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Expression of *Pax-3* is initiated in the early neural plate by posteriorizing signals produced by the organizer and by posterior non-axial mesoderm

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SUMMARY

Pax-3 **is a paired-type homeobox gene that is specifically expressed in the dorsal and posterior neural tube. We have investigated inductive interactions that initiate *Pax-3* transcript expression in the early neural plate. We present several lines of evidence that support a model where *Pax-3* expression is initiated by signals that posteriorize the neuraxis, and then secondarily restricted dorsally in response to dorsal-ventral patterning signals. First, in chick and *Xenopus* gastrulae the onset of *Pax-3* expression occurs in regions fated to become posterior CNS. Second, Hensen's node and posterior non-axial mesoderm which underlies the neural plate induce *Pax-3* expression when combined with presumptive anterior neural plate explants. In contrast, presumptive anterior neural plate explants are not competent to express *Pax-3* in response to dorsalizing signals from epidermal-ectoderm. Third, in a heterospecies explant recombinant assay with *Xenopus* animal caps (ectoderm) as a responding tissue, late, but not early, Hensen's node induces *Pax-3* expression. Chick posterior non-axial mesoderm also induces *Pax-3*, provided that the animal caps are neuralized by treatment with *noggin*. Finally we show that the putative posteriorizing factors, retinoic acid and bFGF, induce *Pax-3* in neuralized animal caps. However, blocking experiments with a dominant-inhibitory FGF receptor and a dominant-inhibitory retinoic acid receptor suggest that *Pax-3* inductive activities arising from Hensen's node and posterior non-axial mesoderm do not strictly depend on FGF or retinoic acid.**

Key words: *Pax-3*, neural plate, A-P patterning, chick, *Xenopus*

INTRODUCTION

Position along the anterior-posterior (A-P) and dorsal-ventral (D-V) axes of the developing vertebrate central nervous system (CNS) is a major determinant of neuronal cell-type. Interactions between the ectoderm and organizer tissue are important in axis determination in the vertebrate CNS (reviewed by Doniach, 1993; Ruiz i Altaba and Jessell, 1993). The organizer produces signals that induce dorsal ectoderm to assume a neural rather than an epidermal fate as well as signals that pattern the CNS along the A-P axis. Derivatives of the organizer, the notochord and prechordal plate, generate signals that mediate D-V patterning. Current models suggest that these patterning signals induce expression of regulatory genes, such as transcription factors, that then endow the CNS with region-specific properties, restricting developmental potential and directing responses to further inductive signaling.

The Pax (paired-type homeobox) genes encode transcription factors whose spatiotemporal expression suggests that they play an important role in pattern formation in the vertebrate CNS (reviewed by Chalepakis et al., 1993). In particular, the expression of *Pax-3*, within a dorsal domain of the hindbrain and spinal cord, is among the earliest known events in D-V patterning of the neural tube (Goulding et al., 1991, 1993; Espeseth et al., 1995). This early expression of *Pax-3* presumably reflects the division of the hindbrain and spinal cord into longitudinal columns along the D-V axis, where floor plate and motor neurons form ventrally, while neural crest and an early population of commissural neurons develop dorsally. A role for *Pax-3* in regional specification of cell-fate along the D-V axis is supported by the observed loss of neural-crest cell derivatives in *Splotch* mice, which carry loss-of-function mutations in the *Pax-3* gene (Epstein et al., 1991; Franz, 1990 and references therein). Moreover, ectopic expression of *Pax-3* in the ventral neural tube of transgenic mice causes a reduction in the number of motor neurons and a suppression of floor plate development (Tremblay et al., 1996). Together these results suggest that *Pax-3* is required to promote development of dorsal cell-types, while its down-regulation ventrally may be necessary for differentiation of floor plate and motor neurons.

The dorsal restriction of *Pax-3* expression in the hindbrain and spinal cord appears to depend directly on signals that pattern the D-V axis of the CNS. *Pax-3* expression is repressed in the ventral neural tube by *sonic hedgehog* (*shh*)-mediated signals from the notochord and floor plate; loss of these signals leads to a ventral shift in the *Pax-3* expression domain and loss of ventral cell-types (Chiang et al. 1996; Liem et al., 1995; Goulding, et al., 1993; see also Espeseth et al., 1995). Dorsalizing signals, produced by epidermal-ectoderm and mimicked

by Bone Morphogenetic Proteins (BMP) 4 and 7, induce expression of neural-crest markers and 'super-induce' expression of *Pax-3* in explants of chick caudal neural plate that already express *Pax-3* (Dickinson et al., 1995; Liem et al., 1995; Selleck and Bronner-Fraser, 1995). Thus, the final pattern of *Pax-3* expression appears to arise in part via repression in ventral neural tube by ventralizing signals like shh, and up-regulation dorsally by a BMP-like signal.

While the *Pax-3* expression pattern is determined in part by D-V patterning, the earliest detectable *Pax-3* transcripts are already restricted to the presumptive posterior neural plate in chicken and *Xenopus* mid-gastrulae, suggesting that this initial expression reflects early A-P patterning. We therefore examined the events that initiate *Pax-3* expression in caudal neural tube. Based on our findings, we propose that *Pax-3* expression is initiated by posteriorizing signals that arise from Hensen's node and from posterior non-axial mesoderm which underlies the neural plate, and that the dorsal restriction of *Pax-3* occurs secondarily in response to D-V patterning signals.

MATERIALS AND METHODS

Embryos

Embryos were obtained from *Xenopus laevis* adult frogs by hormone induced egg-laying and in vitro fertilization using standard methods. *Xenopus* embryos were staged according to Nieuwkoop and Faber (1967). White leghorn hens' eggs and quail eggs were incubated at 38ºC in a humidified, forced-draft incubator. Avian embryos were staged according to Hamburger and Hamilton (1951).

In situ hybridization

Whole-mount in situ hybridization of *Xenopus* embryos was performed according to the method of Harland (1991) with modifications described by Knecht et al. (1995) using digoxigenin-labeled anti-sense RNA probes for *Xenopus Pax-3* (Espeseth et al., 1995) and *engrailed-2* (*en-2*; Hemmati-Brivenlou et al., 1991).

Whole-mount in situ hybridization of chicken embryos was performed according to the method of Wilkinson (1992) using digoxigenin-labeled anti-sense RNA probes for chicken *Pax-3* (Goulding et al., 1993) and chicken *Otx-2* (Bally-Cuif et al.,1995). A chicken *c-qin* digoxigenin-labeled probe was produced from a subclone of the entire *c-qin* coding region (Chang et al., 1995). Stained embryos were either mounted whole in glycerol, or embedded in a mixture of 3.5% agar and 8% sucrose and Vibratome sectioned to 70 μm.

In situ hybridization on 10 μm cryostat sections of collagen-embedded tissue explants was performed as described by Goulding et al. (1993), using ^{35}S-labelled probes for chicken *Pax-3* (Goulding et al., 1993) and chicken *slug* (Nieto et al., 1994).

Isolation, treatment and culturing of *Xenopus* animal caps

Xenopus embryos at the two-cell stage were injected in the animal region of each blastomere with capped synthetic RNAs of *noggin* (0.5 ng; Lamb et al., 1993), *XFD* (0.5 ng; Amaya et al., 1991), dominant negative *ras* [0.5 ng; *ras* p21 (Asn-17)$^{\text{Ha-}ras}$ (Feig and Cooper, 1988), as described by Bhushan et al. (1994)], or dominant negative *xRARγ-1* (1 ng; dn *xRARγ-1* was a generous gift from Dr Bruce Blumberg, and was constructed as described for the dominant negative form of *xRARα-1* by Blumberg et al., 1997). Animal caps were dissected at stage 9. Some caps were treated with 2×10^{-6} M RA, diluted in 0.5× MMR, or 100ng/ml of bFGF (Boehringer Mannhein) or 5 ng/ml recombinant activin (provided by the Vale laboratory) in 0.5× MMR, 0.1% BSA, immediately after dissection. Some animal caps were combined with chick tissues as described below. Animal caps were cultured on agarose-coated Petri dishes in 0.5× MMR containing penicillin/streptomycin until sibling controls reached the appropriate stage as noted.

Isolation of avian tissue explants

Dissections were performed using sharpened tungsten needles. Hensen's nodes were dissected in cold L-15 medium (Gibco-BRL). To isolate posterior non-axial mesoderm (pnm) and head non-axial mesendoderm (hme), explants of approximately 100 μm × 200 μm were dissected at stage 6 from the area lateral to the primitive streak and immediately posterior to the level of Hensen's node, or from the area of the head fold (excluding the midline), respectively (Fig. 3A). Explants were placed briefly (<1 minute) in L-15 medium containing 1 mg/ml Dispase (Boehringer Mannheim), and then transferred to L-15 medium containing 10% heat-inactivated fetal calf serum (L-15/HIFCS), where in the case of pnm, the mesoderm layer was isolated from the endoderm and epiblast, while for hme, the mesoderm and endoderm layers could not be separated and were isolated from the epiblast as a bilayer. Stage 4 presumptive anterior neural plate explants were isolated as approximately 100 μm squares rostral to Hensen's node (Fig. 5A), treated briefly with 1 mg/ml Dispase and then transferred to L-15/HIFCS where contaminating mesendoderm was removed. To isolate epidermal-ectoderm, stage 8 embryos were treated with 1 mg/ml Dispase for 10-15 minutes and then transferred to L-15/HIFCS where mesoderm and endoderm were removed and approximately 200 μm square epidermal-ectoderm explants were cut from an area near the area pellucida and area opaca border (Fig. 7A).

Xenopus/chick explant recombinant cultures

Hensen's node, pnm, or hme explants were 'sandwiched' between two pieces of *Xenopus* animal cap tissue (Fig. 3A). Animal cap/chick tissue recombinants were cultured in 0.5× MMR at room temperature as described above. Before pnm and hme explants were combined with animal caps, they were allowed to recover from Dispase treatment in L-15/HIFCS for 30 minutes at 37°C, and then rinsed twice in L-15.

Culturing of avian explants in collagen

Quail Hensen's node, pnm, hme, or chick epidermal-ectoderm explants were combined with chick neural plate explants immediately after dissection. The tissues were allowed to adhere to one another for 30 minutes at room temperature in L-15/HIFCS. Recombinants were embedded in 10 μl collagen drops, and cultured in MEM medium (Gibco-BRL) plus 10% HIFCS for 20 hours at 37°C, 5% CO_2. Collagen gels were prepared as described by Lumsden and Davies (1983). Some samples included 100 ng/ml of purified recombinant human BMP-4 (Genetics Institute, Cambridge, MA). Identical results for chick/quail recombinant experiments were obtained in defined media conditions using N-2 supplements (Gibco-BRL) (data not shown).

RNase protection

RNA was isolated and analyzed by RNase protection assay (RPA), using ^{32}P-labeled antisense RNA probes, as previously described (Melton et al., 1984; Kintner and Melton, 1987). The probes used to detect *AC100*, *N-CAM*, *EF-1α*, *Otx-2* and *Xbra* RNAs have been described previously (Kintner and Melton, 1987; Ferreiro et al., 1994; Bhushan et al., 1994; Papalopulu and Kintner, 1996). The *Xenopus Pax-3* template was the same as that used for in situ hybridization. RNA samples isolated from 10 animal caps, or 5 animal cap/chick tissue recombinants were analyzed simultaneously with several probes. *EF-1α* expression was used as a loading control.

RT-PCR

Eight independent samples of avian explant recombinants in collagen

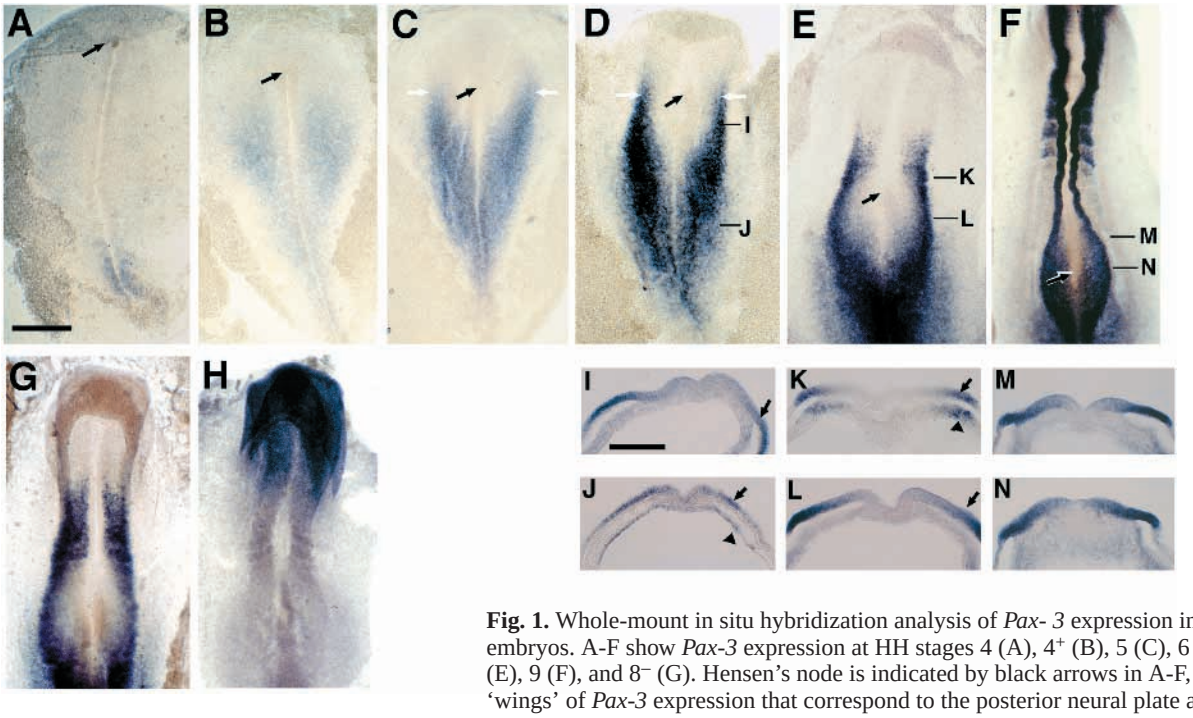

Fig. 1. Whole-mount in situ hybridization analysis of *Pax- 3* expression in chick embryos. A-F show *Pax-3* expression at HH stages 4 (A), 4^+ (B), 5 (C), 6 (D), 7 (E), 9 (F), and 8^- (G). Hensen's node is indicated by black arrows in A-F, and the 'wings' of *Pax-3* expression that correspond to the posterior neural plate are indicated by white arrows in C and D. Comparison of matched embryos at stage 8^-, hybridized with a probe for either *Pax-3* (G) or *Otx-2* (H), indicates that the rostral boundary of *Pax-3* expression approximates to the caudal boundary of *Otx-2* expression. I-N are 70 μm transverse Vibratome sections of *Pax-3*-hybridized embryos at stage 6 (I,J), stage 7 (K,L), and stage 9 (M,N) cut at levels indicated in whole-mount preparations shown in D, E and F, respectively. Sections in I and J show that at stage 6 *Pax-3* expression is primarily in the epiblast (arrows in I and J), with some expression posteriorly in the endoderm (arrowhead in panel J), and no expression in the mesoderm layer. By stage 7, *Pax-3* expression is detected rostral to Hensen's node in paraxial mesoderm of the presumptive occipital somites (arrowhead in K) and in the presumptive lateral neural plate (arrows in K-L). M-N show that *Pax-3* expression is detected uniformly along the M-L axis of the stage 9 open neural plate. *Pax-3* is broadly expressed outside the presumptive neural plate in the posterior epiblast and primitive streak; the functional significance of this expression is unknown. Scale bars, (A-H) 400 μm; (I-N) 200 μm.

gels were pooled together and total RNA was extracted as described by Chomczynski and Sacchi (1987) using the TRI REAGENT (Molecular Research Center, Inc.). RNA was treated with RQ1 DNase (Promega), and then phenol/chloroform extracted. Half of each RNA sample was reverse transcribed using Superscript-RT (Gibco-BRL) and 100 ng of random hexamers (Boehringer Mannheim) in a 20 μl reaction, while the other half was used in a control reaction minus reverse transcriptase. These reactions were subjected to PCR, where 16 μl was used to analyze either *Pax-3* or *Otx-2*, while 2 μl was used to analyze *β-actin*. Each PCR cycle was 94°C for 45 seconds, 55°C for 1 minute, and 72°C for 1 minute. All samples were assayed for 30 cycles. Oligos: Chicken *Otx-2* (Bally-Cuif, et al., 1995): upstream: GGCTCGACCTCCTATTTCGGAG; downstream: AGGAGGTTTG-GTCTTTATAATCC (amplifies a 274 bp fragment).

Chicken *Pax-3* (Goulding et al., 1993) : upstream: GAGAAGCA-GGCTTGTTCTGTA; downstream: GCTCAACTGCTAAATTTC-CAA (amplifies a 267 bp fragment of chicken, but not quail, *Pax-3* 3′ untranslated region).
Chicken *cytoplasmic β-actin* (Kost et al., 1983): upstream: CCAGC-CATGTATGTAGCCATCC; downstream: TCGGGGCACCTGAAC-CTCTCAT (amplifies a 388 bp fragment of both chicken and quail *cytoplasmic β-actin*).

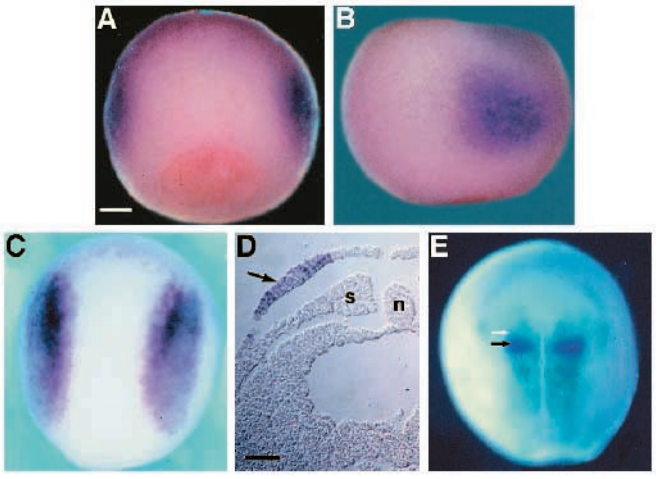

Fig. 2. Whole-mount in situ hybridization analysis of *Pax-3* expression in *Xenopus* embryos. (A) Dorsovegetal and, (B) lateral view at stage 11.5 showing that *Pax-3* is expressed in distinct lateral domains of the presumptive neural plate. (C) Dorsal view at stage 12 showing the refinement of *Pax-3* expression to lateral domains of the neural plate during convergence and extension. (D) Transverse paraffin section of a stage 16 embryo, showing that *Pax-3* expression is restricted to the lateral neural plate (arrow), overlying somitic and lateral plate mesoderm. (E) Dorsoanterior view of a stage 18 embryo hybridized with *Pax-3* (light blue, rostral extent indicated by a white arrow) and *en-2* (purple, indicated by a black arrow) showing that *Pax-3* expression extends just rostral to the mb-hb boundary. n, notochord; s, somite. Scale bars, (A-C, E) 200 μm; (D) 100 μm.

Whole embryo cultures

Embryos were grown in modified New culture (New, 1955). A small incision was made in the endoderm in the region of presumptive prosencephalic neural plate of a stage 4 chick (Fig. 5A), creating a pocket into which a graft of pnm was placed. Care was taken to maintain the orientation of the top and bottom faces of the pnm graft.

RESULTS

Pax-3 is expressed in the presumptive posterior-lateral neural plate of chicken and *Xenopus* gastrulae

Previous studies indicated that *Pax-3* expression within the hindbrain and spinal cord is regulated in part by signals that impose D-V polarity on the neural tube. However, it is not known how *Pax-3* expression is initiated in the neural plate. To begin to characterize the tissue interactions and signals that initiate *Pax-3* expression, we examined by whole-mount in situ hybridization the very early expression of *Pax-3* as it first occurs in the neural plate of chicken and *Xenopus* embryos.

In chicken embryos, *Pax-3* transcript expression was first detected at Hamburger-Hamilton (HH; Hamburger and Hamilton, 1951) stage 4 in the posterior primitive streak (Fig. 1A). By stage 4^+ the *Pax-3* expression domain has spread to include the posterior half of the epiblast and primitive streak (Fig. 1B), and by stage 5 two broad 'wings' of expression are just apparent in the epiblast, on either side of the anterior primitive streak (Fig. 1C). Based on fate mapping studies we interpreted these 'wings' of *Pax-3* expression as corresponding to the posterior neural plate (see Schoenwolf and Sheard, 1990 and references therein). Moreover, *Pax-3* expression appeared to be excluded from regions rostral to Hensen's node that are fated to become anterior neural plate. To determine more precisely the anterior extent of *Pax-3* expression along the A-P axis of the neural plate, we compared expression of *Pax-3* and *Otx-2* in stage matched chicken embryos (Fig. 1G,H and data not shown). Chicken *Otx-2* is expressed in the developing head and exhibits a posterior limit of expression at stage 6 that becomes sharply resolved at the midbrain-hindbrain (mb-hb) boundary by stage 11 (Bally-Cuif et al., 1995). At stage 8^- the anterior boundary of *Pax-3* is close to the posterior boundary of *Otx-2*, suggesting that at these early stages *Pax-3* expression is restricted caudal to the presumptive mb-hb boundary. By late stage 8 to early stage 9, *Pax-3* expression begins to extend further rostrally into the midbrain, but excludes the prosencephalon (data not shown, see Fig. 5B).

Interestingly, this analysis also revealed that *Pax-3* expression appears to be repressed at the midline of the neural plate with different kinetics at different positions along the neuraxis. From stages 5 to 7, when Hensen's node has regressed to the level of prospective hindbrain, the 'wings' of *Pax-3* expression sharpen, and become restricted to the lateral edges of the neural plate, leaving a zone surrounding Hensen's node where *Pax-3* transcript levels are low or undetectable (Fig. 1C-E). Sections of stage 7 embryos, taken immediately caudal to Hensen's node, revealed *Pax-3* expression in a lateral to medial gradient across the presumptive neural plate (Fig. 1E,L). In more rostral sections, *Pax-3* becomes progressively restricted to the lateral edges of the neural plate, as transcripts clear medially, mirroring the formation of the underlying notochord (Fig. 1K). In contrast, at stage 9, when Hensen's node has regressed to the level of the spinal cord, *Pax-3* expression was detected more uniformly along the M-L axis of the open neural plate and did not exhibit lateral restriction until a distance further rostral to Hensen's node, in agreement with a previous report (Fig. 1F,M,N; Liem et al., 1995).

In *Xenopus* gastrula and neural plate stage embryos the earliest expression of *Pax-3* was detected, as in the chick, in broad domains in the posterior and lateral neural plate that become progressively refined to the neural folds during convergence and extension (Fig. 2, see also Espeseth et al., 1995). At stage 11 transient, low-level expression of *Xenopus Pax-3* could be detected across the M-L axis of the presumptive neural plate with higher expression laterally (data not shown). However, the medial expression quickly clears and distinct posterior, lateral expression domains emerge by stage 11.5 (Fig. 2A,B). *Xenopus Pax-3* expression is also restricted in the A-P axis, extending into the midbrain but excluded from the forebrain when compared at neurula stages with expression of *en-2* (Hemmati-Brivanlou et al., 1991) which marks the mb-hb boundary (Fig. 2E).

Thus, in both chick and *Xenopus* embryos the onset of *Pax-3* expression occurs in broad posterior domains which then appear to be repressed at the midline and enhanced at the lateral edges of the neural plate. Taken together, these results suggest that an early signal associated with posteriorization of the neural plate initiates *Pax-3* expression, which is then refined further by D-V patterning signals.

Late but not early Hensen's node induces *Pax-3* expression in *Xenopus* animal caps

To determine whether the initial onset of *Pax-3* expression depends on early A-P patterning of the neural plate, we first asked whether *Pax-3* is induced differentially by early and late organizers, which are known to have different capabilites in terms of inducing A-P neural markers (Storey et al., 1992; Kintner and Dodd, 1991 and references therein). Using a heterospecies tissue recombination approach, we examined the ability of Hensen's nodes from stage 4 and stage 6 embryos to induce *Pax-3* expression in *Xenopus* blastula stage animal caps (ectoderm). This assay offers the distinct advantage that it is conducted at room temperature at which *Xenopus* develops, but growth and differentiation of the chick tissue is arrested. Thus, signals arising from the explanted chick tissues are likely to reflect properties of the stage at which they were isolated. Indeed, stage 4 Hensen's node induces more anterior neural markers while stage 5 node induces posterior markers when combined with *Xenopus* animal caps (Kintner and Dodd, 1991).

Hensen's node and *Xenopus* stage 9 (blastula) animal caps were placed together in 'sandwich' recombinants, incubated to stage 16 (early neurula), and analyzed by RNase protection assay (RPA) (Fig. 3A). Consistent with previous results, both stage 4 and 6 Hensen's nodes exhibited neuralizing activity in that they induced *N-CAM* expression in *Xenopus* ectoderm explants (Fig. 3B; Kintner and Dodd, 1991). We observed that stage 6 but not stage 4 Hensen's node induced *Pax-3* expression in ectoderm (Fig. 3B), implying that *Pax-3* expression is regulated by posteriorization signals produced by the late Hensen's node.

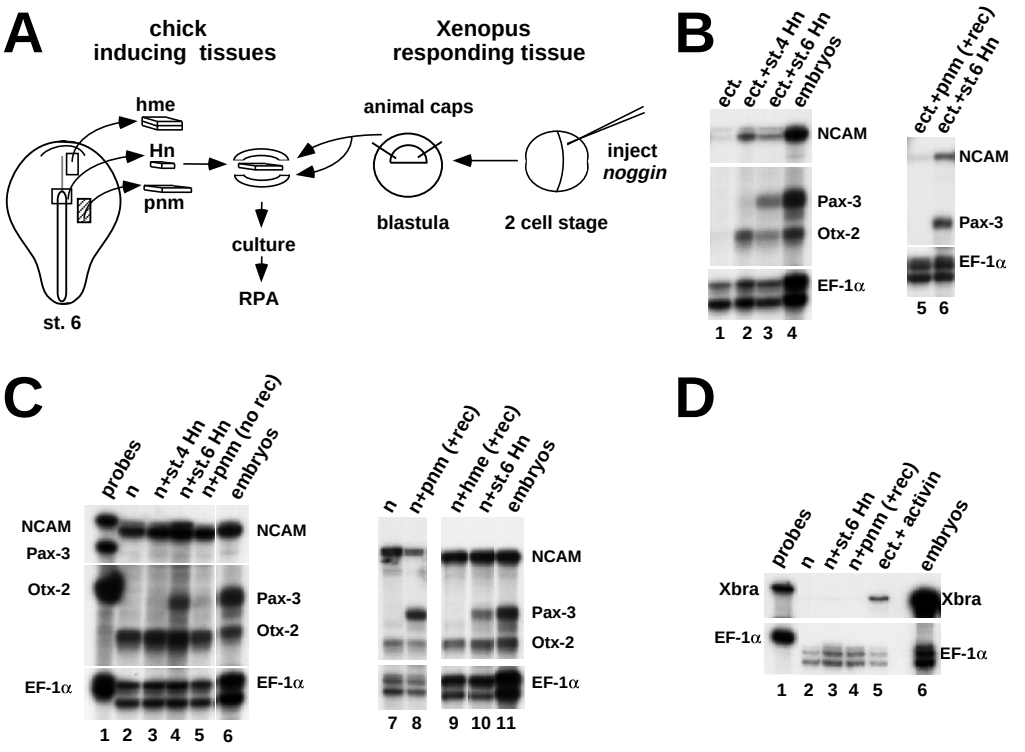

Fig. 3. Chick stage 6 pnm and Hensen's node (Hn) explants induce expression of *Xenopus Pax-3* in *noggin* animal caps. (A) Experimental design. (B) Ectoderm animal caps (lane 1) do not express *Pax-3*, *N-CAM*, or *Otx-2*. Both stage 4 Hn (lane 2) and stage 6 Hn (lane 3), induce expression of *N-CAM* and *Otx-2* in ectoderm, however, only stage 6 Hn (lane 3) induces *Pax-3* expression. We note that the observation that stage 6 Hensen's node induces expression of the anterior marker *Otx-2* may imply that node-derived posteriorizing signals do not completely transform the anterior fate induced by node-derived neuralizing signals. In contrast, compared with stage 6 Hn (lane 6), pnm (lane 5) does not induce *N-CAM* or *Pax-3*. (C) Although stage 16 *Xenopus* embryos express *Pax-3* (lane 6), neuralized *noggin* animal caps of an equivalent stage (lane 2) do not, however they do express the anterior marker, *Otx-2*, and the neural marker, *N-CAM*. Stage 6 Hensen's node (lane 4), but not stage 4 Hn (lane 3), induces *Pax-3* expression in *noggin* animal caps. Pnm combined without a post-dissection recovery period (lane 5) induces only low levels of *Pax-3* expression in *noggin* animal caps, but pnm with a recovery period (lane 8) strongly induces *Pax-3*. Compared with st. 6 Hn (lane 10), hme (with a post-dissection recovery period) does not induce *Pax-3* (lane 9). (D) Stage 6 Hn (lane 3) and pnm (lane 4) do not induce *Xbra* expression in *noggin* animal caps aged to the equivalent of stage 12, however *Xbra* is induced in control animal caps treated with 5 ng/ml of activin (lane 5); in explant recombinants from the same experiment allowed to age until stage 16, both stage 6 Hn and pnm induce *Pax-3* expression as shown in B (data not shown). ect., ectoderm; hme, head mesendoderm; Hn, Hensen's node; pnm, posterior non-axial mesoderm; n, *noggin*; st., stage; rec, recovery at 37°C.

Both Hensen's node and pnm induce *Pax-3* expression in *noggin*-injected animal caps

The results presented above suggest that *Pax-3* expression is initiated via posteriorizing signals, some of which may come from the organizer region. However, expression data from both chick and *Xenopus* show that *Pax-3* transcripts are localized in broad domains of the neural plate in cells located at a distance from organizer tissue (Figs 1C and 2A-B). An alternative source of inductive signals is mesoderm which underlies the *Pax-3* expression domain. To test this idea, *Xenopus* animal caps were combined with explants of stage 6 chick posterior non-axial mesoderm (pnm; mesoderm which underlies the *Pax-3* expression domain in the neural plate is immediately caudal to the level of the node and lateral to the primitive streak, thus we refer to it here as 'posterior non-axial mesoderm'; based on fate mapping studies, this tissue consists of a mixture of mesodermal precursor cells, including segmental and lateral plate, that are indistinguishable in this assay; Schoenwolf et al., 1992 and references therein). Pnm did not induce either *Pax-3* or *N-CAM* expression in *Xenopus* ectoderm (Fig. 3B). We then reasoned that pnm may be able to induce *Pax-3* but only in ectoderm that has been neuralized. To examine this idea, we modified the chick-*Xenopus* recombination assay by using animal caps that were neuralized by *noggin* as the responding tissue.

In agreement with previous reports, *noggin* animal caps formed anterior neural tissue as they expressed the neural marker, *N-CAM*, and the anterior marker, *Otx-2* (Lamb et al., 1993). *Noggin* animal caps did not express *Pax-3* (Fig. 3C), consistent with our observation that, in vivo, *Pax-3* expression is not detected in early anterior neural plate. As inducing tissues, we tested chick stage 6 pnm, stage 4 and 6 Hensen's nodes, and stage 6 head mesendoderm isolated at the level of the head fold (Fig. 3C). Since *noggin* acts as a neural inducer, all types of chick-*Xenopus* recombinants expressed *N-CAM*. As before, we found that stage 6 Hensen's node induced *Pax-3* expression in *noggin* animal caps, whereas stage 4 node did not. In contrast to results obtained when naive animal caps were the responding tissue, we found that pnm was a good inducer of *Pax-3* expression in *noggin* animal caps. Moreover, the *Pax-3* inducing activity from pnm was not a general mesodermal property, since head mesendoderm failed to induce *Pax-3* in *noggin* animal caps. Interestingly, pnm that was isolated using a mild enzymatic treatment lost its inductive activity unless allowed to recover at 37°C for 30 minutes, implying that at least one component of this activity is a protein (Fig. 3C). These results support a model where signals arising from pnm act on overlying neuralized ectoderm to initiate *Pax-3* expression. We did not detect expression of the early mesodermal marker *Xenopus brachyury* (*Xbra*; Smith et al., 1991), suggesting that

the *Pax-3* inductive signals arising from Hensen's node and pnm act directly on *noggin* animal caps, and not through induction of a mesodermal intermediate (Fig. 3D).

Hensen's node and pnm induce *Pax-3* expression in chick neural plate explant cultures

To confirm that the chick-*Xenopus* heterospecies tissue recombinant assay is a faithful model with which to study inductive interactions that regulate *Pax-3* expression, similar experiments were performed using only avian tissues. As a target responding tissue we used stage 4 chick presumptive anterior neural plate, just rostral to Hensen's node, as this tissue should already be neuralized, but should not express *Pax-3* (Fig. 1A; see Storey et al., 1992; Dickinson et al., 1995). Candidate inducing tissues were isolated from quails so that *Pax-3* transcripts in inducing and responding tissues could be distinguished. Explants of chick stage 4 presumptive anterior neural plate were combined with quail stage 6 Hensen's node, pnm, or head mesendoderm and cultured in serum-containing medium within a collagen-gel matrix. Cultures were assayed after 20 hours using RT-PCR analysis (Fig. 4A). Stage 4 anterior neural plate explants alone did not express appreciable levels of *Pax-3*, even after 20 hours in culture, yet they did exhibit an anterior epiblast character in that they expressed *Otx-2* (Fig. 4B). Consistent with results that were obtained with the chick/*Xenopus* assay, stage 6 Hensen's node and pnm induced *Pax-3* expression in stage 4 anterior neural plate explants, whereas stage 6 head mesendoderm did not (Fig. 4B).

To investigate whether these signals can also operate in vivo, we extended our analysis to whole chicken embryos. Pnm explants from stage 6 donor chicks were grafted unilaterally between the epiblast and endoderm in the area of the presumptive anterior neural plate of stage 4 host chicken embryos (Fig. 5A). Approximately 12 hours post-surgery, host embryos were assayed for *Pax-3* expression at stages 7-9 by in situ hybridization. In 13/18 embryos, in which the graft had healed into position beneath the anterior-most neural plate, we detected ectopic *Pax-3* expression on the operated side that extended into the prosencephalon past the rostral *Pax-3* boundary as indicated by comparison with the un-operated side (Fig. 5B). In 6/6 operated embryos, grafts implanted beneath presumptive non-neural ectoderm failed to induce *Pax-3* expression in overlying tissue (data not shown). These results suggest that pnm is capable of providing signals that induce *Pax-3* expression in neuroectoderm, but not in non-neural ectoderm, in vivo.

To determine whether the pnm-mediated induction of ectopic *Pax-3* expression in the prosencephalon reflected posteriorization of the tissue overlying the graft we examined expression of the telencephalic marker, *c-qin*. *c-qin* is the cellular counterpart of the *v-qin* oncogene, and is a putative chicken homolog of rat brain-factor-1 (BF-1), a winged-helix transcription factor that is specifically expressed in the telencephalon (Chang et al., 1995; Tao and Lai, 1992). *c-qin* expression was down-regulated on the operated side in 4/6 of these embryos (Fig. 5C,D), implying that signals from the pnm graft act to posteriorize overlying neuroectoderm, consistent with theories that posteriorization of the neuraxis dominates over anterior development (reviewed by Slack and Tannahill, 1992).

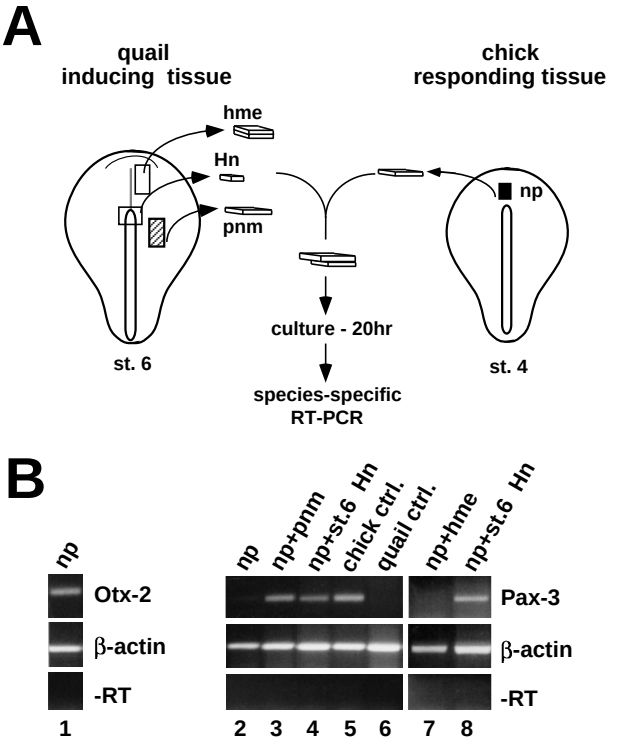

Fig. 4. Stage 6 quail pnm and Hensen's node induce *Pax-3* expression in stage 4 chick presumptive anterior neural plate explants. (A) Experimental design. (B) Ethidium-stained agarose gels of RT-PCR reactions. As a control, RT-PCR was performed on RNA extracted from explants of stage 9 open neural plate to show that *Pax-3* primers specifically amplify chick *Pax-3*, and not quail *Pax-3*, whereas primers for the loading control, *β-actin*, do not distinguish chick and quail (compare lanes 5 and 6). Stage 4 presumptive anterior neural plate does not express appreciable levels of *Pax-3* (lane 2), although it does express the anterior epiblast marker *Otx-2* (lane 1). Pnm (lane 3) and stage 6 Hensen's node (lane 4) both induce *Pax-3* expression in stage 4 presumptive anterior neural plate, however, stage 6 hme (lane 7) fails to display inductive activity compared with stage 6 Hensen's node (lane 8). RT-minus controls using *Otx-2* primers (lane 1) or *Pax-3* primers (lanes 2-8) are shown in the bottom row. *β-actin* RT-minus controls were negative in all cases (data not shown). ctrl., control; hme, head mesendoderm; Hn, Hensen's node; pnm, posterior non-axial mesoderm; np, presumptive anterior neural plate.

Pax-3 expression is induced by putative posteriorization signals

As described above, results obtained using avian explant and whole embryo cultures are in agreement with those from the chick-*Xenopus* 'sandwich' experiments, suggesting that the heterospecies assay provides a good model with which to study regulation of *Pax-3* expression.

To further examine the idea that *Pax-3* expression is induced in response to caudalization of the neuraxis we used neuralized animal caps to specifically test two molecules that are thought to act as posteriorizing signals: FGF and retinoic acid (RA; reviewed by Doniach, 1995; Maden and Holder, 1992). When blastula stage animal caps from *noggin*-injected embryos were treated with bFGF and then allowed to develop to stage 25, *Pax-3* expression was induced (Fig. 6A). To determine whether *Pax-3* inductive signals arising from stage 6 Hensen's node and

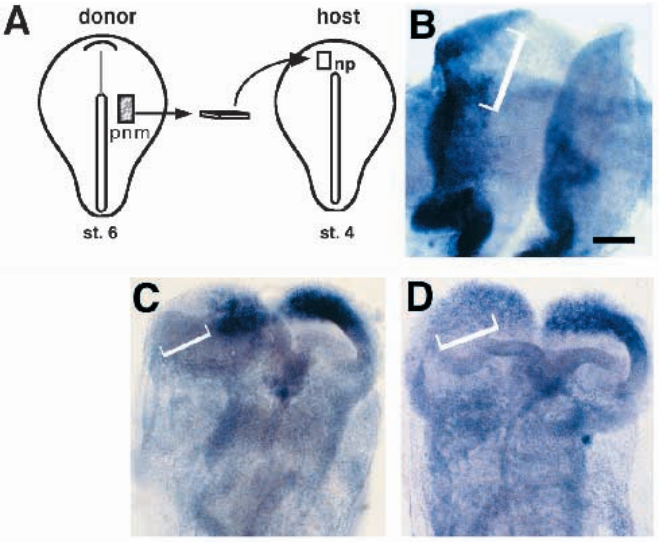

Fig. 5. Grafts of pnm into the presumptive anterior neural plate of chick induce ectopic *Pax-3* expression in the prosencephalon. (A) Experimental design. (B) A host embryo at stage 8 with ectopic *Pax-3* expression detected in the prosencephalon on the operated (left) side (indicated by a white bracket). (C) A host embryo at stage 9 where expression of the telencephalic marker, *c-qin*, is suppressed laterally on the operated (left) side, indicated with a white bracket. (D) A second example of a host embryo at stage 9 where expression of the telencephalic marker, *c-qin*, is strongly reduced on the operated (left) side (indicated with a white bracket). Grafts were not marked, but they could still be distinguished after fixation of the host embryos. Scale bar (B-D) 100 μm.

pnm are FGF-mediated we combined these chick tissues with animal caps isolated from embryos co-injected with *noggin* and a dominant-inhibitory FGF-receptor (*XFD*; Amaya et al., 1991). RPA analysis of explant recombinants showed that *XFD* did not block induction of *Pax-3* expression by either stage 6 Hensen's node or pnm, although *XFD* efficiently blocked *Pax-3* expression induced by exogenous bFGF, and *cardiac actin* (*AC100*) expression induced by activin (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994) (Fig. 6B). Furthermore, a dominant negative form of *ras* (Feig and Cooper, 1988), a small GTP-binding protein that acts in FGF signal transduction, as well as that of a number of other signaling factors, also failed to block induction of *Pax-3* expression by stage 6 node and pnm (Fig. 6B).

The application of RA to developing mice, chicken or *Xenopus* embryos results in transformation of anterior neural tissue to more posterior fates (reviewed by Maden and Holder, 1992). Moreover, ectopic expression of a dominant negative form of the retinoic acid receptor, *xRAR-α1*, in *Xenopus* embryos leads to an enhancement of anterior neural structures at the expense of more posterior ones, suggesting that RA plays an important role in normal development (Blumberg et al. 1997). RA also acts to posteriorize *noggin* animal caps, in that it induces expression of the posterior marker *Hoxb-3* and suppresses expression of the anterior marker *Otx-2* (Papalopulu and Kintner, 1996). We observed that RA induced expression of *Pax-3* both in *noggin* animal caps (Fig. 6A) and in stage 4 chick presumptive anterior neural plate explants (data not shown), consistent with our hypothesis that *Pax-3* expression is regulated by posteriorizing signals. To determine whether *Pax-3* inductive signals arising from stage 6 Hensen's node and

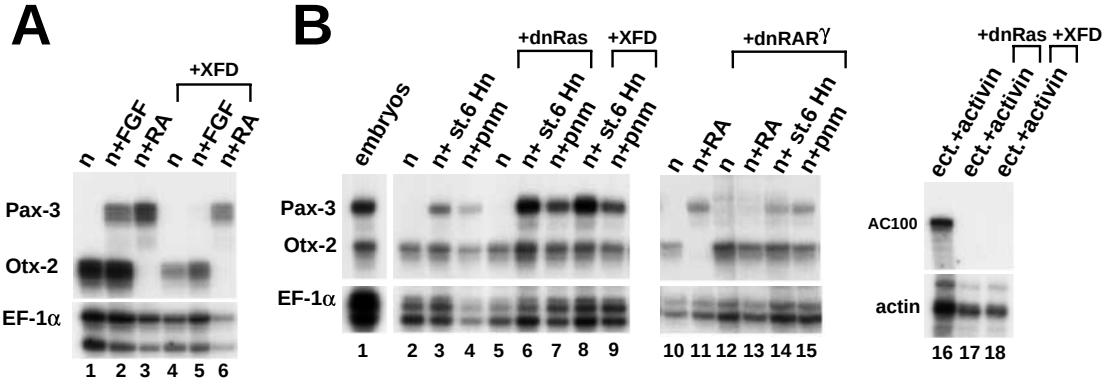

Fig. 6. RA and bFGF induce *Pax-3* expression in *noggin* treated animal caps, however *Pax-3* inductive signals from stage 6 pnm and Hensen's node are not blocked by *XFD*, *dnRas*, or dn *RARγ-1*. (A) Animal caps from blastulae injected only with *noggin* (lanes 1-3), or co-injected with *noggin* and *XFD* (lanes 4-6). Animal caps were left untreated (lanes 1 and 4), or were treated with 2×10^{-6} M RA (lanes 3 and 6), or 100 ng/ml bFGF (lanes 2 and 5), and then allowed to develop to stage 25 when they were analyzed by RPA. RA (lane 3) and bFGF (lane 2) induce *Pax-3* expression in *noggin* animal caps, and *XFD* blocks bFGF-mediated induction of *Pax-3* expression (lane 5), but not RA-mediated *Pax-3* induction. This result indicates that RA-induced expression of *Pax-3* in *noggin* animal caps is not FGF-dependent. (B) Analysis of chick/*Xenopus XFD-*, dn *ras*- and dn *RARγ-1- noggin* animal cap recombinants. Recombinants were allowed to develop until *Xenopus* stage 25 and then analyzed by RPA. Induction of *Pax-3* expression in *noggin* animal caps by chick stage 6 Hensen's node (lane 3) and pnm (lane 4) is not blocked by co-injection of dn *ras* (lanes 6,7), or *XFD* (lanes 8, 9), or dn *RARγ-1* (lanes 14, 15). We note that *Pax-3* expression appears to be upregulated in lanes 6-9, consistent with observations of Kroll and Amaya (1996) that *Pax-3* expression is upregulated in *XFD* transgenic *Xenopus* embryos. Lanes 10-12 show that dn *RARγ-1* blocks induction of *Pax-3* and suppression of *Otx-2* expression in *noggin* animal caps treated with 2×10^{-6} M RA. In addition, induction of *cardiac actin* (*AC100*) in animal caps treated with 5 ng/ml of activin (lane 16) is blocked by co-injection of either *dnRas* (lane 17) or *XFD* (lane 18), showing that both the *dn ras* and *XFD* injected RNAs produce proteins with the expected activity. Cytoskeletal-actin (labeled as *actin*) cross hybridizes with the *AC100* probe, and serves as a loading control in lanes 16-18. dominant negative *ras*, dn *ras*; ect., ectoderm; pnm, posterior non-axial mesoderm; n, *noggin*; st. 6 Hn, stage 6 Hensen's node.

pnm are RA-mediated we made 'sandwich' recombinants using animal caps isolated from embryos co-injected with *noggin* and a dominant-inhibitory form of the *retinoic acid receptor-γ1* (dn *xRAR-γ1*) (Blumberg et al. 1991; Ellinger-Ziegelbauer and Dreyer 1991; see also Blumberg et al. 1997, and Materials and Methods). Analysis of these recombinants showed that dn *xRAR-γ1* failed to block induction of *Pax-3* expression by either stage 6 Hensen's node or pnm, although it was effective at blocking induction of *Pax-3* and suppression of *Otx-2* expression when RA was added exogenously to the culture (Fig. 6B).

These observations imply that although exogenous application of either bFGF or RA induces *Pax-3* expression in neuralized animal caps, *Pax-3* inductive signals arising from two probable in vivo sources, Hensen's node and pnm, are not strictly dependent on FGF, *ras*, or RA.

Epidermal-ectoderm fails to induce *Pax-3* expression in stage 4 chick neural plate explant cultures

Previously it had been shown in chicks that epidermal-ectoderm isolated at either stage 4 or 8 produces dorsalizing signals that induce stage 4 neural plate explants to express markers of neural-crest, including *slug* (Dickinson et al., 1995; Liem et al., 1995; Selleck and Bronner-Fraser, 1995; Nieto et al., 1994). Furthermore, at stage 10, epidermal-ectoderm produces an activity, mimicked by BMP-4 and BMP-7, that 'super-induces' *Pax-3* expression in stage 10 caudal neural plate explants that already express *Pax-3* (Liem et al., 1995). Given our results suggesting that posteriorizing signals from Hensen's node and pnm initiate *Pax-3* expression, we were interested in determining whether dorsalizing signals from epidermal-ectoderm could similarly initiate *Pax-3* expression in early neural plate tissue that does not express *Pax-3*.

Explants of chick stage 4 presumptive anterior neural plate were combined with chick stage 8 epidermal-ectoderm (Fig. 7A). Explant recombinants were cultured for 20 hours in collagen-gel matrices, and then sectioned for in situ hybridization analysis. Expression of *slug* was used as a positive control. Only low or background levels of *Pax-3* and *slug* expression were detected in either stage 4 neural plate or stage 8 epidermal-ectoderm explants, cultured in isolation (Fig. 7B,C; Table 1, and data not shown, see also Dickinson et al., 1995; Liem et al., 1995). Analysis of recombinants revealed that although the epidermal-ectoderm induces robust expression of *slug* in neural plate explants, it does not induce significant levels of *Pax-3* expression in adjacent sections (Fig. 7D-G; Table 1). In addition, treatment of stage 4 neural plate explants with BMP-4 induces *slug* expression, but fails to induce *Pax-3* (Fig. 7H,I; Table 1). These results suggest that, in contrast to stage 10 neural plate explants (Liem et al., 1995), stage 4 neural plate explants are not competent to express *Pax-3* in response to dorsalizing signals from epidermal-ectoderm.

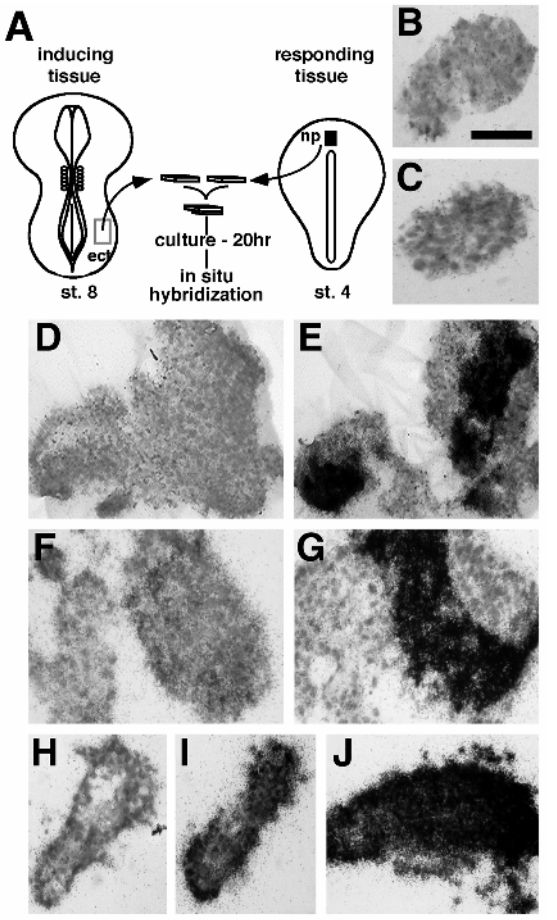

Fig. 7. Ectoderm and BMP-4 do not induce *Pax-3* expression in explants of stage 4 chick presumptive anterior neural plate. (A) Experimental design. (B,C) Sections of stage 4 presumptive anterior neural plate explants cultured alone showing that neither *Pax-3* (B) nor *slug* (C) are expressed. (D,E) Serial sections of a neural plate/epidermal-ectoderm recombinant, showing that epidermal-ectoderm induces *slug* expression (E), but not *Pax-3* expression (D) in an adjacent section (grains of hybridization are black). (F,G) Serial sections of an additional independent sample of a neural plate/epidermal-ectoderm recombinant, showing that epidermal-ectoderm induces *slug* expression (G), but not *Pax-3* expression (F) in an adjacent section. (H,I) Serial sections of a stage 4 presumptive anterior neural plate explant cultured in the presence of 100 ng/ml BMP-4, showing that BMP-4 induces *slug* (I), but not *Pax-3* (H) expression. (J) Section of a stage 9 caudal neural plate explant hybridized as a positive control for the *Pax-3* probe. Scale bar, 50 μm.

DISCUSSION

Initiation of *Pax-3* expression reflects posteriorization of the neuraxis

In this paper we present several lines of evidence that *Pax-3* expression is initiated in the early neural plate by the process of posteriorization. First, *Pax-3* transcripts are restricted to the posterior neural plate in both chick and *Xenopus* gastrula and neurula stage embryos. Second, stage 6 but not stage 4 Hensen's node induces *Pax-3* expression in *Xenopus* naive ectoderm as well as ectoderm neuralized by *noggin*. Signals that posteriorize are thought to be generated by organizer tissue as it ages and gives rise to more posterior mesodermal derivatives (reviewed by Doniach, 1993; Gallera, 1971). Third, two molecules that have been shown to act as posteriorizing agents, RA and bFGF, both induce *Pax-3* expression in neuralized animal caps. We propose that posteriorizing signals that induce *Pax-3* expression also arise from posterior non-axial

Table 1. Epidermal-ectoderm and BMP-4 induce *slug* but not *Pax-3* expression in stage 4 presumptive anterior neural plate explants

Explants	*Pax-3*	*slug*
Stage 4 neural-plate alone	9/9 (–)	12/14 (–) 2/14 (+)
Stage 4 neural-plate + epidermal-ectoderm	10/10 (–)	3/14 (–) 11/14 (+)
Stage 4 neural-plate + BMP-4	5/5 (–)	1/6 (–) 5/6 (+)
Stage 9 caudal neural plate (positive control)	8/8 (+)	n.d.

Fractions of the total number of explant recombinants examined that were positive (+) or negative (–) for *slug* and *Pax-3* expression. The two 'neural plate alone' samples that were scored as positive for *slug* expression had small localized areas of hybridization at the edges of the explants.

mesoderm. Indeed, pnm grafts, placed beneath the presumptive anterior neural plate of cultured chick embryos, induce ectopic *Pax-3* expression in the prosencephalon and appear to posteriorize overlying neuroectoderm in that suppression of the telencephalic marker, *c-qin*, is also observed. In addition, stage 6 pnm combined with *noggin* animal caps induces expression of the mb-hb marker, *en-2* (A.G.B. and C.K., unpublished observation). Cox and Hemmati-Brivenlou (1995) have similarly demonstrated in *Xenopus* that prospective forebrain explants are posteriorized when combined with posterior-dorsal mesoderm.

Regulation of *Pax-3* expression in the early neural plate

If *Pax-3* expression is initiated during neural induction by posteriorization, where do these signals come from and how do they act? Our results indicate that both axial (stage 6 Hensen's node) and non-axial (i.e. pnm) tissues are candidate sources for the in vivo posteriorization signals which induce *Pax-3* expression. Of the two, the pnm is a particularly attractive candidate since it underlies the *Pax-3* expression domain in the neural plate and is thus appropriately positioned to produce signals that induce *Pax-3* expression in vivo. In contrast, the role of Hensen's node is likely to be more complicated given that *Pax-3* expression is absent from the area surrounding the node from stages 5-7 but then moves in close to the node by stage 9. A possible explanation for these observations is that the node could be a source of both inductive and repressive signals. Thus, *Pax-3* expression may be induced along the M-L axis of the neural plate by the combined action of node and pnm derived signals, but rapidly repressed medially by signals from the node, and subsequently from its derivatives, notochord and floor plate. Indeed, the difference between the medial expression of *Pax-3* in the presumptive neural plate at stages 5-7 versus stage 9 (Fig. 1C-F) may be accounted for by the observation that *shh*, which is known to repress *Pax-3* expression (see Introduction), is expressed in Hensen's node from stages 4-7, but is down-regulated by stage 8 (Riddle et al., 1993). Finally, the observation that stage 6 node induces *Pax-3* expression in *noggin* animal caps and chick presumptive anterior neural plate suggests that inductive signals predominate over repressive signals in these in vitro assays.

Pnm acts to induce *Pax-3* expression only in neuralized tissue

Our experiments show that both Hensen's node and pnm isolated from stage 6 embryos produce *Pax-3* inducing signals. However, an important difference between the inducing ability of these two tissues is that stage 6 pnm is only able to induce *Pax-3* expression in neuralized or 'activated' responding tissues, such as *noggin* animal caps, whereas the node can induce *N-CAM* and *Pax-3* in non-neural ectoderm. Interestingly, using the embryonal carcinoma stem cell line, P19, Pruitt (1994) identified a mesodermally derived *Pax-3* inductive activity that is similar to that which we describe here, in that it is most efficient under conditions where neuralization is also induced. These observations are consistent with two-signal models of regional specification of the neuraxis (reviewed by Doniach, 1993). Thus, *Pax-3* expression would be initiated by a combination of an activation step where a neuralizing signal arising from the organizer induces competent ectoderm to take on an anterior neural fate, followed by a transformation step, involving signals from underlying pnm and later stage organizer tissue, where anterior neuroectoderm is progressively transformed into more posterior fates.

Other studies have reported that non-axial mesoderm exhibits poor neural inducing activity, however the ability of these tissues to induce expression of regional markers in competent neuroectoderm was not tested (Hemmati-Brivanlou et al., 1990; Jones and Woodland, 1989). There have been a number of reports of non-organizer mesoderm inducing regionally specific neural markers in the A-P axis, but in these cases the mesoderm also acted as a neural inducer (Ang and Rossant, 1993; Hemmati-Brivanlou et al., 1990). Using the chick-*Xenopus* recombinant assay we have shown that signals that mediate neural induction (arising from organizer tissue) and regionalization (arising both from organizer and pnm) can be uncoupled, since pnm cannot induce neuralization in ectoderm, but can induce *Pax-3* expression in tissue that is already neural. Interestingly, Storey et al. (1995) demonstrated that neural induction and regionalization signals correlate with different prospective cell types in Hensen's node, although in this study these signals were not uncoupled. The observation that both stage 6 Hensen's node and pnm induce *Pax-3* expression leads us to speculate that perhaps cell-types common to both these tissues, such as somitic precursor cells (Selleck and Stern, 1991), may mediate *Pax-3* induction. Indeed, Itasaki et al. (1996) recently showed that somitic mesoderm exhibits a graded ability to posteriorize the hindbrain and reprogram Hox gene expression in chicks. Alternatively, it is possible that Hensen's node and pnm produce different *Pax-3* inductive signals that are spatially restricted in the M-L axis. For instance, the lateral aspect of the pnm is in the right position to provide signals that could act to specifically induce *Pax-3* expression at the lateral edges of the overlying neural plate where neural crest will arise (see Mitani and Okamoto, 1991; Mayor et al., 1995).

What molecules mediate induction of *Pax-3* expression by Hensen's node and pnm?

We have shown that *Pax-3* inductive signals can be mimicked by bFGF and RA, both of which have been previously proposed to be involved in posteriorization of the neuraxis (reviewed by Doniach, 1995; Maden and Holder, 1992). However, it is unclear

whether these molecules have an endogenous role in initiating *Pax-3* expression. Neither a dominant-inhibitory FGF receptor (*XFD*) nor dominant-negative *ras* blocks *Pax-3* induction by Hensen's node and pnm in neuralized animal caps. In addition, it has recently been reported that *XFD* transgenic *Xenopus* embryos exhibit normal patterning in the A-P neuraxis, including expression of *Pax-3*, suggesting that regulation of *Pax-3* expression in vivo also does not require FGF-signaling (Kroll and Amaya, 1996). RA was an attractive candidate for an endogenous inducing factor since stage 6 Hensen's node contains three-fold higher concentration of retinoids than stage 4 Hensen's node (Chen et al., 1992). However, a dominant-negative form of the *xRAR-γ 1* receptor fails to block induction of *Pax-3* by Hensen's node and pnm. In addition, ectopic expression of a dominant-negative *RAR-α1* in *Xenopus* embryos enhances anterior and suppresses expression of posterior neural markers, but it does not alter the *Pax-3* expression pattern (Blumberg et al. 1997; N.P., unpublished observation). Finally, our observation that pnm, isolated using a mild enzymatic treatment, only induces *Pax-3* in *noggin* animal caps if allowed to recover at 37°C, indicates that at least a component of the inductive activity from this tissue is a protein, and is thus unlikely to be mediated by RA alone. Therefore, the nature of the endogenous *Pax-3* inducing signal(s) remains unclear. The possibility that multiple, redundant *Pax-3* inductive signals emanate from Hensen's node and pnm, and thus it is insufficient to block only one will be tested in the future using combinations of dominant negative receptors.

Interestingly, a recent analysis of the murine *Pax-3* promotor by Natoli et al. (1997) shows that regulatory elements sufficient to drive expression of a *lacZ* reporter gene in vivo in the dorsal hindbrain and spinal cord are located within 1.6 kb 5′ to the transcription start, suggesting that the inductive activities we have identified may act to mediate *Pax-3* transcription through this regulatory region.

Presumptive anterior neural plate explants are not competent to express *Pax-3* in response to dorsalizing signals

Liem et al. (1995) demonstrated that dorsalizing signals arising from epidermal-ectoderm, which are mimicked by BMP-4 and BMP-7, can super-induce *Pax-3* expression in stage 10 caudal neural plate explants. We have considered the possibility that *Pax-3* expression is initiated in the early neural plate by signals from flanking presumptive epidermal-ectoderm, a suggestion that is supported by observations that *BMP-4* and *BMP-7* are expressed as early as stage 5 in this region of the epiblast (Watanabe and Le Douarin, 1996; A.G.B. and M.D.G., unpublished observations). However our results showing that epidermal-ectoderm and BMP-4 cannot induce *Pax-3* expression in stage 4 presumptive anterior neural plate explants suggests that these signals alone are not sufficient to induce *Pax-3* until the neural plate acquires competence to respond. Based on these experiments, we speculate that only posteriorized neuroectoderm is competent to express *Pax-3* in response to dorsalizing signals from epidermal-ectoderm. In support of this idea, previous studies have suggested that positional value along the A-P neuraxis can determine how a given cell will respond to D-V patterning signals (see Simon et al., 1995). Indeed, shh induces different ventral neuronal cell types depending upon the A-P character of responding tissue explants (Ericson et al., 1995). Finally, experiments showing that epidermal-ectoderm induces expression of the dorsal marker *Wnt-1* in chick stage 8-10 caudal neural plate, but not stage 4 anterior neural plate suggest that competence is also an important factor in determining how neuroectoderm will respond to dorsalizing signals (Dickinson et al., 1995).

In summary, we propose that *Pax-3* expression is initiated in the early neural plate by posteriorization signals arising from Hensen's node and posterior non-axial mesoderm, and that these activities do not solely depend on either FGF or RA. Taking our results together with those of previous studies, we suggest that in a second step following *Pax-3* initiation by posteriorizing signals, the opposing actions of inductive, dorsalizing signals from epidermal-ectoderm and repressive, ventralizing signals from notochord and floor plate, act to restrict *Pax-3* expression to the dorsal neural tube.

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