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

Anne G. Bang, Nancy Papalopulu*, Chris Kintner and Martyn D. Goulding*
Molecular Neurobiology Laboratory, The Salk Institute for Biological Studies, 10010 North Torrey Pines Rd, La Jolla, CA 92037, USA

*Author for correspondence (e-mail: goulding@axp1.salk.edu)
†Present address: Wellcome/CRC Institute, Tennis Court Road, Cambridge, CB2 1QR, UK

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 suggest 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 induces 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 38°C 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 antisense RNA probes for *Xenopus* Pax-3 (Epeseth et al., 1995) and engrafted-2 (en-2; Hemmati-Brivanlou 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 35% 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 35S-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)Pr-Asn (Feig and Cooper, 1988), as described by Bhushan et al. (1994)), or dominant negative xRAR-1 (1 ng; dn xRAR-1 was a generous gift from Dr Bruce Blumberg, and was constructed as described for the dominant negative form of xRARαβ-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 0.5x MMR, or 100ng/ml of bFGF (Boehringer Mannheim) or 5 ng/ml recombinant activin (provided by the Vale laboratory) in 0.5x 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 0.5x 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), explants of approximately 100 μm x 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 hm, 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 mesoderm was removed. To isolate epidermal-ectoderm, stage 8 explants 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).

**Quail/hybrid explant recombinant cultures**

Hensen’s node, pnm, or hm explants were ‘sandwiched’ between two pieces of *Xenopus* animal cap tissue (Fig. 3A). Animal cap/chick tissue recombinants were cultured in 0.5x MMR at room temperature as described above. Before pnm and hm 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, hm, 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% CO₂. 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 32P-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
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**Pax-3** is initiated by posteriorization

**Materials and Methods**

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: GGCGAGTTGCTTGAGTA; downstream: GCTCACTGTGAATTTCCCAA (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-CATGATGTAGCCCATCC; downstream: TCGGGCAGCTGAACCTCTCAT (amplifies a 388 bp fragment of both chicken and quail **cytoplasmic β-actin**).

**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 the caudal boundary of **Otx-2** expression. I-N are 70 μm transverse Vibratome sections of **Pax-3**-hybridized embryos at stage 6 (IJ), stage 7 (KL), and stage 9 (MN) 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.

**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 ev-2 (Hemmatti-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 capabilities in terms of inducing A-P neural markers (Storey et al., 1992; Kintner and Dodd, 1991 and references therein). Using a heteroepithelial 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 neutralizing 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.
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 neutralized. To examine this idea, we modified the chick-Xenopus recombination assay by using animal caps that were neutralized 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 neutralized 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). ctl., control; hme, head mesendoderm; Hn, Hensen’s node; pnm, posterior non-axial mesendoderm; 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
Pax-3 is initiated by posteriorization

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.

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 RARγ1. (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-, dp ras- and dp RARγ1- 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 dp RARγ1 (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 dp RARγ1 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.

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pmn 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-γ (dn xRAR-γ) (Blumberg et al. 1991; Ellingher-Ziegelmayer and Dreyer 1991; see also Blumberg et al. 1997, and Materials and Methods). Analysis of these recombinants showed that dn xRAR-γ failed to block induction of Pax-3 expression by either stage 6 Hensen’s node or pmn, 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 pmn, 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 dorsaling 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 pmn initiate Pax-3 expression, we were interested in determining whether dorsaling 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 dorsaling signals from epidermal-ectoderm.

**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 Doniaich, 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
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-bb marker, en-2 (A.G.B. and C.K., unpublished observation). Cox and Hemmati-Brivanlou (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.

| 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.

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 carcinosa 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 neutralized 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-γ1 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 nogggin 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 promoter 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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