Identification in *Xenopus* of a structural homologue of the *Drosophila* gene *Snail*

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Summary

We have cloned a *Xenopus* cDNA that is related to *snail*, a gene that is required for mesoderm formation in *Drosophila*. The cDNA encodes a protein that contains five zinc-fingers that closely resemble those of *snail*. In the non-canonical parts of the DNA-binding loop, there is almost 90% homology between *snail* and *xsna*. The corresponding mRNA (*xsna*) is expressed strongly at the start of zygotic transcription simultaneously with the transcription factor EF1α. In early gastrulae, *xsna* is equally distributed between the dorsal and ventral halves of the equatorial zone. The possibility that the capacity to synthesise *xsna* is more localised before the start of zygotic transcription has been investigated by culturing fragments of stage 8 embryos until *xsna* is synthesised. The capacity to synthesise *xsna* at stage 8 is located principally in the dorsal half of the equatorial zone. A small amount of maternal *xsna* is localised in the vegetal hemisphere where zygotic transcription starts. *xsna* is not present in isolated animal caps but can be induced by the mesoderm-inducing factors XTC-MIF and bFGF. Synthesis of *xsna* does not occur autonomously in dispersed cells but is restored when cells reaggregate in the presence of calcium and magnesium.

Key words: *snail*, *Xenopus*, zygotic transcription, mesoderm-inducing factors, zinc-finger proteins.

Introduction

Two distinct systems generate the anterior–posterior and dorsal–ventral axes of *Drosophila* embryos (Anderson, 1990; Levine and Harding, 1990). Some of the zygotic gene products in the anterior–posterior system of *Drosophila* such as homeobox and gap genes have structural homologues in vertebrates that are also concerned functionally with anterior–posterior organisation (Gaunt, 1988; Wilkinson et al. 1989). Just as segmentation in insects and vertebrates was probably established independently, we presume these proteins acquired their role independently. However, structural and functional conservation, such as this, suggests these proteins may have originated in the extremely ancient common ancestors of insects and vertebrates.

Only three known vertebrate genes have structural similarities to members of the *Drosophila* dorsoventral system. The *Xenopus* protein *xtwi* has high homology to *twist*, a zygotic *Drosophila* gene that is positively regulated by the maternal gene *dorsal* (Hopwood et al. 1989). *c-rel*, a chicken oncogene is similar to *dorsal* (Steward, 1987) and the vertebrate growth factor family TGF-β has similarities to *decapentaplegic*, a *Drosophila* zygotic gene that is negatively regulated by *dorsal* (Padgett et al. 1987). Here we report the identification in *Xenopus* of a structural homologue of *snail* another zygotic *Drosophila* gene which like *twist* is positively regulated by *dorsal* and specifies ventral pattern. *snail* encodes a protein with five zinc-finger domains, which is expressed in the ventral furrow and mesoderm of *Drosophila* and is required for mesoderm formation (Simpson, 1985; Boulay et al. 1987). The *Xenopus* homologue of *snail* (*xsna*), described here, has a highly conserved zinc-finger region, and is also associated with sites of mesoderm formation.

Mesoderm formation in amphibia is governed by signals that emanate from the vegetal hemisphere which induce formation of characteristically mesodermal cell types such as muscle. Using animal cap explants as an assay two classes of inducing factors have been identified. These are the transforming growth factor β (TGFβ) family, the most potent of which is a homologue of activin A (Smith et al. 1990) and the fibroblast growth factor (FGF) (Slack et al. 1987; Kimelman and Kirschner, 1987). At least three mesoderm-inducing signals have been operationally identified in early embryos of *Xenopus* (Slack et al. 1989; Smith et al. 1989). Two of these signals are thought to emanate from the vegetal hemisphere. The signal from the dorsal vegetal region (signal 1) induces characteristically dorsal mesodermal tissues (notochord and muscle) while the signal from the ventral vegetal region (signal 2) induces ventral tissues like mesenchyme and blood...
(Dale and Slack, 1987). The third signal is produced by newly induced dorsal mesoderm cells and instructs ventral mesoderm to develop dorsal characteristics (Dale and Slack, 1987). The effects of XTC-MIF (now known to be a homologue of activin A; Smith et al. 1990) and bFGF on animal cap explants suggest that these two substances mimic the dorsal and ventral signals, respectively (Smith et al. 1989; Slack et al. 1989). No candidate molecule for the third signal has yet been identified. The precise relationship between the known factors and the operationally identified signals is, however, quite confused as there may be a number of rather similar candidate molecules. Furthermore, bFGF, which is present in blastulae has no signal sequence (Abraham et al. 1986) and therefore no apparent mechanism of secretion. We show below that xsnA is induced in animal caps by mesoderm-inducing factors.

**Materials and methods**

**Identification of a Xenopus homolog of snail**

A stage 17 library in phage lambda was screened at low stringency (Kintner and Melton, 1987) using the *Drosophila* snail cDNA as a probe (Boulay et al. 1987). Inserts of these clones were subcloned into pSP72 (Kreig and Melton, 1987) and M13 and were sequenced by the dideoxy procedure. The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence databases under the accession number X53450.

**Dissection of embryos, handling of explants**

*Xenopus* eggs were artificially fertilized and dejellied by standard methods. Stages were according to Nieuwkoop and Faber (1969). Embryos were dissected using tungsten needles and the fragments were cultured in full strength NAM on 1% agarose (Slack, 1984; Smith, 1987). Prospective DMZ and VMZ at stage 8 could be dissected accurately in embryos with clear polarity in pigmentation (chosen at the 2-cell stage). The ventral side was distinguished from the dorsal side by greater pigmentation and larger cell size. For the dissections described in Fig. 4A, embryos were arranged with their ventral side to the left and their dorsal to the right. A square of animal cap was then excised such that one diagonal separated ventral and dorsal sides precisely into two equal parts. Vertical cuts were then made at each end of this diagonal to separate DMZ and VMZ. Marginal zone material was then removed from the vegetal pole region by cutting round the yolk mass as shown in Fig. 4A. Explants cultured to the equivalent of stage 20 gave more than 90% of the characteristic phenotype of the two kinds of explants (i.e. DMZ explants elongated axially, while VMZ explants were spherical as described by Dale and Slack (1987). We note that Dale and Slack (1987) believe that the characteristic dorsalizing activity in the DMZ is confined to less than 90° of the circumference of the equator. Our dissections, therefore, are relatively precise as the limits of the DMZ are substantially wider than this. At stage 10.5, VMZ

![Fig. 1. DNA Sequence and deduced translation of xsnA. Boxed area, zinc finger domain.](image-url)
and DMZ were identified unequivocally using the position of the blastopore lip.

Animal caps were dissected and treated with mesoderm-inducing factors as described by Smith (1987).

Single cells were obtained by cultivating embryos in calcium/magnesium-free medium (CMFM; Sargent et al. 1986), from stage 2. These were demembranated at about stage 6 (64-cell stage), dispersed on agarose and cultured as single cells until the equivalent of stage 13 (3 h after stage 10.5). To initiate reaggregation, calcium and magnesium chloride (1 mM) were added to single cell cultures at the equivalent of stage 10.5. The cells were swirled into a heap to facilitate cell-to-cell adhesion and were cultured for a further 3 h.

**Nucleotide protection studies**

Probes for **xsna** were antisense RNA made using SP6 RNA polymerase from nucleotide 692 (Pst) to 529 (Msp) of **xsna** (Fig. 1) in pSP72 (Kreig and Melton, 1987) and gave a protected fragment of 163 bp. EF1α (which is expressed in all embryonic cells when zygotic transcription starts and therefore serves as a control for RNA recovery) (Kreig et al. 1989) was probed using antisense RNA from nucleotides 194 to 100 (Hinf) prepared in pSP72 using SP6 RNA polymerase. Two bands of about this size were always seen and probably indicate the existence of two variants of the EF1α sequence in the **Xenopus** genome. The protected fragment was 94 bp. UTP was used at a specific activity of 800 or 160 μCi per nmole for **xsna** and EF1α, respectively. Solution hybridisation of the two probes was carried out at 45°C for 16 h with the same sample to minimise differences in recovery of the two protected fragments essentially as described by Kreig and Melton (1987). Unhybridised RNA was digested with RNase A and RNase T1. Autoradiograms were usually exposed for 4 days. Most data shown were derived from one third of a sample of 10 caps.

**Results**

**Isolation of a Xenopus cDNA related to the snail gene of Drosophila**

A stage 17 library of Kintner and Melton (1987) was screened at low stringency using **Drosophila snail** as a probe. Sequence data from one purified positive clone indicated that we had identified a zinc-finger protein. This clone was used to rescreen the library for a full-length clone, which was then subcloned and sequenced. A region of high homology with **snail** was found in an open reading frame that began at the first Met codon. The deduced amino acid sequence of this protein, called **xsna**, (259 amino acids) is shorter than **snail**, (390 amino acids). However, the full-length cDNA clone was about the same size as the single transcript observed in Northern blots (1.8 kb) (see below). The finger regions of **snail** and **xsna** have an almost identical layout (Fig. 2) and are 66% homologous in this region overall. Moreover the non-canonical parts of the finger motif are 46% homologous overall, increasing to 90% in the third finger. The % identity of each section intervening between the canonical elements of the protein are shown in Fig. 2. Sections D and E, which are likely to be in the centre of the α-helical DNA-binding region (Gibson et al. 1988), are 84 and 90% identical. The sequence TGEKP, present in the third finger of both proteins, however, is found in many finger proteins (Gibson et al. 1988; Nietfeld et al. 1989). Although there are many published zinc-finger sequences, we are aware of no sequence that approaches this level of similarity to **snail** or **xsna**. We therefore believe that **xsna** is likely to be a true homologue of **snail**. Outside of the finger region, **xsna** has no significant extended homology to **snail** although there are a few small islands of similarity.

**Expression of xsna mRNA in developing embryos**

The entire cDNA was used as a probe to examine **xsna** mRNA accumulation by Northern blot analysis. Only one transcript of about 1.8 kb was found. This was first observed at stage 10, increased to a plateau from stage 12 to 18, and decreased to some extent after stage 25 although the mRNA persisted until at least stage 40 (data not shown). A more sensitive study of the early time course of **xsna** accumulation was made using RNase protection. The rate of synthesis of **xsna** was compared with the rate of synthesis of translation elongation factor EF1α. This mRNA is synthesised as soon as zygotic transcription starts in all embryonic cells (Kreig et al. 1989). It provides an internal standard for

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**Fig. 2.** Relationship with **Drosophila snail** amino acid sequence shown in single letter code. Canonical features of finger proteins in boxes. Identical amino acids (.), conservative substitutions (;). Non-canonical regions A–G with % identity.
comparison of zygotic mRNAs. As EF1α is extremely abundant we have prepared the probe for this mRNA using UTP of one fifth of the specific activity used for xsna, furthermore the protected fragment for EF1α is about one half of the size of xsna so that the relative abundance of xsna appears about 10 times its true abundance relative to EF1α. The RNase protection assay shows the two mRNAs increase simultaneously just after stage 9 (Fig. 3B). The great sensitivity of this method of assaying mRNA reveals that there is also a low level of mRNA of both genes present before the start of zygotic transcription that we presume to be of maternal origin.

Localisation of xsna within the embryo
The location of xsna transcripts within early embryos can be determined quantitatively and with considerable sensitivity using RNase protection to assay mRNA in dissected parts of the embryo. Sets of 10 early gastrulae (stage 10.5) were dissected into animal caps, dorsal and ventral marginal zones (DMZ and VMZ respectively), and vegetal pole regions (Fig. 4A) from which RNA was extracted for RNA protection assays. DMZ and VMZ could be unequivocally identified by orientation relative to the blastopore lip. The bulk of new synthesis of xsna mRNA after the start of zygotic transcription is in the marginal zone. The amount of xsna RNA in the DMZ and VMZ, at stage 10.5 is not significantly different. xsna is almost completely absent from the animal cap although EF1α is present (Fig. 4B, i–l). In the vegetal zone, there is a significant enrichment of xsna relative to EF1α (determined by densitometry, data not shown) compared with the marginal zone although only a small proportion of the total xsna mRNA is in this region (Fig. 4B, l).

A small amount of xsna is present in embryos before zygotic transcription starts. Its distribution in dissected fragments at stage 8 is shown in Fig. 4B (a–d). More than 80% of xsna mRNA at this stage is located in the vegetal hemisphere. Dissections into vegetal and animal pole at earlier stages gave similar results (data not shown).

By the time zygotic transcription starts, it is possible for an initial focus of xsna synthesis to be obscured by induction processes. This possibility was investigated by dissecting embryos at stage 8 before xsna synthesis had started and then culturing these explants until xsna was fully expressed. Prospective DMZ and VMZ were dissected using pigmentation as a marker as described above. The amount of xsna in these explants was taken as an indication of the capacity to synthesise xsna at stage 8 in the dissected regions. The explants were cultured to the equivalent of stage 12 to compensate for the slight reduction in rate of synthesis of EF1α in explants compared with intact embryos. At stage 12 the

Fig. 3. (A) Northern Blot using RNA preparations from oocytes (Oo), eggs and stages 6–18. (B) Nuclease protection study of xsna development in Xenopus embryos. Position of protected fragments shown at side.

Fig. 4. (A) Dissection of embryos at stage 8 and 10.5. Animal caps, vegetal pole, dorsal marginal zone (DMZ) and ventral marginal zone (VMZ) are marked. (B) Location of xsna in early embryos and development of capacity to express xsna. Embryos dissected as in Fig. 4A. (a–d) Stage 8 embryos; (a) AC (b) DMZ (c) VMZ (d) VG. (e–h) Cultured to the equivalent of stage 12; (e) AC, (f) prospective DMZ, (g) prospective VMZ, (h) VG. (i–l) Embryos dissected and processed at stage 10.5; (i) AC (j) DMZ (k) VMZ (l) VG.
DMZ typically contained 4 times more xsna than VMZ explants (Fig. 4B, e–h) while the amount of EF1α in DMZ and VMZ was essentially identical. In contrast, in embryos dissected at the start of gastrulation (stage 10.5) there is no indication of preferential localisation in the DMZ. (Fig. 4B, j–k). The capacity to synthesise xsna is, therefore, localised in the DMZ but not the VMZ before zygotic transcription starts but this soon spreads to the entire marginal zone once zygotic transcription starts.

Effect of mesoderm-inducing factors on xsna synthesis in isolated animal caps
The presence of xsna in the marginal zone and absence from the animal cap of early gastrulae and also the effect of DMZ on VMZ noted above suggests that xsna mRNA synthesis may be regulated by mesoderm-inducing factors. This was investigated by culturing sets of stage 8 animal caps in the presence of the mesoderm-inducing factors XTC-MIF and bFGF at 9 μM and 9.9 μM respectively (equivalent to 1.8 ng and 1 ng respectively of standard pure preparations of the two factors; Smith, 1987; Slack et al. 1987). At these concentrations animal caps are completely induced by morphological criteria, but would not normally give the most extreme tissue-type expression. RNA was extracted from a set of 10 caps at intervals. Both XTC-MIF and bFGF induce xsna significantly at the equivalent of stage 10.5 and strongly at stage 12 whereas control caps have none (Fig. 5). The presence of EF1α in all three sets of caps after stage 10.5 shows that total RNA synthesis is similar in all three samples.

Dependence of xsna synthesis on cell-to-cell contact
In Xenopus embryogenesis, some gene products are expressed in a cell autonomous fashion while others require cell contact (Sargent et al. 1986). To distinguish between these alternatives, the rate of synthesis of xsna has been examined in dispersed embryonic cells cultured in media lacking calcium and magnesium (CMFM, see methods) or after aggregation has been initiated by restoration of calcium and magnesium (Fig. 6). When dispersed cells were cultured in CMFM, there was no significant synthesis of xsna while EF1α continued to be synthesised until the equivalent of stage 13. Addition of calcium and magnesium at any time after stage 10 restored synthesis of xsna and this correlated with aggregation of the cells. On restoration of calcium and magnesium, xsna synthesis took place over a two hour period (data not shown).

Discussion
We have described a Xenopus mRNA, xsna, that encodes a protein that is very similar to Drosophila snail in the putative DNA-binding domain that contains five zinc-fingers. The finger regions of xsna and snail resemble each other more closely than either resemble any of the many zinc-finger containing proteins that exist (Gibson et al. 1988; Nietfeld et al. 1989). Although the possibility of the two genes evolving independently cannot be excluded, it seems more likely that the DNA-binding regions of xsna and snail are truly homologous, an observation that adds to the increasing body of information that suggests that the genetic systems that determine the two major axes of Drosophila are evolutionarily conserved in vertebrates and may be the legacy of an extremely ancient common ancestor. One may speculate that homology in the DNA-binding region and lack of it in the remainder of the protein indicates that the conserved function is the capacity to regulate specifically the target genes of these putative transcriptional activators. It may be significant too, that although the anatomy of regions of mesoderm formation in Drosophila and Xenopus is dissimilar, the putative Xenopus homologues of twist and snail (xtwi and xsna) are associated with these sites in Xenopus (Hopwood et al. 1989). Conversely, xsna is not found solely on the ventral side of the embryo as snail is, in Drosophila.

At stage 10.5, the bulk of both xsna and EF1α was
localised in the marginal zone. We were a little surprised to discover that EFlα was not more abundant in the vegetal zone as this region probably represents at least 30% of the volume of the embryo. There has been very few investigations of the location of housekeeping mRNAs in early embryos of *Xenopus*. However, an early autoradiographic study by Bachvarova et al. (1966) indicated that newly synthesised mRNA was located predominantly in the equatorial region of blastulae at the start of zygotic transcription and in the developing mesoderm in stage 10.5 gastrulae. An RNA with a maternal component would probably have been more abundant in the vegetal region and would therefore have given a false impression of the specificity of location of *xsna*. We therefore believe that a zygotic location of this gene in the vegetal region is probably a maternal effect.

Although only a small part of the *xsna* mRNA at stage 10.5 is located in the vegetal region, it is more enriched relative to EFlα than in the marginal zone. This may, in part, be due to the maternal contribution found in vegetal cells at stage 8. However, if, as we suggest below, *xsna* is synthesised in response to inducing factor emanating from the vegetal pole, the site of *xsna* synthesis may be contigous with the vegetal pole and would therefore tend to enrich this fraction. This is currently being investigated by in situ hybridisation.

The behaviour of *xsna* at the start of zygotic transcription can largely be explained in terms of the current view of the three-signal model of mesoderm induction as developed by Slack et al. (1989) and Smith et al. (1989) (see Introduction). The relative abundance of *xsna* in the DMZ rather than in VMZ explants cultured from stage 8 to 12 indicates that the initial capacity to synthesise *xsna* is located in the same region as the source of the dorso-vegetal signal (signal 1). In view of the inducibility of *xsna* by XTC-MIF, which mimics the dorsal signal when applied to animal caps, we infer that the dorso-vegetal signal controls *xsna* and is active before zygotic transcription starts in establishing the capacity to synthesise *xsna* in contiguous tissues.

In intact embryos the amount of *xsna* in the DMZ and VMZ is equal by stage 10.5. We believe that this shows that *xsna* expression in the VMZ is increased as a result of its proximity to the DMZ. This is reminiscent of the ‘dorsalisation’ of tissues to the ventral side effected by the blastopore lip region (Smith and Slack, 1983), and may indicate that *xsna* expression in the VMZ is controlled by the third ‘signal’. The possibility that dorsalisation is achieved by cell migration from the DMZ to the VMZ is thought to be unlikely (Smith and Slack, 1983).

The rate of synthesis of *xsna* in VMZ explants is very low compared with DMZ explants. This suggests that signal 2 which emanates from the ventral-vegetal region (Slack et al. 1989) does not induce *xsna*. Paradoxically, FGF, which is the best candidate at present for signal 2, is able to induce *xsna* in animal caps. We have no explanation for this, but we note that Slack et al. (1989) have recognised that bFGF has no signal sequence (Abraham et al. 1986), and no apparent mechanism of secretion, and therefore may not be signal 2 in vivo. As more inducing factors are discovered the paradox should be resolved. We would predict that the real in vivo ventral mesoderm inducer would not induce *xsna* in animal caps.

Induction of *xsna* in animal caps by the two mesoderm-inducing factors and the inferred response to the third dorsalising signal suggests that synthesis of *xsna* can be a response to more than one signal. Furthermore the timing of the *xsna* response in animal caps suggests that induction is not an ‘immediate early’ response to mesoderm-inducing factors as with *Mix.1* (Rosa, 1989) but takes place over several hours. There is no contradiction between this observation and the observed rapid onset of synthesis at the start of zygotic transcription, as the tissues that respond probably have developed the capacity to produce *xsna* at an earlier time.

The dependence of *xsna* synthesis on cell-to-cell contact and the lack of autonomous expression of *xsna* is consistent with its inducibility by mesoderm-inducing factors. Thus the capacity to synthesise *xsna* could be lost from dispersed cells when a diffusible factor becomes too dilute. In addition, there may be a requirement for cell-to-cell contact or a community effect as, for actin in muscle differentiation (Gurdon et al. 1985; Gurdon, 1988) or even a specific requirement for divalent metal ions. These alternatives are being investigated.

Whilst there is no real anatomical homology between *Drosophila* and *Xenopus* early embryos, the presence of the structurally homologous snail and *xsna* at sites of involution and of presumptive mesoderm formation in early embryos supports the idea that the system determining dorsoventral pattern identified in *Drosophila* exists in vertebrates in a greatly modified form.

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