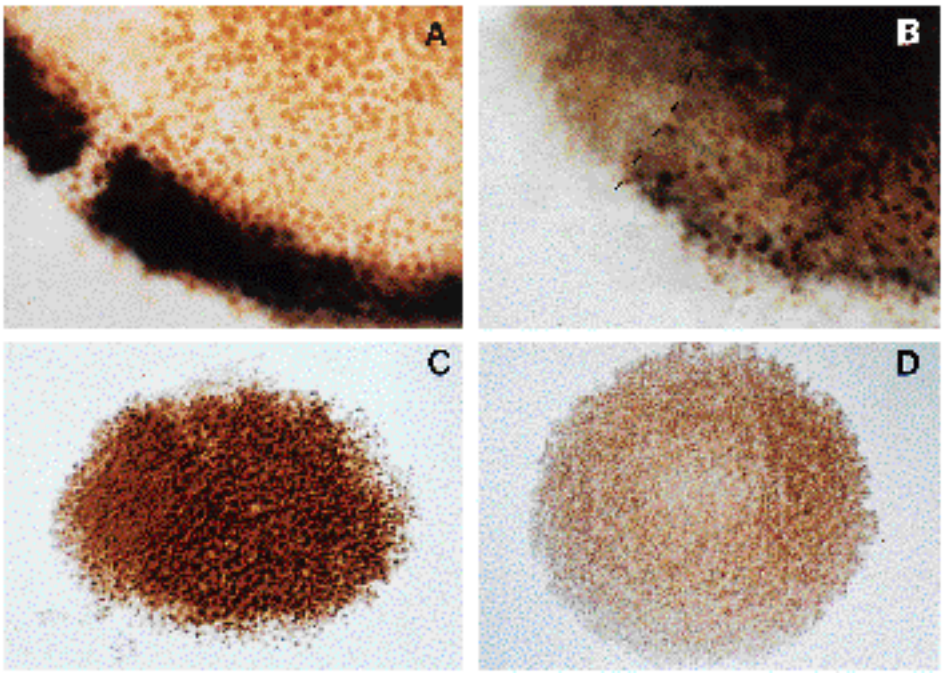


Fig. 9. *sna-1* expression after ectopic expression of *ntl* in intact embryos (A,B; stained for Ntl protein and *sna-1* mRNA) and after activin A treatment of animal caps (C,D; stained for Sna-1 protein). (A) Germ ring-stage embryo. Ntl protein is ectopically expressed in animal regions of the embryo. *sna-1* expression is restricted to the germ ring, its endogenous expression domain. (B) Sphere stage. Ntl protein is ectopically expressed and overexpressed in the right part of the embryo (separated by dashes). *sna-1* is more strongly expressed at the margin of the right part of the embryo. (C) Expression of Sna-1 protein upon incubation of the animal cap for 5 hours in medium containing activin A. (D) No Sna-1 expression upon incubation in control medium. The animal caps are slightly spread.

after they have migrated to ventrolateral positions. Thus, zebrafish *sna-1* may provide only part of the function of *Xenopus xsna* and mouse *msna*.

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Note added in proof

The sequence shown in Fig. 1 has been submitted to the EMBL database and has the accession number X 74790.

We would like to apologize for using the incorrect abbreviation of the gene name in this paper. According to the zebrafish naming conventions it should be *sna1* instead of *sna-1*.