INTRODUCTION

Many genes known to participate in Drosophila embryogenesis have homologues in vertebrates that are also involved in early development. Xenopus (Xsna) and mouse (Sna) genes that are structural homologues of Drosophila snail have been described. (Sargent and Bennett, 1990; Nieto et al., 1992; Smith et al., 1992). These encode highly conserved zinc finger motifs (five in Xenopus or four in mouse) that are expressed, like snail, only zygotically. Xsna is expressed in the marginal zone of Xenopus blastulae and expression is induced by the mesoderm-inducing factors activin A and fibroblast growth factor (Sargent and Bennett, 1990). Sna, the mouse homologue of Xsna appears in two major lineages, mesoderm and neural crest (Nieto et al., 1992). In this report, we show that Xsna also has a complex expression pattern that is broadly similar but, in addition, we demonstrate transitional states in the development of the expression pattern not evident in the mouse. We show that ectodermal expression of Xsna, at a very early stage, identifies a band surrounding the prospective neural plate that we designate the neural plate border. This becomes the neural folds and thereafter is incorporated into the neural crest cells during their subsequent migration.

The role of the Xsna promoter in creating this pattern of expression has been investigated by injecting fertilised eggs with constructs containing the 5' upstream sequence of the gene fused to a reporter. An element of 115 base pairs (−160 to −45 relative to the transcriptional start) is sufficient to drive appropriate reporter gene expression. The promoter does not contain a TATA or CAAT box and does not have a high GC content, but RNA synthesis starts precisely at 33 bases upstream to the translational start. The start sequence can be deleted so that transcription is initiated elsewhere without affecting the expression pattern. The distribution of Xsna promoter activity within the embryo, examined using β-galactosidase (β-gal) fusions, is similar to that of the endogenous mRNA seen by in situ hybridisation. The contribution of elements within the 5' sequence have been assessed by comparing the expression patterns of constructs that have deletions in this region. Sequences from −112 to −97 are required for mesodermal expression and sequences from −96 to −44 are required for ectodermal expression. The behaviour of the injected promoter constructs differ in one important respect from the endogenous gene in that expression in an animal cap assay is not inducible by mesoderm-inducing factors but is inducible by cells of the vegetal pole.

Key words: CAT, β-gal, in situ hybridisation, mesoderm, neural crest, neural folds, neural plate border, neural tube roof, Xsna promoter, Xenopus

SUMMARY

Distinct elements of the xsna promoter are required for mesodermal and ectodermal expression

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Xsna, the Xenopus homologue of Drosophila snail, is expressed in both mesoderm and ectoderm. Expression occurs in all mesoderm initially but is down regulated in a tissue-specific fashion at the end of gastrulation in a way that reveals the subdivision of the mesoderm before its derivatives are overtly differentiated. Xsna is also expressed in the ectoderm of the prospective neural fold from stage 11, in a distinct band of cells surrounding the prospective neural plate, which we designate the neural plate border. The deep and superficial ectoderm compartments labelled by Xsna represent the prospective neural crest and the prospective roof of the neural tube, respectively. Xsna expression persists in neural crest cells during their subsequent migration.

The role of the Xsna promoter in creating this pattern of expression has been investigated by injecting fertilised eggs with constructs containing the 5' upstream sequence of the gene fused to a reporter. An element of 115 base pairs (−160 to −45 relative to the transcriptional start) is sufficient to drive appropriate reporter gene expression. The promoter does not contain a TATA or CAAT box and does not have a high GC content, but RNA synthesis starts precisely at 33 bases upstream to the translational start. The start sequence can be deleted so that transcription is initiated elsewhere without affecting the expression pattern. The distribution of Xsna promoter activity within the embryo, examined using β-galactosidase (β-gal) fusions, is similar to that of the endogenous mRNA seen by in situ hybridisation. The contribution of elements within the 5' sequence have been assessed by comparing the expression patterns of constructs that have deletions in this region. Sequences from −112 to −97 are required for mesodermal expression and sequences from −96 to −44 are required for ectodermal expression. The behaviour of the injected promoter constructs differ in one important respect from the endogenous gene in that expression in an animal cap assay is not inducible by mesoderm-inducing factors but is inducible by cells of the vegetal pole.

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than 200 base pairs is sufficient to drive reporter gene expression and this contains distinct sequence elements required for either mesodermal or ectodermal expression.

MATERIALS AND METHODS

Embryos, explants and protein labelling

*Xenopus* embryos were obtained by artificial fertilization, dejellied in 2% cysteine (Smith and Slack, 1983), reared in 10% normal amphibian medium (NAM: Slack, 1984) and staged according to Nieuwkoop and Faber (1967). Animal caps were prepared and cultured (Sargent and Bennett, 1990) with activin A (20 ng/ml) or bFGF (9 ng/ml). Conjugates of vegetal pole and animal caps were prepared as described by Nieuwkoop (1969). Protein synthesis was measured by determining the amount of [35S]methionine (25 µCi per ml of culture medium) incorporated into trichloroacetic acid-insoluble material.

Radioactive in situ hybridisation

The procedures for in situ hybridisation (including fixation of embryos, embedding, sectioning and the high-stringency wash) were carried out exactly as described for mouse (Wilkinson and Green, 1990). 35S-labelled antisense and sense transcripts corresponding to nucleotides 1120-1800 of the 3'-untranslated region of *Xsna* mRNA (Sargent and Bennett, 1990) were used. To avoid confusing silver grains with melanin particles, sections were bleached in 1.5% H2O2 in PBS for 4 hours over a light box at room temperature immediately after dewaxing and rehydration (N. Hopwood, personal communication). Developed slides were stained with toluidine blue and photographed on Kodak Ektachrome (64T) using a Zeiss Axiophot photomicroscope.

Isolation of genomic clones containing *Xsna* sequences

An EMBL4 genomic clone bank kindly provided by Tom Sargent (NIH) was screened using the full-length *Xsna* cDNA sequence. The clones obtained fell into two classes on the basis of their restriction patterns. Subclones containing *Xsna* sequences were prepared in pSP72 by standard methods. The most useful of these contained a 5 kb fragment with about 2 kb upstream to the translational start and the cDNA sequences 1-1200. Restriction mapping and sequencing was carried out by standard methods and used to establish the position of the two introns. The sequence of 2 kb of the upstream region together with the introns has been deposited at EMBL (Accession Number X74329).

Construction of expression plasmids

A *HindIII* site was introduced at position 66 in the first exon of *Xsna* cDNA by PCR. Promoter sequences were cloned in the *HindIII* site of pSP-CAT (a derivative of pSP72 containing the CAT gene in the PvuII site, kindly provided by Peter Vize). For the ID-45/+74 construct (Fig. 4) an EcoR1-SphI fragment (−2000 to −45; Fig. 2) was cloned into the *EcoR1-SphI* sites of pSP72. A β-gal reporter was constructed from the CAT constructs by inserting an expression cassette (kindly provided by Richard Harland) into the CAT construct linearised at the *HindIII* site. This contained the thymidine kinase leader sequence, a nuclear localisation sequence from the rat glucocorticoid receptor fused to β-gal and 2 kb of rabbit globin 3′ tail. Deletions of the promoter sequence were prepared either by restriction digestes, BAL31 resection or by using PCR with oligonucleotides containing restriction sites at their 5′ ends.

Primer extension

The transcriptional start of the endogenous *Xsna* gene was determined by primer extension (Maniatis et al., 1982) using poly-nucleotide kinase end-labelled oligonucleotides that are complementary to bases 77-96 in the coding sequence of *Xsna* (Sargent and Bennett, 1990). RNA was extracted as described previously (Sargent and Bennett, 1990).

Microinjection procedure

Fertilized eggs were dejellied and injected with 7-9 nl of DNA (25 ng/µl) in 5% Ficoll in 75% NAM and kept in this medium until stage 6-8. Injections were made in the animal half during the first cell cycle or in both blastomeres during the second cell cycle. No difference in expression of the reporter genes was obtained if embryos were injected in the vegetal half. The pattern of β-gal expression after injection of plasmid DNA was affected by the temperature at which the embryos were incubated. At less than 18°C, development was normal and the expression of β-gal from promoter fusion vectors rationally reflected the distribution of *Xsna* shown by in situ hybridisation. At higher temperatures, embryos developed irregularly, sometimes with aberrant gastrulation and strong irregular β-gal staining. The lower incubation temperature allowed more time for injection before the first cleavage. The maximum amount of DNA that could be injected without detrimental consequences to the embryo was 200 pg in 8 nl but expression was substantially reduced if the amount of DNA injected was reduced to 100 pg. If the amount injected exceeded 300 pg per embryo, an artificial expression was seen in the epidermis. The concentration of DNA injected was therefore determined precisely (Cesarone et al., 1979). Injected DNA was linearised (usually with *KpnI*) before injection at a site 5′ to promoter. Individual clutches of eggs varied considerably in the amount of expression and in the extent to which linear DNA gave superior expression to circular DNA although linear DNA was usually better than circular DNA.

Chloramphenicol transacylase (CAT) assays

Homogenates of embryos were prepared in 0.25 M Tris-HCl, pH 8 containing phenylmethyl sulphonyl fluoride (1 mM) and amido-phenylmethyl sulphonyl fluoride (1 mM) and were assayed for chloramphenicol transacylase activity (Gorman et al., 1982).

Histochemical staining of β-gal

Injected embryos were fixed for 2 hours in 4% formaldehyde in standard PBS containing MgCl2 (2 mM) and stained with X-gal (bromo-chloro indole galactoside) for 2 hours (Dannenberg and Suga, 1981). Some embryos were bleached using 2% hydrogen peroxide in PBS illuminated by fluorescent light for 3-6 hours.

Preparation of sections for histochemistry

Two methods were used to examine β-gal staining in thin sections (1 µm). Embryos were postfixed in 4% formaldehyde, dehydrated, cleared in 1,2,3,4-tetrahydroxiphthaleine, embedded in wax and the sections stained in 0.2% eosin yellow. Alternatively, embryos were postfixed in Karnovsky’s fixative, dehydrated, osmicated and embedded in Araldite for sectioning.

RESULTS

Expression of *Xsna* in developing mesoderm

*Xsna* transcripts were first detected by in situ hybridisation during stage 9 on the dorsal side of embryos (oriented by their pigmentation during embedding), in the lowest tier of the marginal zone, adjacent to the vegetal yolk mass (Fig. 1A). These labelled cells are the presumptive most anterior mesoderm (prechordal) which begin to move around the internal blastopore lip before the external blastopore lip has formed (Nieuwkoop and Faber, 1967). At stage 11, the
hybridisation signal was of similar intensity dorsally and ventrally and was present in all involuted mesoderm but not in mesoderm that was still to involute (Fig. 1B). The boundary between the expressing and non-expressing mesoderm was sharp and was in the blastopore lip (Fig. 1B).

At stage 12, the prospective notochord was identifiable by the disappearance of Xsna mRNA (Fig. 1C, n) before histologically recognisable notochord could be seen. Xsna transcripts were present throughout the paraxial mesoderm until somite formation and then disappeared from the myotome compartment of each somite before it rotated. The entire lateral plate mesoderm contained Xsna mRNA until the early-tailbud stages, when transcripts disappeared from the anterior dorsal regions at which the pronephroi form (Fig. 1E, pn).

**Xsna expression in the ectoderm**

Xsna transcripts were detected in regions of the ectoderm from stage 11 (Fig. 1B) when the ectoderm had been reduced by epiboly to two layers of cells, termed superficial and deep (Fig. 1C, sf and dp). Transverse sections showed expression in two almost adjacent stripes, one in each layer, on each side of the dorsal midline. The superficial layer stripe was slightly more dorsal than the deep layer stripe. The stripes were only a few cells wide in cross-section and had sharp boundaries (Fig. 1C, arrows). At stage 18, the superficial ectodermal layer that expresses Xsna was present in the approaching tips of the neural folds (Fig. 1D) and was incorporated into the neural tube roof by stage 23 (not shown). The premigratory cephalic neural crest (c) and superficial ectoderm cells, on the tips of the neural folds (f) are also labelled. (E) Stage 23. Transverse anterior trunk section. Absence of Xsna transcripts from notochord (n), myotome (my) and pronephros anlage (pn) in the dorsal lateral plate, while transcripts are still detected in the dermatome (dm), sclerotome (s) and the rest of the lateral plate (lp). Transcripts present in roofplate and trunk neural crest (rp + c) in and above the closed neural tube, respectively. (F) Stage 39. Horizontal head section showing branchial arches (ba) with branchial arch cartilages (bc) containing Xsna transcripts. a, anterior; p, posterior.

**Identification of the Xsna gene promoter**

The 5 kb EcoR1 fragment of genomic DNA used to isolate the Xsna promoter contained about 2 kb of 5' sequence, the coding sequence and introns of 0.75 and 1.0 kb at positions 100 and 619, respectively (Fig. 2). The sequences at the exon/intron boundaries conform to published consensus sequences (Padgett et al., 1986) with the exception of the first intron which is followed by T rather than G (Fig. 2). A TATA-like sequence was not found in the region 5' to the
first exon. However, the presence of a promoter in the 2 kb of 5′ sequence was demonstrated by showing that this sequence, including 60 bases of the first exon, could drive CAT expression when injected into fertilized eggs of Xenopus. Constructs in which the promoter was cloned adjacent to the CAT gene (i.e. in the HindIII site of pSP72) gave the strongest expression. Constructs containing parts of the SP72 polylinker between the promoter and CAT showed weaker expression. Expression of promoter-CAT constructs started at stage 10 and increased continuously, matching the increase in Xsna mRNA seen previously (Sargent and Bennett, 1990) and by in situ hybridisations (shown above).

The start of transcription was mapped by primer extension using RNA from embryos at stages 12 and 20 and an end-labelled primer that was complementary to bases 77-96 in the coding sequence. One major extension product of 129 bases was obtained from stage 12 RNA and RNA from heads and bodies of stage 20 embryos (Fig. 3). This indicates that there is a precise start of transcription 33 bases upstream to the translational start which is the same throughout early development and is the same in tissues expressing Xsna predominantly in ectoderm (heads) or mesoderm (bodies). No sequences resembling TATA or CAAT boxes were found at the typical sites of these motifs at −32 or −100 bases, respectively, upstream to this point or at any other point in the 5′ untranslated region (Fig. 2).

The translational start could be deleted (Construct ID-45/+74, Fig. 4) without any effect on the amount or pattern of expression (data not shown).

The effect of deletions of the 5′ region on CAT expression in developing embryos was assessed relative to the full length promoter (Fig. 4). Injected embryos were assayed at stage 11. Deletion of a 1.8 kb EcoR1-Sma fragment (to −161 Fig. 2) had no significant effect on CAT expression but deletion of 60 bases more from the 5′ end reduced expression considerably and removal of 70 bases abolished expression. The vector without an insert had no significant non-specific expression when assayed at this stage, although at later stages there was significant non-specific expression.

**Spatial pattern of promoter expression**

To investigate the spatial expression pattern controlled by promoter sequences, we have used a β-gal reporter and its substrate X-gal to examine expression histochemically. Constructs containing a 2 kb 5′ region, gave an expression pattern that corresponds in most respects to that found by in situ hybridisation (shown above).

Fig. 2. Organisation of the Xenopus snail gene. (A). Sequence of 5′ region of Xsna. Sequences in bold are possible sites for Oct1, Sp-1, B2 in TF111A promoter and AP4 (a-d) respectively (e) Translational start of endogenous Xsna. Numbers relate to transcriptional start (0). Lower case letters, sequence of CAT leader. (B). Sequences at splice sites. Upper case letters, Xsna cDNA sequence with numbers indicating position in cDNA of bold-lettered bases.

Fig. 3. Start site of transcription. Extension products of an oligonucleotide complimentary to bases 77-96 of Xsna were obtained by reverse transcription using total RNA from (a) stage 12 embryos (b) stage 20 heads (c) stage 20 posterior parts. A DNA sequence using the same primer is shown in parallel (NB, the sequence tracks show the complement of the Xsna promoter). The translational start is at +33.
The examples shown are representative of embryos with the most complete pattern. During gastrulation, expression was observed in mesoderm above the blastopore lip (Fig. 5A) (seen by focusing through the ectoderm and in cross sections of embryos; Fig. 5B). Expression was most obvious on the dorsal side and was progressively reduced towards the ventral side. At this stage, there was very little difference between the dorsal and ventral expression of the endogenous gene (Fig. 1B) although there was at an earlier stage (Fig. 1A). Clear discrepancies between the expression pattern of the endogenous gene, analyzed by in situ hybridisation, and the β-gal expression pattern were evident at this stage. In two experiments (involving 294 embryos), about 26% of injected embryos expressed β-gal in the dorsal animal cap region with rather less on the ventral side, in contrast to the lack of expression of the endogenous gene in this tissue (Fig. 1A,B). There was also expression in the suprablastoporal endoderm in about 5% of injected embryos, a tissue that also does not express the endogenous gene (Fig. 1B). In the same set of embryos, 47% and 26% of the embryos expressed in the dorsal and ventral mesoderm, respectively.

At stage 11, ectodermal expression occurred in a continuous stripe on the lateral aspects of the embryo, extending from a region close to the blastopore lip (probably the limit of involution) to a position high on the dorsal side in the best examples (Fig. 5C). At stage 13, these stripes were associated with neural folds (Fig. 5E) and the neural plate was not stained. In thin sections, the lateral plate and somitic mesoderm was stained (Fig. 5D), together with the prospective neural folds. Although expression appears notably mosaic in section, β-gal-staining cells were visible in the superficial and deep ectoderm. By stage 19, when the neural tube was almost closed, β-gal staining was evident in the roof of the neural tube and the neural folds (Fig. 5G), which are derived from the superficial and deep ectodermal layers, respectively. At the tailbud stage, there was strong staining of the cephalic neural crest (Fig. 5H) but the much less developed trunk neural crest was also visible. At the tadpole stage, the branchial arches, which are derived from cephalic neural crest, are stained and the somites appear as striking rows of stained nuclei as a result of translocation of β-gal to the nucleus (mediated by the nuclear localisation sequence, see Methods).

**Effect of truncated promoter sequences on β-gal expression**

The expression pattern of reporter constructs with truncated promoter sequences was examined in terms of the contributions to the phenotype of mesoderm and tissues derived from the neural folds. If any β-gal-positive cells were found in these tissues, the embryo was scored as positive (Fig. 6). As the percentage of stained embryos observed was approximately correlated with the intensity of staining, we believe the former was a more practical measure of activity. Truncation from −160 to −112 caused significant but more-or-less equal reduction in expression in both ectodermal and mesodermal expression but truncation to −96 almost completely prevented mesodermal expression with no effect on neural fold expression. The sequences between −112 and −96 were therefore designated the M region and the sequences between −96 and −45 the E region. As we found with CAT constructs (Fig. 4), truncation to −93 greatly reduced expression although a small amount of ectodermal expression persisted. The bases −96 to −45 must therefore include a basal element required for all Xsna expression and we presume a sequence that determines ectodermal expression specifically. We have attempted, unsuccessfully, to delete sequences in this region to obtain expression in mesoderm only (Fig. 6; ID −92−89). This may suggest that there is some overlap in the function of sequences that are ectoderm specific and the basal promoter.

The spatial pattern of expression of the ectoderm-specific promoter (Δ−96) was examined as for the full-length constructs (Fig. 7). In thin sections at stage 11, β-gal expression is entirely in the ectoderm (prospective neural folds; Fig. 7A) and in the horseshoe-shaped presumptive neural folds on the dorsal aspect of the embryo at stage 11.5 (Fig. 7B) and in the neural folds at stage 13 (Fig. 7C).
Fig. 5. Patterns of β-gal expression in embryos obtained from fertilized eggs injected with promoter constructs. Fertilised eggs were injected with the full-length promoter (2 kb) except D and E which were injected with Δ −160. No significant difference was observed between the two constructs. b, blastopore; d, dorsal midline; e, endoderm; v, ventral. (A) Stage 10.25. β-gal expression in mesoderm (m). (B) Stage 10.5, cut through dorsal midline. Expression in mesoderm (m) and in animal cap ectoderm. (C) Stage 11. β-gal expression in prospective neural folds (n) on lateral aspect of embryo. (D) Stage 13. β-gal expression seen in thin sections in the prospective neural folds (n) and lateral plate mesoderm (lp). Black triangles, expression in superficial ectoderm; white triangles expression in deep ectoderm although some superficial layer cells are also labelled. (E) Stage 13. β-gal expression in neural fold but not in neural plate (np) or ventral ectoderm. (F) Stage 14. Transverse section through middle of embryo showing β-gal expression in lateral plate (lp) and in the left side somite. (G) Stage 19. β-gal expression in roof of neural tube (n) and in the cephalic neural crest (cn); (bleached embryo). (H) Stage 25. Lateral view of expression in cephalic (cn) and trunk neural crest (tn). (I) Stage 33/34. β-gal expression in somites (s) and in the branchial arches (ba).
The expression pattern generated by the ecderm-specific promoter is independent of the site of injection

The specificity of the ecderm-specific promoter was shown by injecting constructs containing it into specific blastomeres, which are approximately prospective mesoderm, neural folds and epidermis, respectively (Dale and Slack, 1987). The complete short promoter (Δ −160) and the ecderm-specific promoter (E-promoter) fused to β-gal (Δ −96) were injected at the 32-cell stage into the blastomeres C1, A2 and A4. The resulting phenotypes are shown in Fig. 8. The E-promoter was expressed most strongly in the neural folds when injected at the 1-cell stage or in blastomere A2 and only weakly or not at all when injected into prospective epidermal or mesodermal blastomeres, showing that the E Promoter is active in neural fold but is not active in mesoderm. In contrast, the expression pattern generated by the complete promoter was characteristic of the blas-
tomere injected. In blastomere C1, expression was predominantly mesodermal (95%), in A2 predominantly neural fold (82%) and in A4 predominantly epidermal (83%). Expression in epidermis in these experiments was considerably higher than expected. One possible explanation of this is that, because the cells injected are very small, the DNA concentration within the cell has exceeded the amount that causes nonspecific expression (see Materials and Methods).

**Effect of mesoderm-inducing factors on expression of promoter-reporter constructs**

The amount of Xsna in animal cap explants from stage 8 embryos is very low but is increased when cultured with soluble mesoderm-inducing factors, such as activin A or bFGF (Sargent and Bennett, 1990), or in conjugates with the vegetal poles of stage 8 embryos. The inducibility of Xsna promoter-CAT fusions has been investigated in animal cap explants prepared from embryos derived from eggs injected with promoter-CAT fusion plasmids (Fig. 9). The explants cultured alone express a very low level of CAT. There is a small but significant increase in CAT activity in the presence of mesoderm-inducing factors (activin, bFGF or both) that is attributable to the activity of the promoter, as the vector lacking the promoter (pSPCAT) has no significant CAT expression (Fig. 4). However, the difference between the treated and untreated controls is very small compared with the induction of endogenous Xsna reported previously (Sargent and Bennett, 1990). We believe this increase reflects a slight stimulation of the rate of protein synthesis mediated by the growth factors. Protein synthesis, measured by incorporation of [35S]methionine, is about 20-30% higher in caps treated with the mesoderm-inducing factors than in the untreated control (data not shown). In contrast, when the vegetal pole was used as an inducer in a conjugate (Nieuwkoop, 1969), we found strong CAT expression (Fig. 9). These results indicate that the 2 kb promoter-reporter constructs, described here, lack an element that confers inducibility by activin A or bFGF but are able to respond to another signal emanating from the vegetal pole.

**DISCUSSION**

**Comparison of the expression patterns of Xsna and Sna**

The vertebrate homologues of snail, Sna and Xsna, are expressed in a complex pattern in the mesoderm and ectoderm. Although the patterns are broadly similar, we describe transitional states in the development of the expression pattern in *Xenopus* that are not seen in mouse (Nieto et al., 1992). Xsna mRNA first appears in the pre-chordal mesoderm (the prospective most anterior mesoderm), as it is beginning to move around the internal blastopore lip before external blastopore formation (Nieuwkoop and Faber, 1967). It appears in all cells as they involute but is subsequently down-regulated in a tissue-specific fashion. This is more evident in *Xenopus* than mouse and reveals the subdivision of the mesoderm into its derivatives before they are overtly differentiated, e.g. notochord by stage 12; each myotome before rotation and the pronephroi in early tailbud. Expression of Xsna is therefore characteristic of undifferentiated mesoderm and may indicate that inactivation of Xsna is necessary to permit expression of tissue-specific genes, as in *Drosophila* where certain genes are repressed by snail and expressed only where snail is not expressed (Leptin, 1991).

**Ectodermal expression of Xsna : the neural plate border**

In the mouse, ectodermal Sna mRNA is found almost entirely in delaminating neural crest cells. Any expression of Sna in an epithelium before delamination can only be very brief whereas Xsna is present in an ectodermal sheet for a considerable time before neural crest migration. The earliest appearance of Xsna, at stage 11, seen in transverse section, is as stripes at almost 90˚ to the dorsal midline. Reconstructions from serial sections of stage 12 and 14 embryos (data not shown) show these stripes curve around the
anterior dorsal surface and extend posteriorly along the lateral surfaces to the blastopore creating a sharp border to the presumptive neural plate. During neurulation, the stripes of Xsna expression approach the dorsal midline outlining the familiar keyhole shape of the neural folds at stage 14. An early study of the histology, ultrastructure and fate of the prospective mid-trunk neur ectoderm of stage 13 Xenopus revealed the presence of a distinct compartment, flanking the prospective neural plate in each ectodermal layer (Schroeder, 1970). During neural tube closure, the superficial ectodermal compartment fuses on the dorsal midline to become the roof of the neural tube and the deep ectodermal compartment is just excluded from the neural tube and becomes the neural crest. Here we report that Xsna mRNA is a precise marker of these two compartments and that they are present from stage 11, substantially earlier than suggested by Schroeder (1970). Furthermore, the reconstructions show these compartments surround the entire neural plate. In the anterior, they become the anterior transverse fold which is incorporated into forebrain and does not generate crest (Eagleson and Harris, 1990). We designate this band of cells the neural plate border. It is not revealed in its entirety by any other available molecular markers; however, expression of a number of ectodermal markers end at sharp boundaries, which may be either dorsal or ventral sides of the neural plate border (Balak et al., 1987; Kintner and Melton, 1987; Akers et al., 1986; Jones and Woodland, 1989; Jamrich et al., 1987; Itoh et al., 1988; London et al., 1988; Rosa et al., 1988; Angres et al., 1991; Levi et al., 1991).

The Xsna promoter

The promoter of Xsna has been identified by its capacity to drive either CAT or β-gal expression in embryos injected with suitable constructs. A 2 kb promoter driving β-gal expression gives a pattern of expression in the best stained specimens in mesoderm and ectoderm that corresponds in most respects to that found by in situ hybridisation. Mesodermal expression starts at stage 10 and appears stronger on the prospective dorsal side than on the ventral side as for the endogenous gene at stage 9. The ectodermal pattern of β-gal expression at stage 11, of wide bands at about 75-90˚ to the dorsal midline, also matches the pattern of ectodermal expression deduced from serially sectioned embryos. The lower end of these bands are found close to the lateral blastopore lip (probably the limit of involution). β-gal is also expressed in the anterior neural fold. We are unable to distinguish between the contributions of the deep and superficial layers at this stage using intact embryos but, in thin sections, there is expression in both layers. During late neurulation, these contributions are clearly distinguished as the roof of the neural tube and the cranial and trunk neural crest (Fig. 5G) while the neural plate does not express.

Significant discrepancies have been observed between the β-gal patterns and the in situ hybridisation pattern in four respects. (a) Expression persists in the somites long after the endogenous gene has turned off. (b) There is β-gal expression in the animal cap at stage 10 where no Xsna expression is detectable by in situ hybridisation although small amounts of Xsna mRNA are detectable by RNase protection (Sargent and Bennett, 1990). (c) There is occasional β-gal expression (about 5% of cases) in injected embryos in the tissue above the blastopore lip (the supra-blastoporal endoderm), in which endogenous Xsna is not detectable by in situ hybridisation until after involution. (d) There is no apparent induction of reporter genes in animal cap explants in response to activin A or bFGF.

As injected β-gal protein survives in embryos for several days (unpublished observations), the persistent expression in somites but not other mesodermal tissues may be explained in terms of longevity of the fusion protein in this tissue. We believe that the other instances of anomalous expression are explained by the lack of control elements that normally limit expression so that there is expression in parts of the embryo (such as the animal cap and the supra-blastoporal endoderm) which do not normally express the endogenous gene at significant levels. There are a number of recorded instances of promoter constructs of developmental genes that lack elements that modulate the stability of the mRNA and which consequently express where the endogenous gene product is not usually detected (Colberg-Poley et al., 1987; Gagnon et al., 1992). There are indications that a change in expression of two mesodermal genes in early embryogenesis, mediated by mesoderm-inducing factors, may be mediated by changes in the stability of the mRNA (Rupp and Weintraub, 1991; Dassow et al., 1993). Possibly, the lack of inducibility of Xsna promoter-reporter constructs by activin and bFGF could be explained by postulating that the effect of mesoderm-inducing factors on the gene is to stabilise the transcripts as in the case of myoD (Rupp and Weintraub, 1991). If mesoderm-inducing factors cause stabilisation of the mRNA of Xsna, it raises the question; what induces Xsna when zygotic transcription starts? Our observation of induction of promoter-reporters by the vegetal pole suggests that another kind of inducing signal emanates from the vegetal pole in addition to activin. Thus there are probably two kinds of regulatory influences affecting the level of Xsna mRNA; one an induction affecting initiation of mRNA synthesis and another affecting the stability of the processed mRNA mediated by the mesoderm-inducing factors. The synthesis of Mix.I (Rosa, 1989), Xbra (Smith et al., 1991) and Goosecoid (Cho et al., 1991) are all earlier responses to activin in animal cap assays than the appearance of Xsna. Furthermore, as artificially increased expression of Xbra in animal cap explants, obtained by microinjection of mRNA, causes expression of Xsna without the need for mesoderm-inducing factors (Cunliffe and Smith, 1992), it is clear that the influences regulating Xsna expression are complex.

Organisation of the Xsna promoter

The two major features of the expression pattern of endogenous Xsna and of the promoter-β-gal constructs (i.e. in mesoderm and neural folds) can be attributed to two separate elements in the promoter. When the M element is deleted there is only ectodermal expression. Attempts to obtain mesodermal expression without ectodermal expression by construction of internal deletions within ectodermal promoter have so far failed and therefore we are unable formally to distinguish between the part played by a putative ectodermal element and the basal promoter.

The most notable feature of the promoter, apart from its
small size, is the absence of known transcription factor binding sites. Unlike many promoters that lack a TATA box, the Xsnu promoter is not GC-rich and does not have Sp1 sites near the 3′ end (Blake et al., 1990; Magin et al., 1992). It contains only two motifs (Fig. 2) that may correspond to a known transcription factor binding site AP4 (−55; Mermod et al., 1988) and the B2 promoter element of TF111A (−91; Scotto et al., 1989). Many TATA-less promoters do not have precisely determined start sequences (Blake et al., 1990) or, if they do, they have a specific sequence at ‘start’ that is required for initiation (O’Shea-Greenfield and Smale, 1992; Wiley et al., 1992). The Xsnu promoter initiates transcription at a fixed distance (45 bases) from the Sp1 site but the entire sequence from −45 to the translational start can be replaced by part of the reporter sequence with no reduction in expression. We presume that there must be a cryptic TF11D recognition sequence in the promoter but its spatial relationship with ‘start’ is different from TATA-containing promoters and, indeed, from those TATA-less promoters that require TF11D for initiation at 30 bases downstream (Wiley et al., 1992).

Upstream to the M region-specific sequences there is an element that increases ectodermal and mesodermal expression. It includes recognition sequences for Sp-1 (Briggs et al., 1985) and Oct1 (Mattaj et al., 1985; La Bella et al., 1988) in reverse of their usual orientation.

The availability of a promoter-reporter construct that expresses only in the neural plate border provides us with a method for studying induction of the presumptive neural folds. We hope to use them to establish the source and identity of the inducer within the embryo.

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REFERENCES


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