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Developmental expression of the *Xenopus int-2* (FGF-3) gene: activation by mesodermal and neural induction

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Summary

We have used a probe specific for the *Xenopus* homologue of the mammalian proto-oncogene *int-2* (FGF-3) to examine the temporal and spatial expression pattern of the gene during *Xenopus* development. *int-2* is expressed from just before the onset of gastrulation through to prelarval stages. In the early gastrula, it is expressed around the blastopore lip. This is maintained in the posterior third of the prospective mesoderm and neuroectoderm in the neurula. A second expression domain in the anterior third of the neuroectoderm alone appears in the late gastrula, which later resolves into the optic vesicles, hypothalamus and midbrain-hindbrain junction region. Further domains of expression arise in tailbud to prelarval embryos, including the stomodeal mesenchyme, the endoderm of the pharyngeal pouches and the cranial ganglia flanking the otocyst.

It is shown, by treatment of blastula ectoderm with bFGF and activin, that *int-2* can be expressed in response to mesoderm induction. By heterotypic grafting of gastrula ectoderm into axolotl neural plate, we have also demonstrated that *int-2* can be expressed in response to neural induction.

These results suggest that *int-2* has multiple functions in development, including an early role in patterning of the anteroposterior body axis and a later role in the development of the tail, brain-derived structures and other epithelia.

Key words: *Xenopus*, *int-2* (FGF-3), mesoderm induction, neural induction.

Introduction

The *int-2* oncogene (alternative name FGF-3) was first described as a common proviral integration site in tumours induced by mouse mammary tumour virus (MMTV) (Peters et al., 1983; Dickson et al., 1984). It belongs to the fibroblast growth factor (FGF) family (Dickson and Peters, 1987) that comprises at least seven members sharing 30 to 60% homology over a 120 amino acid central core region (Goldfarb, 1990). bFGF (FGF-2) is the prototype of this family and, along with aFGF (FGF-1), is unusual in that it does not contain a secretory signal sequence and is therefore not secreted, however, in some situations, bFGF may be secreted at low levels by a novel mechanism (Kandel et al., 1991). The other FGFs, such as kFGF (FGF-4), contain hydrophobic signal sequences and are capable of secretion (Fuller-Pace et al., 1991; Talarico and Basilico, 1991). int-2 also contains a signal sequence and is secreted efficiently from stably transformed mouse fibroblasts (Kiefer et al., 1991). Nuclear located forms of int-2 protein can also be produced by translation from an upstream CUG codon but their functional significance remains to be established (Acland et al., 1990).

int-2 is rarely expressed in adult tissues, but proviral insertion by MMTV can activate its expression in the mammary gland where normally it is transcriptionally silent (Dickson et al., 1984). *int-2* is expressed in embryonal carcinoma and embryonal stem cells that have been induced to differentiate (Jakobovits et al., 1986; Mansour and Martin, 1988; Smith et al., 1988; Wilkinson et al., 1988) and displays complex expression patterns during mouse embryogenesis (Jakobovits et al., 1986; Wilkinson et al., 1988, 1989; Niswander and Martin, 1992). In the prestreak mouse embryo, transcripts are found at a low level in parietal endoderm (Niswander and Martin, 1992). During gastrulation, expression is localized to migrating mesodermal cells that have left the primitive streak and also to extraembryonic mesoderm (Wilkinson et al., 1988; Niswander and Martin, 1992). Later sites of expression include rhombomeres r5 and r6 in the hindbrain adjacent to the developing otocyst (Wilkinson, 1990), the endoderm of the pharyngeal pouches, the developing cerebellum, the retina, the sensory region of the inner ear and the tooth mesenchyme (Wilkinson et al., 1989; Niswander and Martin, 1992).

To examine further the developmental functions of *int-2*, we have exploited the *Xenopus* embryo as a more amenable system for experimental embryology. There is compelling

evidence to suggest that members of the FGF family play an important role in early regional specification. For example, many heterologous FGFs, including int-2, show mesoderm-inducing activity (Slack et al., 1987; Paterno et al., 1989), FGF receptors are known to be expressed in early developmental stages (Gillespie et al., 1989; Musci et al., 1990; Friesel and Dawid, 1991) and interference with FGF receptor function disrupts the body pattern (Amaya et al., 1991). We have now analyzed *int-2* in order to assess its role in inductive events within the early *Xenopus* embryo. We present a complete expression pattern and show how the expression of *int-2* is affected by specific inductive signals. Our results suggest that *int-2*, rather than being involved early in mesoderm induction, has a role to play in signalling events in the gastrula and neurula stages of development.

Materials and methods

Embryos and factor treatments

Embryos were produced following the procedures outlined previously (Godsave et al., 1988). Recombinant bovine activin A was a gift of D. Huylebroek, Innogenetics, Belgium. Recombinant *Xenopus* bFGF was prepared by bacterial expression using the clone produced by Kimelman et al., 1988. Both factors, at 10 units/ml, were used to induce mesoderm in animal cap explants dissected from stage 8 to 9 blastula embryos. Explants were cultured to the required stage in 0.5× Normal Amphibian Medium (NAM) in agarose-coated wells of Terasaki plates (Sterilin) (Godsave et al., 1988).

Heterotypic grafting experiments

Axolotl neurulae at stage 13 were placed in 1× NAM, 0.01% trypsin (type IX, Sigma), and a small square of neural plate was removed from the prospective hindbrain region leaving the archenteron roof tissue in place. An excess of soybean trypsin inhibitor (Sigma) was added and a similar size square of stage 10 *Xenopus* gastrula ectoderm was grafted into the hole. Glass bridges, made from coverslip fragments, were used to hold the grafts until sufficient healing had occurred. The host embryos were then cultured to the required stage in 0.1× NAM in agar-coated dishes.

Library screening and probes

A fragment of the murine *int-2* gene covering exon II was used to screen a stage 17 *Xenopus* λgt10 cDNA library at low stringency. Approximately 3×10^5 phages were grown to subconfluence and transfered to Hybond-N. After denaturation and fixing (Sambrook et al., 1989), filters were hybridized with oligolabelled DNA probes synthesized to a specific activity of ~10^9cts/minute/μg (Pharmacia). Hybridization was carried out in 4 × SSC, 40% formamide, 2× Denhardts, and 50 μg/ml yeast tRNA at 37°C for 16 hours. The final stringency of washing was 2× SSC, 0.1% SDS at 50°C. After exposure to XAR-5 X-Omat film (Kodak), positive phages were selected for subcloning by standard procedures (Sambrook et al., 1989). DNA sequencing was performed using Sequenase kits (USB). The *Xenopus int-2* probe used in this study is a 297 base pair cDNA fragment cloned into the transcription vector pBluescriptII KS+ (Stratagene). The sequence of this clone (presented in Fig. 1) has been submitted to the EMBL database (accession number X65237). Details of other clones will be published elsewhere (Close, M. J., Kiefer, P. Halley, C., Dickson, C. Peters, G., unpublished data) The *Xenopus* ornithine decarboxylase (ODC) probe used as a internal control has been described previously (Isaacs et al., 1992). Antisense transcripts from the ODC plasmid linearized with *Bgl*II protect a 91 base pair hybrid in RNAase protection analysis.

Northern blot analysis

Total RNA was prepared by a modified LiCl/urea precipitation technique (Isaacs et al., 1992) and poly $(A)^+$ RNA was purified by oligo(dT) chromatography (Sambrook et al., 1989) or by poly (A) tract magnetic capture technology (Promega). RNA was separated on formaldehyde-agarose denaturing gels (Sambrook et al., 1989) and transfered to Hybond-N in a vacuum blotting apparatus (Hoefer). Fixation of RNA was accomplished by UV cross-linking in a Stratalinker (Stratagene) or by baking at 80°C for 1 hour. Oligolabelled DNA insert probes were synthesized to a specific activity of ~10^9cts/minute/μg (Pharmacia). Hybridization and washing to a final stringency of 0.1× SSPE at 65°C was performed as described by Sambrook et al., 1989. Filters were exposed at −70°C to preflashed XAR-5 X-Omat film (Kodak) for 14 days.

RNAase protection analysis

RNAase protection analysis was performed as described earlier (Isaacs et al., 1992), with a modified RNAase digestion step. RNAase digestion was performed for 20 minutes at 37°C using RNAase A (Boehringer Mannheim) at a concentration of 32 μg/ml and RNAase T1 (BRL) at a concentration of 560 units/ml. Exposure times for *int-2* and ODC were approximately 10-14 and 1-3 days, respectively.

In situ hybridization analysis

In situ hybridization experiments on *Xenopus* embryos using ^{35}S-labelled antisense RNA probes were carried out as previously described (Isaacs et al., 1992). Slides were exposed for 10 to 14 days.

Results

Sequence

A murine *int-2* (FGF-3) probe encompassing exon II (Smith et al., 1988) was used to screen, at low stringency, a *Xenopus* stage 17 neurula cDNA library. This probe was chosen empirically since it gave the best cross-species signals in genomic blots (data not shown). Positive phages were isolated and subjected to DNA sequence analysis. Fig. 1 illustrates the DNA sequence of one of the clones along with its derived amino acid sequence and a comparison of the *Xenopus* amino acid sequence with both the mouse and human *int-2* sequences is shown below. From this it can be calculated that the percentage identity of the *Xenopus* amino acid sequence to both the mammalian sequences is ~81% which rises to ~90% if chemically similar amino acids are regarded as equivalent. The match to other known *Xenopus* FGFs is ~40% for eFGF (Isaacs et al., 1992) and ~34% for bFGF (Kimelman et al., 1988), making it unlikely that these mRNAs will cross-hybridize in the expression study described here. The closest match to other known mammalian FGFs is ~45% for keratinocyte growth factor (FGF-7). Although we acknowledge the possibility that another *int-2* homologue may exist due to the pseudotetraploidy of the *Xenopus laevis* genome, this cDNA probe detects both “sets”

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          TGCGACCCCAGGCAGAGACGAGATGCTGGGGGACGTGGAGGGGTTTACGAACATCTTGGA
Xenopus   C  D  P  R  Q  R  R  D  A  G  G  R  G  G  V  Y  E  H  L  G
  Human   P  G  A  *  L  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
  Mouse   P  G  T  *  L  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

          GGCGCCCCTCGAAACAGAAAACTTTACTGCGCTACTAAGTACCACCTACAGATACACCTC
Xenopus   G  A  P  R  N  R  K  L  Y  C  A  T  K  Y  H  L  Q  I  H  L
  Human   *  *  *  *  R  *  *  *  *  *  *  *  *  *  *  *  L  *  P
  Mouse   *  *  *  *  R  *  *  *  *  *  *  *  *  *  *  *  L  *  P

          AACGGCAAGATTAACGGGACTCTGGAGAAAAACAGCGTATTTAGTATTTTAGAAATAACC
Xenopus   N  G  K  I  N  G  T  L  E  K  N  S  V  F  S  I  L  E  I  T
  Human   S  *  R  V  *  *  S  *  *  -  *  *  A  Y  *  *  *  *  *  *
  Mouse   S  *  R  V  *  *  S  *  *  -  *  *  A  Y  *  *  *  *  *  *

          GCCGTGGATGTGGGAATTGTTGCCATCAAAGGGCTGTTTTCTGGGAGATATCTGGCCATG
Xenopus   A  V  D  V  G  I  V  A  I  K  G  L  F  S  G  R  Y  L  A  M
  Human   *  *  E  *  *  *  *  *  *  R  *  *  *  *  *  *  *  *  *  *
  Mouse   *  *  E  *  *  V  *  *  *  *  *  *  *  *  *  *  *  *  *  *

          AACCAAAGAGGGAGACTTTACGCCTCGGAAACATACAACCCTGAATGTGAGTTCGTG
Xenopus   N  Q  R  G  R  L  Y  A  S  E  T  Y  N  P  E  C  E  F  V
  Human   *  K  *  *  *  *  *  *  *  H  *  S  A  *  *  *  *  *
  Mouse   *  K  *  *  *  *  *  *  D  H  *  *  A  *  *  *  *  *
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Fig. 1. DNA sequence of the *Xenopus int-2* clone spanning putative exon II. The derived amino acid sequence is indicated below together with alignments to both the human and mouse *int-2* amino acid sequences. Stars refer to sequence conservation with the *Xenopus* sequence. If the genomic structure of *int-2* is conserved between mammals and amphibians then the underlined nucleotides represent exon II.

of genomic fragments in Southern blots (Close, M. J., Kiefer, P. Halley, C., Dickson, C. Peters, G., unpublished data).

Temporal expression

To begin to address the developmental role of *int-2* during *Xenopus* embryogenesis, it is essential to know both its temporal and spatial expression. In this study, we have used RNA blotting, RNAase protection analysis and *in situ* hybridization to provide a detailed expression pattern. The probe used in all experiments described below corresponds to the entire sequence indicated in Fig. 1. This probe crosses the presumed boundaries between exons I, II and III, and, by analogy with the mouse gene structure and organization (Mansour and Martin, 1988; Smith et al., 1988), it is likely to be common to all *int-2* transcripts. Fig. 2A shows the result of a RNA blot, bearing poly $(A)^+$ RNA from successive developmental stages, hybridized with the *Xenopus* probe. It can be seen that *int-2* transcripts are not maternal, the first transcripts appear from the late blastula stages onwards. Initially, a single ~1.6kb *int-2* mRNA is present at a low level, however, by the mid-gastrula stage at least three more abundant *int-2* transcripts of ~1.6, 1.9 and 2.2 kb have accumulated. The largest of these disappears in neurula and tailbud embryos.

The finding that *int-2* RNA is not maternal has been confirmed using a sensitive RNAase protection assay. In all such experiments, we have adopted an ornithine decarboxylase (ODC) probe for an internal reference standard. ODC appears to be expressed at quantitatively equivalent levels both in different embryo stages and between different embryo parts (Isaacs et al., 1992). Fig. 2B shows the result of such an experiment and it can be seen that *int-2* just begins to accumulate by the late blastula stage (this gel was deliberately overexposed to highlight this) and reaches maximal levels by the gastrula stage. High levels of *int-2* expression are then maintained through all stages of development analyzed. A crude estimate of *int-2* RNA abundance within the embryo, based on relative exposure time to ODC, suggests that it falls in the rare to moderately abundant class of RNAs. There is not enough *int-2* RNA to be detected on RNA blots that have been prepared using total RNA.

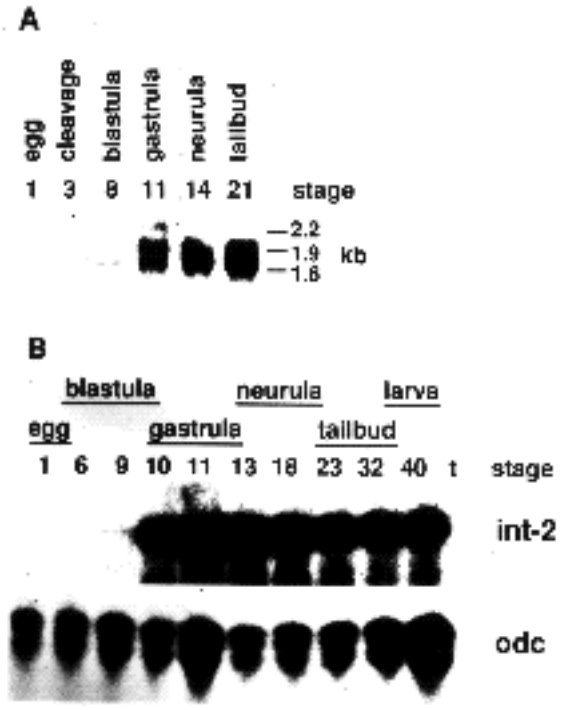

Fig. 2. Expression of *int-2* RNA during development. (A) Developmental stage series RNA blot hybridized with a *int-2* probe. 3 μg of poly $(A)^+$ RNA was loaded per lane. Approximate sizes were estimated from an RNA standards (BRL) used as a marker track and from the 18S and 28S rRNA sizes. Indicated sizes are in kilobases. Exposure time was ~10 days. (B) RNAase protection analysis using 20 μg of total RNA from different developmental stages. Exposure time for *int-2* was ~14 days and for the internal ODC control it was ~16 hours. Stage numbers correspond to that of the normal table (Nieuwkoop and Faber, 1967). No bands are detected in the tRNA negative control lane (t) with either *int-2* or ODC probes.

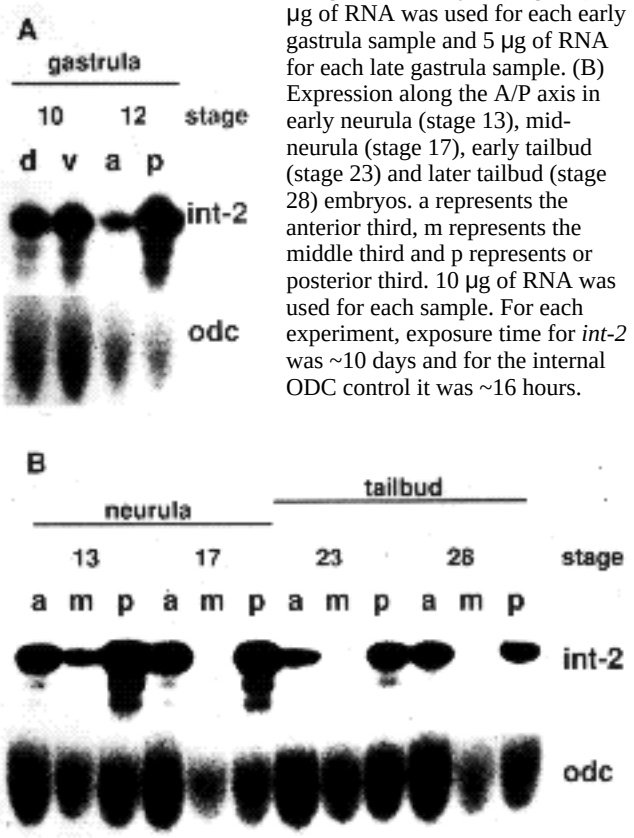

Fig. 3. *int-2* expression in dissected embryo pieces analyzed by RNAase protection analysis. (A) Expression in the dorsal quarters (d) and ventral three quarters (v) of early gastrula (stage 10) and expression in the anterior two thirds (a) and posterior third (p) of late gastrula embryos (stage 12). 10 μg of RNA was used for each early gastrula sample and 5 μg of RNA for each late gastrula sample. (B) Expression along the A/P axis in early neurula (stage 13), mid-neurula (stage 17), early tailbud (stage 23) and later tailbud (stage 28) embryos. a represents the anterior third, m represents the middle third and p represents or posterior third. 10 μg of RNA was used for each sample. For each experiment, exposure time for *int-2* was ~10 days and for the internal ODC control it was ~16 hours.

Spatial localization

We have used both RNAase protection analysis and *in situ* hybridization to different embryo stages to establish the spatial expression pattern of *int-2*. These two techniques are complementary: RNAase protection analysis on dissected embryo pieces affords a very sensitive but crude localization assay, whereas *in situ* hybridization gives a precise description of localization but is inherently less sensitive.

RNAase protection on embryo pieces

int-2 RNA begins to accumulate from the late blastula stage and approaches maximal levels during gastrulation. Fig. 3A shows the result of an RNAase protection analysis on gastrula stage embryos. Stage 10 gastrula embryos (dorsal lip clearly visible) were dissected into two pieces: a ventral piece encompassing three quarters of the circumference and a dorsal piece encompassing the remaining quarter. It can be seen that there are significant amounts of *int-2* RNA present in both pieces, therefore the activation of *int-2* during development does not appear to be restricted to either the dorsal or ventral side of the embryo. Two pieces were taken from late gastrula embryos for analysis by dissection into anterior two thirds and posterior third pieces. The anterior piece contains some involuting mesoderm that has not yet reached its final position and the posterior piece contains the blastopore that is yet to close fully. Fig. 3A shows that there is a significantly greater amount of *int-2* RNA in the posterior than the anterior piece; however, it is notable that the anterior piece contains some RNA.

For the analysis on neurula to tailbud stages, embryos were dissected into thirds along the A/P axis. In neurula stages, the anterior (a) third contains most of the presumptive forebrain and midbrain, the middle third (m) contains most of the hindbrain while the posterior third (p) gives rise to the rest of the body axis from spinal cord to tailbud (Eagleson and Harris, 1990). This over-representation of brain structures in the length of the A/P axis arises because at these early stages the posterior has not finished expanding to its fullest extent. On the other hand, for dissections at the tailbud stages, the anterior piece (a) contains head structures to about the level of the hindbrain/spinal cord junction and the posterior piece (p) contains the tailbud. The trunk piece (m) is the rest of the embryo and contains the bulk of the somites (the other two pieces will contain a little somitic material). *int-2* expression in each of these three pieces at the different stages is shown in Fig. 3B. It can be seen that in early neurula embryos most of the *int-2* RNA is localized to both the anterior and posterior third of the axis. From the mid-neurula to tailbud stages, it is clear that *int-2* expression is localized predominantly to both the anterior and posterior thirds with very little being present in the middle. These results show that there are two distinct domains of *int-2* expression, one is in the prospective brain area and the other in the prospective tailbud. This pattern of expression is established in the early neurula and maintained through to the tailbud stages.

In situ hybridization

We are able to detect *int-2* expression from the mid-gastrula stage, when it is expressed around the entire circumference of the blastopore in both the ectodermal and mesodermal cell layers (Fig. 4A). Even though gastrula and neurula stages contain comparable amounts of *int-2* RNA, as assessed by RNAase protection, there seems to be a reduced sensitivity in gastrula stage in situs. Perhaps, the large size of the cells together with the large amount of yolk result in the target RNA being more diffuse or less accessible to the probe.

By the early neurula stage, two distinct domains of *int-2* expression have become established. Fig. 4B shows a low-power view, in a whole, slightly parasagittal, section from a stage 13 embryo (as here, the yolky endodermal mass often falls out of sections but this is not a major site of *int-2* expression). One domain of expression is in the posterior, just above the closing blastopore, where *int-2* RNA is localized to both the mesodermal and ectodermal component. It can be seen that the ectodermal expression extends further along the A/P axis than the mesodermal expression. The other domain is in the anterior, just behind the thickened region of the neuroectoderm. These two highly expressing regions are maintained through neurulation. Fig. 4C shows a higher power view of the neuroectodermal expression in a mid-neurula (stage 15) embryo and Fig. 4D shows the posterior expression at the equivalent stage. A transverse section through the anterior of a later neurula is shown in Fig. 4E. It can be seen that *int-2* expression is localized specifically to the neural rather than the epidermal component of the ectodermal germ layer.

We have attempted to find out where these two domains lie

in terms of the normal fate map of the neurula (Eagleson and Harris, 1990). It is not possible to be very precise because the published map shows only a single stage viewed from a single orientation but we conclude that the *int-2* expression in the posterior will encompass much of the prospective trunk and tail and that the anterior domain will span a number of prospective regions in the midbrain and hindbrain.

In the late neurula, the anterior domain of *int-2* expression appears to become more narrow than in the early stages (compare Fig. 4C and F) and also it has become restricted to the more ventral part of the neural tube. In the very early tailbud stage (stage 25), the anterior expression is much reduced whereas the posterior is still strongly expressing (Fig. 4G); however, the anterior domain is now joined by expression in the developing optic cups (as shown in the frontal section in Fig. 4H).

By the tailbud and prelarval stages of development (stage 34 onwards), expression of *int-2* has become quite complex. These multiple sites of *int-2* expression are shown in Fig. 5A-E). In the brain, there is a site of expression at the junction of the hindbrain and midbrain that appears to be restricted to the ventral half of this area. The view in Fig. 5A shows this localization in a frontal section and confirms that it is not next to the ear (the *int-2* positive ganglion just anterior to the ear is becoming visible in the bottom half of the picture). Fig. 5A also shows that the anterior part of the forebrain expresses increased levels of *int-2*. Another area of the brain that also has increased levels of *int-2* RNA is the region forming the tip of the depression of the diencephalon known as the infundibulum (Fig. 5B). This appears to be restricted more to the posterior and sides of the brain wall and would thus correspond to the developing hypothalamus. Fig. 5B also shows a patch of expression in an area of head mesenchyme lying just above the cement gland. The patch of expression at the midbrain-hindbrain junction can barely be seen in this almost medial section because it is very much less intense in the dorsal midline than at more lateral levels.

A very intense signal of *int-2* expression appears in the two cranial ganglia adjacent to the otocyst (Fig. 5C). The anterior positive signal probably comes from the acoustic ganglion although in *Xenopus* this is intertwined with the facial ganglion to form the acousticofacial complex, and the posterior signal comes from the glossopharyngeal ganglion. From these and results from later stages, it can also be seen that the thickened (sensorial) region of the otocyst expresses *int-2*. The endodermal lining of the pharyngeal pouches also expresses high levels of *int-2* RNA and this is clearly seen in more lateral sections (Fig. 5D).

In the posterior of the tailbud and prelarval stages, two sites of expression are found (Fig. 5E). There is a stripe that is localized to certain mesenchymal cells of the fin. The other, very intense, site is in the tailbud itself. It can be seen that both the overlying ectodermal component and the underlying tailbud blastema are strongly positive.

In summary, these results have confirmed and extended those gained using RNAase protection analysis. The seemingly simple pattern of *int-2* expression in the early embryo becomes a very intricate pattern of expression in later embryos.

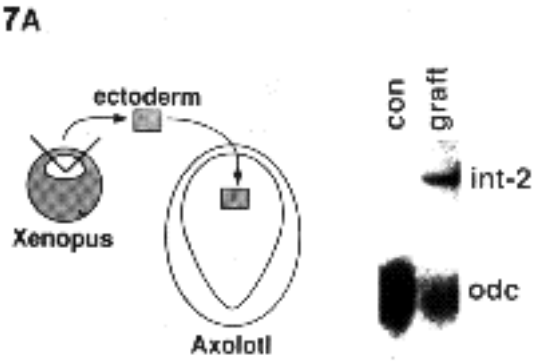

Fig. 7. *int-2* expression is induced by neural induction. Stage 10 *Xenopus* gastrula ectoderm was transplanted into the neural plate of an axolotl at the early neurula stage and cultured before harvesting for RNAase protection analysis or *in situ* hybridization. (A) RNAase protection analysis on grafted embryos or on controls (con). Controls were the equivalent numbers of ectoderms and axolotl embryos cultured separately and then combined for RNA extraction. 16 μg of RNA was used per sample. Exposure time for *int-2* and for the internal ODC control was ~14 days. (B) (Colour plate) In situ hybridization analysis on axolotl embryos grafted with *Xenopus* gastrula ectoderm and cultured to the mid-neurula stage (i.e. to the age of control *Xenopus* embryos). Dark-field view. (C) Same section viewed through bright field optics. The *Xenopus* tissue is easily discernible due to the smaller size of the cells. White dots represent silver grains whereas the orange-pink dots represents endogenous pigment granules. No signal was detected with negative control probes (data not shown). Scale bars correspond to 100 μm.

int-2 expression can be provoked by mesodermal and neural induction

How is the early bipolar pattern of *int-2* expression established? Intuitively, it would seem that the posterior site of expression in the mesoderm should arise through mesodermal induction and that anterior site of expression in the neuroectoderm should arise through neural induction. The next series of experiments were performed to test these proposals directly.

Fig. 6A and B shows the result of an assay for *int-2* expression in mesodermally induced animal cap explants. Treatment of animal caps with either the dorsal type mesoderm-inducing factor, activin, or the ventral type mesoderm-inducing factor, bFGF, results in similarly elevated levels of *int-2* RNA relative to control untreated caps. (In Fig. 6A the apparent elevation of signal in the activin track compared with the FGF track is due to more RNA having been loaded.) The *int-2* response to mesoderm induction can be detected as early as the mid-gastrula embryo, a time when the mesoderm has yet to migrate to its final position (Fig. 6A), and perhaps corresponds with the expression in the dorsal and ventral halves of the embryo (Figs 3A and 4A). *int-2* levels remain elevated, again to a roughly equivalent extent, in the explants grown to the late neurula stage. This later expression might correspond with that in the developing tailbud shown in Fig. 5E. Due to convergence towards the dorsal midline, the tailbud is formed from both the dorsal and ventral regions of the embryo both of which were expressing *int-2* in the gastrula.

One way to test whether neural induction is necessary for

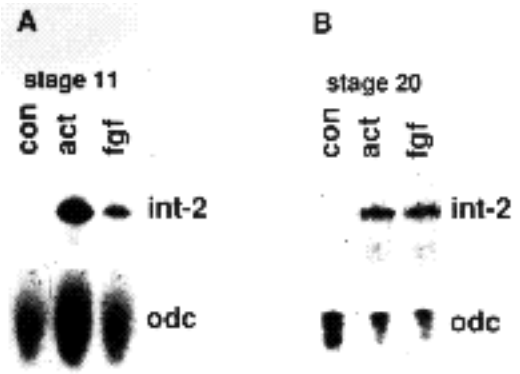

Fig. 6. *int-2* expression is induced by mesoderm induction. Stage 8 blastula ectoderm was treated with either 10 units/ml of activin (act) or bFGF (fgf) or incubated alone (con) then cultured before harvesting for RNAase protection analysis. (A) Analysis at the mid-gastrula stage (stage 11) ~5 μg of RNA was used per sample. Exposure time for *int-2* was ~10 days and for the internal ODC control it was ~16 hours. (B) Analysis at the very early tailbud stage (stage 20). 4 μg of RNA was used per sample. Exposure time for *int-2* was ~10 days and for the internal ODC control it was ~3 days

the expression of a particular gene is to dissect inducing dorsal mesoderm from gastrula embryos and recombine it with gastrula ectoderm (eg Dixon and Kintner, 1989; Sharpe and Gurdon, 1990). After a period of culture, recombinants are assayed by RNAase protection for induced expression of the gene relative to control ectoderm grown in isolation. In the case of *int-2*, this approach is complicated by the fact that *int-2* itself is expressed in the posterior of the inducing mesoderm (Fig. 4A) and therefore makes problematic the clean dissection of mesoderm that does not express *int-2*. Also, this method has a disadvantage that it only assays the appositional component of the neural inducing signal and not the tangential inducing component that has been the subject of much recent attention (Dixon and Kintner, 1989; Sharpe and Gurdon, 1990). For these reasons, we have adopted an alternative neural inducing assay that involves the transplantation of *Xenopus* gastrula ectoderm into the anterior neural plate of an early neurula stage axolotl embryo. This approach is possible because the *Xenopus int-2* probe does not cross react with axolotl material and also has the advantage that both the appositional and tangential neural inducing signals can be assayed simultaneously. A small square of neural ectoderm was removed approximately four fifths of the way along the A/P axis from the posterior end of axolotl early neurula (equivalent to *Xenopus* stage 13) and stage 10 *Xenopus* gastrula ectoderm was transplanted into this site. This site was selected on the basis of the normal expression pattern in *Xenopus* on the assumption that axolotl *int-2* would be induced at a similar position along the A/P axis. The heterotypic grafts were then allowed to grow until the donor *Xenopus* ectoderm had reached the mid-neurula stage (stage 15) to allow induction to take place. Samples were then harvested for RNAase protection analysis or *in situ* hybridization.

Results from this type of neural inducing assay are shown in Fig. 7. By RNAase protection, it is found that competent *Xenopus* ectoderm grafted into axolotl neural plate does indeed express significant levels of *int-2* relative to ectoderm cultured in isolation (Fig. 7A). This is confirmed by the in situ hybridization analysis shown in Fig. 7B and C. There is a very strong site of *int-2* expression in the grafted embryos (Fig. 7B) that specifically co-localizes to the grafted *Xenopus* but not host axolotl ectodermal cells within the neural plate (Fig. 7C). The *Xenopus* cells can easily be identified by the small size of their nuclei. No significant hybridization of the *Xenopus int-2* probe to control axolotl neural plate is observed (Fig. 7B and data not shown). This experiment provides strong support for the proposal that neural induction is responsible for the activation of *int-2* expression in the anterior neural ectoderm.

Discussion

Possible functions

The absence of maternal expression makes it unlikely that *int-2* is responsible for mesoderm induction *in vivo* even though *int-2* protein shows this activity when applied to animal caps (Paterno et al., 1989). This is because the timing of mesoderm induction requires that the molecules responsible should be synthesized off maternal rather than zygotic messages (Jones and Woodland, 1987; Smith et al., 1989; New and Smith, 1990). The mesoderm-inducing activity of this FGF-related protein probably results from the activation of the maternally coded and uniformly distributed FGF receptors (Gillespie et al., 1989; Musci et al., 1990; Friesel and Dawid, 1991).

int-2 can be turned on by mesoderm induction and in fact the earliest zygotic expression is found in the blastopore region. Since *int-2* is not expressed in the mesoderm after it has involuted the expression must be transient, being maintained only while cells remain near the blastopore, in the most posterior part of the forming axis. In this respect, it resembles eFGF, a newly identified molecule similar to both mammalian kFGF and FGF-6 (Isaacs et al., 1992). We have recently proposed that specification of anteroposterior body levels occurs during gastrulation and depends on a dominant signal arising from the posterior end (Slack and Tannahill, 1992). So either or both *int-2* and eFGF are candidates for posteriorizing factors during axis formation. The posterior zone of expression persists for many hours, eventually becoming concentrated into the undifferentiated blastema known as the tailbud. We might speculate that the factors are needed in the tailbud to maintain the cells in an undifferentiated condition.

The expression of *Xenopus int-2* in the neuroectoderm can be detected well before the neural tube begins to elevate right through to the end of neurulation. We have shown that neural induction leads to activation of *int-2* in a situation in which both appositional and tangential signals are present. It will be of interest to ascertain if both signals are in fact required, or whether one will do. Initially, the neural patch is rather broad, spanning the prospective midbrain and hindbrain, before eventually narrowing and breaking up into at least two subdomains including the midbrain-hindbrain junction and the hypothalamus. We can often see expression adjacent to the epidermal thickening of the otic placode at the very early tailbud stages (stage 23-25). This is somewhat variable since

it is about the time that the large neural domain is breaking up but it is consistent with the widely held idea that *int-2* is responsible in some capacity for proper morphogenesis of the otocyst (Wilkinson et al., 1988). It has been shown that otic vesicle closure is inhibited through the application of antisense oligonucleotides or antibodies against *int-2* (Represa et al., 1991). These suggestive experiments must be regarded as provisional since the reagents used were specific for mammalian *int-2* whereas the embryological material was avian, a system in which *int-2* has not been characterized.

The later expression domains all seem to be associated with interactions between cell sheets, particularly foldings and fusions of epithelia. In the brain, there is expression in the optic cups, which become apposed to the epidermis during lens induction; in the base of the infundibulum, which cooperates with the pharyngeal roof endoderm in the formation of the pituitary; and at the midbrain-hindbrain border, where there is a characteristic kink in the neuroepithelium. Elsewhere in the head there is expression in two small patches of mesenchyme near the stomodeum, where epidermal and endodermal epithelia fuse together to form the mouth; and in the pharyngeal pouches, where endodermal and epidermal epithelia fuse to form gill slits.

Comparison with the mouse

The temporal expression of *Xenopus int-2* bears some similarity with that in the mouse. In both organisms, *int-2* is transcribed with multiple transcripts from the onset of gastrulation (Wilkinson et al., 1988). There are also resemblances in the spatial expression pattern. During gastrulation, mouse *int-2* is found in mesodermal cells that are undergoing migration (Wilkinson et al., 1988; Niswander and Martin, 1992) and is probably homologous to the blastopore-associated region in *Xenopus*. Early in the mouse, at approximately 8.5 days postfertilization (E8.5), *int-2* is expressed in a broad domain spanning the hindbrain and part of the midbrain. One day later, this becomes restricted adjacent to the otocyst and is down regulated by the time the otic vesicle closes (Wilkinson et al., 1988 Wilkinson, 1990; Ian McKay personal communication). This situation is similar to that in neurula to tailbud stage *Xenopus* embryos.

Later, there are other common sites of *int-2* expression between mouse and *Xenopus* that include the endoderm of the pharyngeal pouches and the otic vesicle. The conservation of so many expression domains, especially over the early period of body plan formation, strongly suggests that *int-2* plays an important role in the development of vertebrates in general. There are, however, also species-specific differences. In particular, over the developmental period that we have studied, the infundibulum, the optic cups, the midbrain-hindbrain junction and the cranial ganglia VIII and IX are all positive in *Xenopus* but not in the mouse.

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Side one of tip-in, facing p. 4

Fig. 4. In situ hybridization analysis of *int-2* up to the tailbud stage. In all plates white dots represent silver grains whereas orange-pink dots represents endogenous pigment granules. No signal was detected with negative control probes (data not shown). Scale bars correspond to 100 μm. (A) Stage 11, mid-gastrula. View of dorsal and ventral lips, dorsal is to the top. Arrows indicate the blastopore lips where *int-2* expression is just visible above background. (B) Early neurula (stage 13) in parasagittal section. Whole view. (C) Mid-neurula (stage 15) in parasagittal section. Anterior detail. (D) Mid-neurula (stage 15) in parasagittal section. Posterior detail. (E) Mid-neurula (stage 17) in transverse section. Anterior detail. (F) Late neurula (stage 20) in parasagittal section. Anterior detail. (G) Very early tailbud (stage 25) in frontal section. Posterior detail. (H) Tailbud (stage 25) in parasagittal section. Anterior detail.

Side two of tip-in, facing p. 5

Fig. 5. In situ hybridization analysis of *int-2* in tailbud and prelarval stages. In all plates white dots represent silver grains whereas orange-pink dots represents endogenous pigment granules. No signal was detected with negative control probes (data not shown). Scale bars correspond to 100 μm. (A) Stage 34 in frontal section. The midbrain/hindbrain junction and forebrain are positive. (B) Stage 34 in (slightly) parasagittal section. The infundibulum (top arrow) and a patch of head mesenchyme (lower arrow) are positive. (C) Stage 40 in parasagittal section. The cranial ganglia and ear are positive. (D) Stage 34 in parasagittal section. The pharyngeal pouches are positive. In this section the whiteness at the anterior end is a mounting artifact. (E) Stage 34 in parasagittal section. The tailbud and a stripe of fin mesenchyme are positive.