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Isolation of *Sna*, a mouse gene homologous to the *Drosophila* genes *snail* and *escargot*: its expression pattern suggests multiple roles during postimplantation development

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Summary

The *Drosophila* gene *snail* encodes a zinc-finger protein that is required zygotically for mesoderm formation. *Snail* acts as a transcriptional repressor during the period of mesoderm formation by preventing expression of mesectodermal and ectodermal genes in the mesoderm anlage. A *Xenopus* homolog (*xsnail*) of *snail* has been cloned and it too is expressed early in the mesodermal germ layer. We have isolated cDNA clones of a mouse gene (termed *Sna*) closely related to *snail* and *xsnail* and another *Drosophila* gene termed *escargot* that also encodes a zinc-finger protein. *Sna* encodes a 264 amino acid protein that contains four zinc fingers. Developmental RNA blot analysis showed that *Sna* transcripts are expressed throughout postimplantation development. Analysis of the spatial and temporal localization of *Sna* transcripts by in situ hybridization to both whole-mount and sectioned embryos revealed that, in the gastrulating embryo, *Sna* is expressed throughout the primitive streak and in the entire mesodermal germ layer. By 9.5 days post coitum (dpc) *Sna* is expressed at high levels in cephalic neural crest and limb bud mesenchyme. In fact, by 10.5 dpc *Sna* expression is observed in most mesenchymal cells, whether of neural crest or mesodermal origin. Later in gestation, high levels of *Sna* expression are observed in condensing cartilage and in the mesenchymal component of several tissues (lung, kidney, teeth and vibrissae) that undergo epithelial-mesenchymal inductive interactions during development. These results suggest multiple roles for the *Sna* gene in gastrulation and organogenesis during murine development.

Key words: gastrulation, mesenchyme, mesoderm formation, neural crest, *snail*, *twist*, *escargot*.

Introduction

Gastrulation in a wide variety of organisms involves the transformation of an embryo with one or two embryonic germ layers into an embryo with three embryonic germ layers (ectoderm, endoderm and mesoderm). Although the morphogenetic changes that result in mesoderm formation vary greatly among individuals of different species, recent work has shown that several genes involved in mesoderm formation have been conserved during evolution.

In *Drosophila*, classical genetic studies have identified the genes involved in determining cell fates along the dorsoventral axis of the embryo, including the most ventral region which forms mesoderm (for reviews, see Anderson, 1987; Rushlow and Arora, 1990; St. Johnston and Nüsslein-Volhard, 1992). The maternal morphogen *dorsal*, which is related to the *rel* family of vertebrate transcription factors (for review, see Rushlow and Warrior, 1992), acts in the ventral region of the embryo to activate the expression of two zygotic genes, *twist* and *snail*, which are known to be required for mesoderm formation. Embryos homozygous for null mutations of *twist* and *snail* fail to form a normal ventral furrow, form no mesodermal germ layer and die late in embryogenesis (Simpson, 1983; Grau et al., 1984; Leptin and Grunewald, 1990). *Twist* is a transcriptional activator of mesoderm-specific genes, while *snail* acts to maintain proper germ layer boundaries by repressing the expression within the mesoderm of regulatory genes of the lateral mesectoderm and neuroectoderm (Kosman et al., 1991; Leptin, 1991; Nambu et al., 1990). Molecular analysis of the *twist* and *snail* genes indicates that they encode proteins of the basic helix-loop-helix and zinc-finger families, respectively (Boulay et al., 1987; Thisse et al., 1988).

In vertebrates, the molecular aspects of mesoderm formation have been studied most extensively in *Xenopus* (for reviews, see Hopwood, 1990; Jessell and Melton, 1992; Smith, 1989). These studies have suggested that diffusible peptide growth factors initiate mesoderm formation in vivo. These signaling molecules emanate from the endoderm of the vegetal hemisphere to induce mesoderm in the neighboring marginal zone. During in vitro animal cap explant studies, members of the fibroblast growth factor (FGF),

transforming growth factor β (TGFβ) and *Wnt* gene families have all been shown to induce mesoderm structures. Some of these signaling molecules have been shown to be present in the embryo at the time of mesoderm induction (Isaacs et al., 1992; Kimelman et al., 1988; Slack and Isaacs, 1989; Thomsen et al., 1990). In addition, studies with a dominant-negative FGF receptor provide strong evidence for a FGF-like factor being involved in mesoderm induction in vivo (Amaya et al., 1991).

In an attempt to identify which mesoderm-specific regulatory genes might respond to these signaling molecules, the cloning of *Xenopus* homologs to mesoderm regulatory genes of *Drosophila* has been undertaken. Homologs to *twist* and *snail* have been identified in *Xenopus* (Hopwood et al., 1989; Sargent and Bennett, 1990). Both genes are expressed in the marginal zone and respond to mesoderm inducing factors in the animal cap explant studies. The identification of such homologs in *Xenopus* suggest that there may be a conservation of required regulatory genes in evolutionarily divergent modes of mesoderm formation.

Less is known about the molecular aspects of mesoderm formation in chick and mouse embryos (reviewed by Stern, 1992). Recent work on the chick has shown that peptide growth factors, which induce mesoderm in the *Xenopus* animal cap explant studies, have some comparable effects on early chick embryos. For example, chick epiblasts cultured in the presence of conditioned medium from an activin-secreting tissue culture cell line develop axial structures including a notochord and bilaterally symmetric somites (Mitrani et al., 1990). This study also demonstrated that transcripts for activin β_B, but not activin β_A, were present at the stage when axial mesoderm induction takes place in the chick (Mitrani et al., 1990). Recently, it was shown that localized application of activin to the chick blastoderm can generate ectopic axes (Ziv et al., 1992). A number of recent experiments suggest that mesoderm induction and formation of the embryonic axis (i.e., generation of the primitive streak) are different and separable events in the chick, and may involve different inducing factors (reviewed by Stern, 1992).

In the mouse, studies to localize RNA and protein expression of several peptide growth factors have been performed. RNA localization studies have shown that three members of the mammalian FGF family, *Fgf-3* (*int-2*), *Fgf-4* and *Fgf-5* are expressed in the primitive streak region of the gastrulating mouse embryo (Haub and Goldfarb, 1991; Hébert et al., 1991; Niswander and Martin, 1992; Wilkinson et al., 1988). Similar studies analyzing the localization of RNA transcripts of TGFβ1, TGFβ2, activin β_A, activin β_B and inhibin α were recently performed (Manova et al., 1992). Transcripts from three of these genes (TGFβ1, TGFβ2 and activin β_A) were expressed at a time consistent with their products having a role in mesoderm formation. However, these genes were not expressed in the embryo but were expressed in the uterine decidua (Manova et al., 1992). These results, however, may be in contradiction with the finding of TGFβ2 protein in the gastrulating mouse embryo, particularly in the visceral endoderm (Slager et al., 1991).

The isolation of murine homologs of genes important for development in other organisms has been a valuable addition to the array of techniques that can be used to study mammalian development (for reviews, see Gridley, 1991; Kessel and Gruss, 1990; Rossant and Hopkins, 1992). As mentioned above, *Xenopus* homologs of both *snail* and *twist* have been reported (Hopwood et al., 1989; Sargent and Bennett, 1990), and a mouse homolog of *twist* (*mtwist*) has also recently been cloned (Wolf et al., 1991). However, the temporal and spatial distribution of *mtwist* in the early embryo suggests that it is involved in the specification of specific subpopulations of mesodermal cells and not in the initiation of mesoderm formation. We describe in this paper the cloning of *Sna*, a mouse homolog of the *Drosophila* genes *snail* and *escargot* and the *Xenopus* gene *xsnail*. An analysis by in situ hybridization of the spatial and temporal localization of *Sna* RNA expression suggests multiple roles for the *Sna* gene in gastrulation and organogenesis during postimplantation mouse development.

Materials and methods

Embryo isolation

Embryos were obtained from natural matings of C57Bl/6 mice. The day on which the vaginal plug was detected was designated 0.5 dpc.

PCR amplification and cloning of zinc-finger domains from 8.5 dpc embryo cDNA

Total RNA was isolated from 8.5 dpc embryos by the acid guanidinium thiocyanate-phenol-chloroform technique (Chomczynski and Sacchi, 1987) using RNazol B (Biotecx). First strand cDNA was synthesized from 10 μg total RNA using oligo (dT) priming and the Superscript cDNA synthesis kit (BRL) according to the manufacturer's recommendations. Zinc-finger domain cDNAs were amplified using degenerate oligonucleotide primers corresponding to the amino acids TGEKPF (5′) and SRMSLL (3′), with additional 5′ nucleotides coding for the restriction sites *Eco*RI (5′ primer) and *Sal*I (3′ primer). The primer sequences were:

5′ primer - GTGAATTCAC(GATC)GG(GATC)GA(AG)C(CT)-(GATC)TT;
3′ primer - GTCGACCTG(GATC)A(AG)(GATC)A(AG)-(GATC)GACAT(GATC)C(TG)(GATC)GA.

Standard PCR amplification was performed with 10% of the first strand cDNA using the following cycling paramaters: denaturation, 95°C, 25 seconds; annealing, 58°C, 30 seconds; elongation, 72°C, 30 seconds for 40 cycles. The PCR product was gel-purified, extracted twice with phenol-chloroform, and ethanol precipitated with 20 μg of glycogen as carrier. The precipitate was then resuspended and digested with *Eco*RI and *Sal*I and cloned into pGem7(zf+) (Promega). The inserts of the clones were sequenced by the dideoxy technique using T7 and SP6 primers.

cDNA library screening

The insert from a subclone of the amplified PCR product showing a high degree of homology to the *Drosophila* and *Xenopus snail* genes was purified from vector sequences and labelled with ^{32}P using a random prime labelling kit (Pharmacia). Replica filter lifts of approximately 7.5×10^5 clones of an 8.5 dpc mouse embryo cDNA library (Fahrner et al., 1987) were hybridized with 5×10^5 cts/minute/ml of the labelled insert. Hybridization was performed overnight at 65°C in 5× SSC, 5× Denhardt's, 0.5% SDS and 100 μg/ml denatured salmon testes DNA. Filters were washed twice for 15 minutes at room temperature in 2× SSC, 0.5% SDS, and

twice for 30 minutes at 65°C in 0.2× SSC, 0.5% SDS. Individual hybridizing clones were plaque-purified and phage inserts were subcloned into pGEM7(zf+) (Promega). The longest clone was sequenced on both strands by the dideoxy technique using specific oligonucleotide primers.

In situ hybridization

In situ hybridization of embryo sections was performed as previously described (Franco del Amo et al., 1992; Smith and Gridley, 1992). The probe for *Sna* included nucleotides 730 to 1200 of the *Sna* cDNA. A probe for the *Brachyury* (*T*) gene was generously provided by Bernhard Herrmann (described by Herrmann, 1991). Plasmids were linearized with the appropriate enzymes, and ^{35}S-labelled sense and antisense transcripts were generated by run-off transcription.

Whole-mount in situ hybridization was performed using procedures similar to those described for embryos of other organisms (Tautz and Pfeifle, 1989; Hemmati-Brivanlou et al., 1990), using a protocol kindly supplied by David Wilkinson (Wilkinson, 1992).

Results

PCR amplification of mouse zinc-finger sequences homologous to snail

A comparison of the published amino acid sequences of the *Drosophila* (Boulay et al., 1987) and *Xenopus* (Sargent and Bennett, 1990) *snail* genes (hereafter referred to as *snail* and *xsnail*, respectively) revealed extensive amino acid homology within the zinc-finger domains. To clone a mouse homolog of *snail*, we chose two of these conserved peptide regions for the creation of degenerate oligonucleotide primers for PCR amplification. The 5′ primer encompassed the peptide sequence TGEKPF, which is found in many zinc-finger proteins (Gibson et al., 1988; Nietfeld et al., 1989). The 3′ oligonucleotide primer encompassed the peptide sequence SRMSLL which appears to be unique to the *snail* homologs. Both *snail* (Boulay et al., 1987) and *xsnail* (Sargent and Bennett, 1990) are expressed early in the mesoderm anlage. Therefore, assuming that the mouse homolog would also be expressed early in the mesoderm we used first strand cDNA made from 8.5 dpc embryo RNA as the template for the degenerate oligonucleotide-based PCR amplification (see Materials and methods). The PCR amplification products were cloned and 30 of these subclones were sequenced. Several of the sequenced clones were identical. Examination of this sequence indicated that it encoded a zinc-finger domain highly homologous to the zinc-finger domain of *snail* (77% amino acid identity) and *xsnail* (86% amino acid identity) (data not shown).

Isolation of a cDNA clone encoding a homolog of snail

The PCR clone that was highly homologous to the *snail* genes was used to screen an 8.5 dpc cDNA library (Fahrner et al., 1987). Of 7.5×10^5 phage clones screened, 8 positive clones were identified, plaque purified and subcloned. Inserts from these clones ranged in size from 700 bp to 1400 bp. Restriction enzyme mapping and partial nucleotide sequence analysis indicated that these inserts formed a set of overlapping cDNA clones. The nucleotide and predicted amino acid sequence of a composite of two of the clones is presented in Fig. 1. A region of high amino acid homology with the amino terminus of the *xsnail* gene (Sargent and Bennett, 1990) was found in an open reading frame that began at the first Met codon (Fig. 2). The nucleotide sequence around this first Met codon also conforms well with the consensus sequence for eukaryotic translation initiation sites (Kozak, 1987). This suggests that this methionine codon represents the translation initiation codon for this cDNA clone. This open reading frame encoded a protein of 264 amino acids, which is similar in size to the 259 amino acid open reading frame encoding *xsnail*. Both of these predicted polypeptides are considerably smaller than the 390 amino acid polypeptide encoded by *snail.* Comparison of the amino acid sequence encoded by this mouse gene with the amino acid sequence of *xsnail* (Fig. 2) revealed two main areas of sequence conservation. The region of strongest sequence identity is the zinc-finger domain at the carboxy terminus of the protein. Another region of strong sequence conservation exists at the amino terminus of the protein. Other more limited regions of homology are scattered throughout the remaining areas of the protein. Because of the extensive amino acid conservation with *xsnail* and the observed expression pattern of the gene (discussed below), we have termed this mouse gene *Sna*.

Comparison of the amino acid sequence of the zinc-finger region of *Sna* with the zinc-finger regions encoded by the genes *snail*, *xsnail* and *escargot* (a recently identified *Drosophila* gene closely related to *snail;* Whiteley et al., 1992) reveals that the zinc-finger domain encoded by *xsnail, escargot* and *Sna* are more closely related to each other than they are to *snail* (Fig. 3). In the region of these proteins upstream (amino-terminal) of the zinc-finger domain, amino acid sequence homologies can be observed between snail and escargot (Whiteley et al., 1992) and between Sna and xsnail (Fig. 2), but no significant homologies in this region are observed when comparing the *Drosophila* proteins with the vertebrate proteins. The comparison of the zinc-finger domains (Fig. 3) also reveals that the protein encoded by the *Sna* gene only has four zinc fingers, instead of the five zinc fingers of *snail*, *xsnail* and *escargot*. The region of the first zinc finger has lost the conserved cysteine and histidine residues characteristic of zinc fingers of the Cys_2/His_2 class, although a few of the non-canonical amino acid residues in the first zinc finger are conserved with *xsnail*.

Spatial and temporal localization of Sna *expression*

We analyzed by northern blotting the steady state expression level of *Sna* during postimplantation mouse development, and detected a single *Sna* transcript of approximately 1.7 kb (data not shown). Levels of *Sna* RNA remained relatively constant from 7.5 dpc through 14.5 dpc. To analyze further the embryonic expression pattern of *Sna*, and in particular to address the question of a possible role for *Sna* during mesoderm formation in the mouse, we analyzed, by in situ hybridization, the spatial and temporal localization of *Sna* transcripts during postimplantation development.

We performed in situ hybridization analysis on both whole-mount embryos from 7.5 dpc to 9.5 dpc and to sections of embryos from 7.5 dpc through 10.5 dpc and at 13.5

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   1  TCGCTCTGGCCAACATGCCGCGCTCCTTCCTGGTCAGGAAGCCGTCCGACCCCCGCCGGA
                 M  P  R  S  F  L  V  R  K  P  S  D  P  R  R  K   16

  61  AGCCCAACTATAGCGAGCTGCAGGACGCGTGTGTGGAGTTCACCTTCCAGCAGCCCTACG
       P  N  Y  S  E  L  Q  D  A  C  V  E  F  T  F  Q  Q  P  Y  D   36

 121  ACCAGGCCCACCTGCTGGCCGCCATCCCTCCGCCCGAGGTCCTCAACCCCGCCGCTTCGC
       Q  A  H  L  L  A  A  I  P  P  P  E  V  L  N  P  A  A  S  L   56

 181  TGCCCACCCTCATCTGGGACTCTCTCCTGGTACCCCAAGTGCGGCCGGTTGCCTGGGCCA
       P  T  L  I  W  D  S  L  L  V  P  Q  V  R  P  V  A  W  A  T   76

 241  CCCTCCCGCTGCGGGAGAGCCCCAAGGCCGTAGAGCTGACCTCGCTGTCCGATGAGGACA
       L  P  L  R  E  S  P  K  A  V  E  L  T  S  L  S  D  E  D  S   96

 301  GTGGCAAAAGCTCCCAGCCGCCCAGCCCGCCCTCGCCGGCGCCGTCGTCCTTCTCGTCCA
       G  K  S  S  Q  P  P  S  P  P  S  P  A  P  S  S  F  S  S  T  116

 361  CCTCGGCCTCCTCCCTGGAGGCCGAGGCCTTCATCGCCTTCCCTGGCTTGGGCCAACTTC
       S  A  S  S  L  E  A  E  A  F  I  A  F  P  G  L  G  Q  L  P  136

 421  CCAAGCAGCTGGCCAGGCTCTCGGTGGCCAAGGACCCCCAGTCGCGGAAGATCTTCAACT
       K  Q  L  A  R  L  S  V  A  K  D  P  Q  S  R  K  I  F  N  C  156

 481  GCAAATATTGTAACAAGGAGTACCTCAGCCTGGGCGCTCTGAAGATGCACATCCGAAGCC
       K  Y  C  N  K  E  Y  L  S  L  G  A  L  K  M  H  I  R  S  H  176

 541  ACACGCTGCCTTGTGTCTGCACGACCTGTGGAAAGGCCTTCTCTAGGCCCTGGCTGCTTC
       T  L  P  C  V  C  T  T  C  G  K  A  F  S  R  P  W  L  L  Q  196

 601  AGGGCCACGTCCGCACCCACACTGGTGAGAAGCCATTCTCCTGCTCCCACTGCAACCGTG
       G  H  V  R  T  H  T  G  E  K  P  F  S  C  S  H  C  N  R  A  216

 661  CTTTTGCTGACCGCTCCAACCTGCGTGCCCACCTCCAAACCCACTCGGATGTGAAGAGAT
       F  A  D  R  S  N  L  R  A  H  L  Q  T  H  S  D  V  K  R  Y  236

 721  ACCAGTGCCAGGCCTGTGCCCGAACCTTCTCCCGCATGTCCTTGCTCCACAAGCACCAAG
       Q  C  Q  A  C  A  R  T  F  S  R  M  S  L  L  H  K  H  Q  E  256

 781  AGTCTGGCTGCTCCGGAGGCCCTCGCTGACCCTGCTACCTCCCCATCCTCGCTGGCATCT
       S  G  C  S  G  G  P  R                                      264

 841  TCCCGGAGCTCACCCTCCTCCTCACTGCCAGGACTCCTTCCAGCCTTGGTCCGGGGACCT

 901  GTGGCGTCCATGTCTGGACCTGGTTCCTGCTTGGCTCTCTTGGTGGCCTTTGCCGCAGGT

 961  GGCTGATGGAGTGCCTTTGTACCCGCCCAGAGCCTCCTACCCCTCAGTATTCATGAGGTG

1021  TAGCCTCTGGACACAGCTGCTTCGAGCCATAGAACTAAAGCCAACCCACTGGCTGGGAAG

1081  CTTGAACCCCGCTCAGGGACCCCACTTCCCTACCTCCCTCAAGGACCCTTCAGGCCACCT

1141  TCTTTGAGGTACAACAGACTATGCAATAGTTCCCCTCCCCCCCACCCCGTCCAGCTGTAA

1201  CCATGCCTCAGCAGGGTGGTTACTGGACACATGTCCAGGTGCCCCTGGGCCTGGGCAACT

1261  GTTTCAGCCCCCGCCCCCATTTGTCTGGTGACACCTGTTTCACAGCAGTTTAACTGTCTC

1321  AGAAGGGACCATGAATAATGGCCATCACTTGTTAGGGGCCAAGTGGGGTGCTTCAGCCTG

1381  GCCAATGTGTCTCCCAGAACTATTTTGGGGGCCCAACAGGTGGCCCCGGGAGAAAGATGT

1441  TTACATTTTAAAGGTATTTATATAGTAAGCAGCATTTTGTATAGTTAATATGTACAGTTT

1501  ATTGATATTCAATAAAATGGTTAATTTATATACTAAAAAAA  1541
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Fig. 1. Nucleotide and deduced amino acid sequence of the predicted *Sna* cDNA. This sequence is a composite of two cDNA clones, clone 5-15 (nucleotides 1-1357) and clone 21-7 (nucleotides 702-1538). The sequence ends in a run of seven A residues which may be part of the poly(A) tail. Eighteen nucleotides upstream of this stretch of A residues is a consensus poly(A) addition site (underlined). Nucleotide numbering is shown to the left, and predicted amino acid numbering is shown to the right. This nucleotide sequence will appear in the EMBL/GenBank/DDBJ databases under the accession number M95604.

dpc and 16.5 dpc. The antisense riboprobe used in these studies was generated from nucleotides 730 to 1200 of the *Sna* cDNA clone. This region includes the last zinc finger as well as approximately 400 nucleotides of 3′ untranslated sequence. Identical hybridization results were obtained using antisense riboprobes from the 5′ end of the cDNA, as well as an antisense riboprobe made from the clone of the initial PCR product (not shown). Control hybridization of embryo sections with sense-strand riboprobes did not show any signal (not shown).

Hybridization of the antisense *Sna* riboprobe to transverse and sagittal sections of 7.5 dpc embryos revealed high levels of expression in parietal endoderm, in the primitive streak and in mesoderm migrating away from the primitive streak (Fig. 4B,C). At this stage all mesoderm appeared to express high levels of *Sna*. This was confirmed by examination of whole-mount embryos hybridized with a *Sna* antisense riboprobe, which revealed *Sna* expression throughout the primitive streak, in the migrating mesodermal wings and in part of the ectoplacental cone (Fig. 4A). By comparison, hybridization of transverse sections of 7.5 dpc embryos with an antisense riboprobe for the *Brachyury* (*T*) gene revealed high levels of expression in the primitive streak and in mesoderm medial to the streak, but *T* gene expression was not observed in more lateral mesoderm (Fig. 4D and Herrmann, 1991; Wilkinson et al., 1990).

At 8.5 dpc, high levels of *Sna* expression were observed in the allantois, in cephalic mesenchyme and in both presomitic and somitic mesoderm (Fig. 5). To assess whether *Sna* transcripts were expressed in cephalic mesenchyme of both mesodermal and neural crest derivation, sections of a 7-somite, 8.5 dpc embryo were hybridized with antisense riboprobes for *Sna* and for cellular retinoic acid binding protein I (CRABP I). CRABP I has been shown to be a marker for both cranial and trunk neural crest in mice and chicks (Maden and Holder, 1992; Ruberte et al., 1991). In mouse embryos, prior to the 4-somite stage CRABP I is expressed in cranial mesenchyme of mesodermal origin, but by the 7-somite stage mesodermal expression has diminished and CRABP I expression appears to be specific for

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  Sna       MPRSFLVRKP SDPRRKPNYS ELQDACVEFT FQQPYDQAHL LAAIPPPEVL
 Xsna       MPRSFLVKKH FSASKKPNYS ELESQTV.YI SPFIYDK... FPVIPQPEIL
Consensus   MPRSFLV-K- -----KPNYS EL----V--- ----YD---- ---IP-PE-L

  Sna       NPAASLPTLI WDSLLV.... ...PQVRPVA WATLPLRESP KAVELTSLSD
 Xsna       STGAYYTPLV WDTGLLTTFF TSESDYKKSP ISPSSSDDSS KPLDLTSFSS
Consensus   ---A----L- WD--L----- ---------- --------S- K---LTS-S-

  Sna       ED.SGKSSQP PSPPSPAPSS FSSTSASSLE AEAFIAFPGL GQLPKQLARL
 Xsna       EDEGGKTSDP PSPASSATE. .AEKFQCNLC SKSYSTFAGL SK.HKQLHC.
Consensus   ED--GK-S-P PSP-S-A--- --------L- ------F-GL ----KQL---

  Sna       SVAKDPQSRK IFNCKYCNKE YLSLGALKMH IRSHTLPCVC TTCGKAFSRP
 Xsna       ....DSQTRK SFSCKYCEKE YVSLGALKMH IRSHTLPCVC KICGKAFSRP
Consensus   ----D-Q-RK -F-CKYC-KE Y-SLGALKMH IRSHTLPCVC --CGKAFSRP

  Sna       WLLQGHVRTH TGEKPFSCSH CNRAFADRSN LRAHLQTHSD VKRYQCQACA
 Xsna       WLLQGHIRTH TGEKPFSCTH CNRAFADRSN LRAHLQTHSD VKKYQCKSCS
Consensus   WLLQGH-RTH TGEKPFSC-H CNRAFADRSN LRAHLQTHSD VK-YQC--C-

  Sna       RTFSRMSLLH KHQESGCSGG PR
 Xsna       RTFSRMSLLH KHEETGCTVA H
Consensus   RTFSRMSLLH KH-E-GC--- -
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Fig. 2. Alignment and comparison of the predicted amino acid sequences of *Sna* and *xsnail*. The sequences were aligned with the GAP program of the GCG Sequence Analysis Software Package (Devereaux et al., 1984). Gaps inserted into the sequences are indicated by a dot (.). Amino acid residues identical between the two sequences are shown on the consensus sequence on the bottom line. The *xsnail* sequence was obtained from Sargent and Bennett, 1990.

neural crest (Ruberte et al., 1991). Hybridization of serial, near-adjacent sections with both riboprobes demonstrated that *Sna* is expressed in migrating cranial neural crest (Fig. 6).

Examination of whole-mount embryos at 9.5 dpc hybridized with the *Sna* antisense riboprobe (Fig. 7A) revealed high levels of expression in regions colonized by cephalic neural crest, in somites, in the forelimb bud and in tail bud mesenchyme. In the forelimb bud *Sna* was expressed at high levels throughout the mesenchyme but was absent from the apical ectodermal ridge (Fig. 7B). In somites *Sna* expression was not present in dermatome, but was present in myotome (Fig. 7C,D and data not shown) and was expressed at higher levels in sclerotome (Fig. 7D). By 10.5 dpc, a majority of mesenchymal cells, whether of neural crest or of mesodermal origin, expressed high levels of *Sna* RNA (Fig. 7E). Neuroepithelium in the brain and spinal cord did not express *Sna* (Fig. 7E), nor did neuronal derivatives of neural crest such as trigeminal or dorsal root ganglia (Fig. 7E).

Later in gestation, the overall level of *Sna* expression diminished but high levels of expression were maintained in condensing cartilage, in the choroid plexus and in the mesenchymal component of several tissues that undergo reciprocal epithelial-mesenchymal inductive interactions during their development (Fig. 8). *Sna* expression was observed both in cells undergoing intramembranous bone formation, characteristic of the skull (Fig. 8A), and in cells undergoing endochondral bone formation, characteristic of the axial and appendicular skeleton (Fig. 8E,G). At 13.5 dpc, another site of *Sna* expression was the choroid plexus (Fig. 8A,B), which serves to secrete cerebrospinal fluid into the ventricles of the brain. *Sna* expression was observed in the mesenchymal layer of the choroid plexus and in mesenchyme that appeared to be poised to migrate into the involuting choroidal epithelium (Fig. 8B).

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snail     F K C D E C Q K M Y S T S M G L S K . H R Q F H C P A A E C N . Q E K K T
escargot  Y Q - P D - - - S - - - F S - - T - . - Q - - - - - - - - G - . - V - - S
xsnail    - Q - N L - S - S - - - F A - - - - . - K - L - - D S . . . . . - T R - S
Sna       S A S S L E A E A F I A F P - - G Q L P K - L A R L S V A K D P - S R - I

snail     H S C E E C G K L Y T T I G A L K M H I R T H T L P
escargot  F - - K D - D - T - V S L - - - - - - - - - - - - -
xsnail    F - - K Y - E - E - V S L - - - - - - - - - S - - - -
Sna       F N - K Y - N - E - L S L - - - - - - - - - S - - - -

snail     C K C P I C G K A F S R P W L L Q G H I R T H T G E K P
escargot  - - - N L - - - - - - - - - - - - - - - - - - - - - -
xsnail    - V - K - - - - - - - - - - - - - - - - - - - - - - - -
Sna       - V - T T - - - - - - - - - - - - - V - - - - - - - - -

snail     F Q C P D C P R S F A D R S N L R A H Q Q T H V D V K K
escargot  - S - Q H - H - A - - - - - - - - - - L - - - S - I - -
xsnail    - S - T H - N - A - - - - - - - - - - L - - - S - - - -
Sna       - S - S H - N - A - - - - - - - - - - L - - - S - - - R

snail     Y A C Q V C H K S F S R M S L L N K H S S S N C T I T I A *
escargot  - S - T S - S - T - - - - - - - - T - - - E G G - P G G S A G S S S S S S E L N Y A G Y A E P *
xsnail    - Q - K S - S R T - - - - - - - - H - - E E T G - - V A H *
Sna       - Q - - A - A R T - - - - - - - - H - - Q E - G - S G G P R *
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Fig. 3. Comparison of zinc-finger domains of *snail*-related genes. The amino acid sequence of the zinc-finger region of *snail* (Boulay et al., 1987) is compared to the zinc-finger regions of the related genes *escargot* (Whiteley et al., 1992), *xsnail* (Sargent and Bennett, 1990) and *Sna*. Zinc fingers are boxed in grey. Identity to the *snail* sequence is indicated by a dash (−). Gaps inserted into the sequences to maximize homology are indicated by a dot (.). Amino acid residues that differ from the *snail* sequence but are common to *escargot*, *xsnail*, and *Sna* are boxed in white. Note that the *Sna* gene has lost the first of the five zinc fingers conserved in the other family members. The translation stop signal at the carboxy terminus of these proteins is indicated with an asterisk.

At 13.5 dpc, *Sna* was expressed at high levels in metanephrogenic mesenchyme of the kidney (Fig. 8D), in lung mesenchyme (Fig. 8E) and in condensing mesenchyme beneath the first molar tooth bud (Fig. 8C). At 16.5 dpc *Sna* was expressed at high levels in mesenchyme in the snout, particularly mesenchymal condensations around the developing whisker follicles (Fig. 8F). These mesenchymal condensations will go on to form the dermal papillae. The development of all these tissues has been shown to involve a series of reciprocal inductive interactions between the epithelial and mesenchymal cell layers (Hardy, 1992; Lumsden, 1988; Saxén, 1987; Thesleff et al., 1989). The maintenance of *Sna* expression in these tissues suggests that *Sna* might be involved in signals emanating from mesenchyme during these reciprocal inductive interactions.

Discussion

In this report we have presented the cloning and postimplantation expression pattern of a mouse gene (*Sna*) homologous to the *Drosophila* genes *snail* and *escargot* and the *Xenopus* gene *xsnail*. After submission of this report, we discovered that Nieto et al. (1992) had also submitted a manuscript describing the cloning and expression pattern of this gene. The results they report are very similar to our results.

A mouse homolog of snail *or* escargot*?*

While analysis of the amino acid sequences in the zinc-finger region of *snail*, *escargot*, *xsnail* and *Sna* clearly indicates that *escargot*, *xsnail* and *Sna* form a group more closely related to each other than they are to *snail* (Fig. 3), the expression patterns support the notion that both *xsnail* and *Sna* are functionally more closely related to *snail*. *Snail* is expressed early in *Drosophila* embryogenesis throughout the mesoderm anlage (Alberga et al., 1991; Leptin, 1991), while *escargot* is expressed in a complex and rapidly changing pattern, primarily in cells of ectodermal origin (Whiteley et al., 1992). While we cannot exclude the possibility that a gene with greater homology to *snail* exists in the mouse genome, neither the PCR amplification reaction with degenerate oligonucleotides nor our screen of the 8.5 dpc cDNA library with the probe cloned from the PCR amplification identified such a gene.

RNA blot analysis demonstrated that *Sna* is expressed throughout postimplantation development. This maintenance of *Sna* expression after initial formation of the mesoderm is similar to that observed for *xsnail*. Both our results with *Sna* and those of *xsnail* would indicate a role for vertebrate *snail* genes well after the initial specification of the mesoderm lineage. Such a role is supported by an analysis of the spatial and temporal localization of *Sna* transcripts during development.

Expression pattern of Sna *in the gastrulating embryo suggests a role in mesoderm formation*

Sna is expressed throughout the primitive streak and the entire mesodermal germ layer at 7.5 dpc. This expression pattern suggests that *Sna* may be required both for the initial formation of the mesodermal germ layer as well as its maintenance. This pattern of expression can be contrasted to that of *Brachyury* (*T*) at the same stage, which is expressed in the primitive streak and in mesoderm medial to the streak, but is not expressed in more lateral mesoderm (Fig. 4D, and Hermann, 1991; Wilkinson et al., 1990). Mutant alleles of *T* suggest that it is required for proper notochord formation and axial development (Hermann, 1991; Yanagisawa et al., 1981). Examination of *Sna* expression in *T* mutants will help determine if *Sna* acts upstream or downstream of *T* during mesoderm formation in the mouse.

Sna *expression during organogenesis*

By 10.5 dpc, most mesenchymal cells in the embryo, whether of mesodermal or neural crest derivation, express *Sna*. Cranial neural crest contributes to structures in the head, such as connective tissue and skeletal elements, that are exclusively of mesodermal origin in the trunk (Noden, 1988). Thus, expression of *Sna* in mesenchymal cells of both mesodermal and neural crest origin may reflect the similar fates that will be adopted by these cells. At 8.5 dpc expression levels appear to be highest in cephalic mesenchyme and in presomitic mesoderm. *Sna* expression is maintained as somites condense, but expression becomes progressively confined to the scleratome, which adopts a mesenchymal configuration, and is lost from the dermatome, which maintains an epithelial configuration.

It is interesting to compare the RNA expression pattern we have observed for *Sna* and the pattern reported for *mtwist* (Wolf et al., 1991). *Mtwist* was not detected in mesoderm or the primitive streak at 7.5 dpc, but was first detected in somites at around 8.0 dpc. Subsequently, *mtwist* and *Sna* were both expressed in most mesenchymal cells, particularly those in the branchial arches, limb buds and scleratome. This is reminiscent of the common expression domain of *snail* and *twist* in the prospective mesoderm of the cellular blastoderm-stage *Drosophila* embryo (Leptin, 1991), and may indicate that the *Sna* and *mtwist* genes share regulatory elements responsible for expression after initial mesoderm specification.

Later in gestation, *Sna* expression becomes progressively confined to cartilage and the mesenchymal component of several tissues that undergo reciprocal epithelial-mesenchymal inductive interactions during their development. This localization of *Sna* transcripts suggests that *Sna* may have multiple roles during postimplantation development, and may be involved in chondrogenesis and in epithelial-mesenchymal inductive interactions.

Does Sna *encode a transcription factor?*

Snail has been shown to function as a transcriptional repressor during early Drosophila embryogenesis (Kosman et al., 1991; Leptin, 1991; Nambu et al., 1990). One of the genes whose transcription is negatively regulated by snail protein is *single-minded* (*sim*; Leptin, 1991; Nambu et al., 1990). Recently, binding of recombinant snail protein to the promoter region of the *sim* gene was demonstrated (Kasai et al., 1992). DNAase I footprint analysis showed that the recombinant snail protein protected a 14-bp consensus sequence that was present nine times in the regulatory region of the *sim* gene (Kasai et al., 1992).

There are no data at present to suggest whether or not

Sna protein acts as a transcriptional repressor during mouse embryogenesis. However, given that there is essentially no amino acid homology between snail and Sna upstream of the zinc-finger domain, it is possible that Sna might function just as well as a transcriptional activator. We expect that the Sna protein could bind DNA, although the fact that the Sna protein only has four zinc fingers, rather than the five zinc fingers of the snail protein, may affect its binding specificity and/or affinity. It will be interesting to determine if recombinant Sna protein can bind and protect the same sites as snail protein in the *sim* regulatory region. Experiments are in progress to answer some of these questions.

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Fig. 8. Expression of *Sna* in cartilage and tissues undergoing epithelial-mesenchymal inductive interactions. All sections were hybridized with the antisense *Sna* riboprobe. (A-E) Sections of a 13.5 dpc embryo. (F,G) Sections of a 16.5 dpc embryo. (A) *Sna* is expressed at high levels in condensing nasal and mandibular cartilage (arrows), as well as tongue mesenchyme and the choroid plexus (asterisk). (B) High levels of *Sna* expression are observed in mesenchyme migrating into the involuting choroid plexus neuroepithelium. (C) Intense *Sna* expression is observed in condensing mesenchyme beneath the first molar tooth bud. (D) *Sna* is expressed in metanephric mesenchyme, but not in glomerular epithelium (arrow). (E) *Sna* is expressed in lung mesenchyme but is not expressed in bronchial epithelium. *Sna* expression is also observed in condensing cartilage of the prevertebrae, but not in spinal ganglia. (F) In the snout, *Sna* expression is strongest in mesenchyme condensing around vibrissa follicles (arrow). (G) *Sna* expression is observed in cartilage of the hindfoot plate. Magnifications: (A,G) 25×; (B) 100×; (C,D,F) 200×; (E) 50×. Abbreviations: l, lung; m, first molar tooth bud; pv, prevertebra; sg, spinal ganglion; t, tongue.

Fig. 4. Expression of the *Sna* and *T* genes at 7.5 dpc. (A) Hybridization of a whole-mount embryo with an antisense *Sna* riboprobe. The purple color indicates detection of *Sna* RNA transcripts. Expression is observed throughout the primitive streak, in the advancing mesodermal wings and in part of the ectoplacental cone.The arrowhead indicates the anterior limit of migration of the mesoderm. (B,C) Transverse (B) and slightly oblique sagittal (C) sections hybridized with an antisense *Sna* riboprobe. High levels of *Sna* expression are observed throughout the mesoderm, in the primitive streak and in parietal endoderm cells (arrowheads). (D) Transverse section of an embryo similar to that in B hybridized with an antisense *T* riboprobe. High levels of *T* gene expression are observed in the primitive streak and in mesoderm adjacent to the streak, but not in more lateral mesoderm. Abbreviations: ec, ectoplacental cone; ee, embryonic ectoderm; m, mesoderm; ps, primitive streak.

Fig. 6. Expression of *Sna* and CRABP I in cranial neural crest. Near-adjacent serial transverse sections of a 7-somite, 8.5 dpc embryo were hybridized with antisense riboprobes for *Sna* (A,C) or CRABP I (B,D). (A,B) *Sna* transcripts are observed in all cephalic mesenchyme, while CRABP I transcripts are not observed in cephalic mesenchyme of mesodermal origin (closed arrow in B). (C,D) Higher magnification view of sections shown in A and B. Neural crest delaminating from the lateral edge of the hindbrain neuroepithelium (open arrows) express both *Sna* and CRABP I transcripts. As previously observed (Ruberte et al., 1991) CRABP I is expressed at very high levels in the hindbrain, with strong labelling both apically and basally in the neuroepithelium and in migrating neural crest. Magnifications: (A,B) 200×; (C,D) 400×. Abbreviations: g, foregut; h, neural epithelium of the caudal hindbrain.

Fig. 5. Expression of *Sna* at 8.5 dpc. (A,D) Hybridization of whole-mount embryos. High levels of expression are observed in cephalic mesenchyme in the headfold region, in somites (arrowheads), in presomitic mesoderm and in the allantois. (B,C,E) Transverse (B,C) and sagittal (E) sections hybridized with an antisense *Sna* riboprobe. High levels of expression are observed in cephalic mesenchyme and in presomitic mesoderm. No expression is observed in neuroepithelium. Abbreviations: al, allantois; cm, cephalic mesenchyme; hf, headfold; pm, presomitic mesoderm.

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Fig. 7. Expression of *Sna* at 9.5 and 10.5 dpc. (A) Hybridization of a 9.5 dpc whole-mount embryo. High levels of expression are observed in tail bud mesenchyme, in somites (open arrowheads) and in the forelimb bud. Expression is also observed in areas colonized by cephalic neural crest, particularly the branchial arches (closed arrowhead). (B-D) Hybridization of 9.5 dpc embryo sections with an antisense *Sna* riboprobe. (B) Transverse section through the region of the forelimb buds. Very high levels of *Sna* expression are observed throughout the limb bud mesenchyme, but no expression is observed in the apical ectodermal ridge. Expression is also observed in paraxial and lateral mesoderm. Expression is not observed in the spinal cord and in the gut endoderm. (C) Parasagittal section. *Sna* expression is observed in most mesenchymal cells, whether of neural crest or of mesodermal origin. Expression is entirely absent from neuroepithelium. The white arrow indicates the region enlarged in D. (D) In the somites, *Sna* is expressed at highest levels in the scleratome, at lower levels in the myotome and appears to be largely absent from the dermatome. (E,F) Hybridization of 10.5 dpc embryo sections with an antisense *Sna* riboprobe. (E) Parasagittal section. The expression is similar to that observed at 9.5 dpc. Note that neuronal derivatives of both cranial and trunk neural crest, such as trigeminal and dorsal root ganglia, do not express *Sna* at this stage. The white arrow indicates the region enlarged in F. (F) High levels of expression are observed in mesenchyme surrounding the epithelial layer of a developing lung bud. Magnifications: (C,E) 25×; (B) 100×; (D) 200×; (F) 400×. Abbreviations: aer, apical ectodermal ridge; d, dermatome; fb, forelimb bud; m, myotome; sc, scleratome.