Induction of the prospective neural crest of *Xenopus*

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SUMMARY

The earliest sign of the prospective neural crest of *Xenopus* is the expression of the ectodermal component of *Xsna* (the *Xenopus* homologue of *snail*) in a low arc on the dorsal aspect of stage 11 embryos, which subsequently assumes the horseshoe shape characteristic of the neural folds as the convergence-extension movements shape the neural plate. A related zinc-finger gene called *Slug* (*Xslu*) is expressed specifically in this tissue (i.e. the prospective crest) when the convergence extension movements are completed. Subsequently, *Xslu* is found in pre- and post-migratory cranial and trunk neural crest and also in lateral plate mesoderm after stage 17. Both *Xslu* and *Xsna* are induced by mesoderm from the dorsal or lateral marginal zone but not from the ventral marginal zone. From stage 10.5, explants of the prospective neural crest, which is underlain with tissue, are able to express *Xslu*. However expression of *Xsna* is not apparently specified until stage 12 and further contact with the inducer is required to raise the level of expression to that seen later in development. *Xslu* is specified at a later time. Embryos injected with noggin mRNA at the 1-cell stage or with plasmids driving noggin expression after the start of zygotic transcription express *Xslu* in a ring surrounding the embryo on the ventroposterior side. We suggest this indicates (a) that noggin interacts with another signal that is present throughout the ventral side of the embryo and (b) that *Xslu* is unable to express in the neural plate either because of the absence of a co-inducer or by a positive prohibition of expression. The ventral co-inducer, in the presence of overexpressed noggin, seems to generate an anterior/posterior pattern in the ventral part of the embryo comparable to that seen in neural crest of normal embryos. We suggest that the prospective neural crest is induced in normal embryos in the ectoderm that overlies the junction of the domains that express noggin and *Xwnt-8*. In support of this, we show animal cap explants from blastulae and gastrulae, treated with bFGF and noggin express *Xslu* but not NCAM although the mesoderm marker *Xbra* is also expressed. Explants treated with noggin alone express NCAM only. An indication that induction of the neural plate border is regulated independently of the neural plate is obtained from experiments using ultraviolet irradiation in the pre-cleavage period. At certain doses, the cranial crest domains are not separated into lateral masses and there is a reduction in the size of the neural plate.

Key words: neural crest, neural folds, neural plate border, bFGF, noggin, NCAM, *Xbra*, *Xslu*, *Xsna*, *Xwnt-8*, *Xenopus*, ultraviolet

INTRODUCTION

The emergence of neural crest in the evolution of vertebrates is of special importance because of its supposed role in facilitating cephalisation (Gans and Northcott, 1983). Substantial differences distinguish the organisation of neural crest in the major classes of vertebrates although the presumptive neural crest is always derived from a tissue at the edge of the neural plate. Thus in the mouse, neural crest cells are found delaminating from the neuroepithelium before the neural tube is closed and are only briefly part of an epithelium (Nieto et al., 1992), whereas in the chick a premigratory form of neural crest exists in the most dorsal part of the closed neural tube. Dorsal neural tube cells can become neurons in the peripheral or central nervous system and furthermore, if this tissue is ablated, the adjoining tissue is able to reconstitute the neural crest (Scherson et al., 1993). In amphibia, the neural folds mark the edge of the neural plate and contain the premigratory crest (Schroeder, 1970). The premigratory cephalic crest is typically massive compared with the trunk crest and never comes to that seen in neural crest of normal embryos. We suggest that the prospective neural crest is induced in normal embryos in the ectoderm that overlies the junction of the domains that express noggin and *Xwnt-8*. In support of this, we show animal cap explants from blastulae and gastrulae, treated with bFGF and noggin express *Xslu* but not NCAM although the mesoderm marker *Xbra* is also expressed. Explants treated with noggin alone express NCAM only. An indication that induction of the neural plate border is regulated independently of the neural plate is obtained from experiments using ultraviolet irradiation in the pre-cleavage period. At certain doses, the cranial crest domains are not separated into lateral masses and there is a reduction in the size of the neural plate. (Essex et al., 1993). Changes in the expression pattern of *Xsna* reflect the dynamics of neurulation in a striking way. *Xsna* expression in the neural plate border first appears as a low arc on the dorsal aspect of the embryo at stage 11, when the convergence-extension movements that
shape the neural plate are at an early stage. The appearance of ectodermal Xsna in a distinct domain of prospective neural fold before neural plate formation is complete, indicates that induction of the border should be considered independently of formation of the neural plate.

Pioneering studies on Urodèles suggested that induction of the neural crest could be mediated by underlying tissues, independently of the neural plate. The archenteron roof underlying the neural folds was able to induce neural crest derivatives principally, whereas the medial part of the archenteron roof tended to induce neural plate and less neural crest (Raven and Kloos, 1945). More recently, the capacity to induce melanophores (a derivative of neural crest) without neurulation by co-culture of ventral ectoderm and lateral mesoderm but not dorsal mesoderm has been demonstrated (Mitani and Okamoto, 1991). The induction of neural crest Xwnt by mesoderm has been demonstrated (Hopwood et al., 1989). In contrast, other investigators have argued that the signals passing through the plane of the ectoderm induce neural folds and placodes as its potency is reduced below a critical level (Albers, 1987) or when qualitative changes occur in the competence of ectoderm to respond (Servetnick and Grainger, 1991). Neural fold and neural crest, derived from ectodermal or neuroectodermal material, respectively, are induced when neural plate and ectoderm are juxtaposed experimentally (Moury and Jacobson, 1990). Two recent investigations suggest the position of the neural plate border may be regulated by the balance of two opposing inducing signals that emanate from the neural plate and ectoderm (Coffman et al., 1993; Zhang and Jacobson, 1993).

Neural crest induction has often been assessed using melanophores as a marker although these are expressed a considerable time after the primary inductive event. In this investigation, we have used molecular markers (Xsna and Xslu) that are expressed early to study induction of the prospective neural crest.

MATERIALS AND METHODS

Isolation of Xslu
A stage 17 Xenopus cDNA library in lambda GT10 was screened (Kintner and Melton, 1987) using a chicken slug clone (Nieto et al., 1994) as a probe. Inserts of these clones were subcloned into pSP72 (Krieg and Melton, 1987) and were sequenced by the double stranded dideoxy procedure. The sequence has been deposited with EMBL (Accession number X80269).

Whole-mount in situ hybridisation
Digoxigenin containing RNA probes were prepared from the Xslu cDNA sequence. The sequences from 968 to 1701 were used to prepare sense and antisense probes. This sequence has no similarity to the Xsna sequence and would not be expected to hybridise with Xsna sequences. Sense transcripts consistently gave no staining. Other expression patterns were obtained using antisense transcripts of sequences 1200-1800 and 0-723 of Xsna and NCAM, respectively. Specimens were prepared, hybridised and stained by the method of Harland (1991). We found that after a 1 hour fixation, proteinase K and RNAse treatment were unnecessary as neither treatment improved the result and mesodermal signals such as Xsna were not adversely affected (Essex et al., 1993). After colour development, embryos were washed in methanol for 1 hour, to reduce background and enhance blue colour. Some stained specimens was bleached, after colour development without effect on the blue colour, by placing them in hydrogen peroxide, formamide, SSC (1%, 5%, 0.5x respectively) over a fluorescent light for about 1 hour. In some cases, specimens were cleared with tetrahydroanaphthol to obtain better photographic images. Some stained embryos were sectioned in wax and counterstained with eosin for microscopic examination. In situ hybridisation has been used to demonstrate Xslu in conjugates so that the number of expressing conjugates can be determined.

Embryos and explants
Xenopus embryos were obtained as described previously (Mayor et al., 1993) and staged according to Nieuwkoop and Faber (1967). Animal caps, dissected at stage 8 or stage 10.5, were cultured in 1x NAM (Slack, 1984) supplemented with FGF or noggin protein, as indicated, until the equivalent of stage 17.

Preparation of fate map of stage 10+ embryos
Dots of Nile Blue (one per embryo) were made on the surface of stage 10+ Xenopus embryos by applying the dye for a few seconds, using a glass microneedle, under an dissecting microscope in 0.8 NAM and washed with a micromanipulator. In most cases, the vitelline membranes were still present but a similar result was obtained using demembranated embryos. Embryos were orientated in a well to apply the dye. The positions of these dots were recorded, using a camera lucida, and plotted as described by Keller (1975) and their subsequent fate was determined by examining embryos at stages between 12 and 25.

To mark the prospective neural plate border for specification experiments, the animal caps of the embryos were stained to include the prospective folds only. This was done using a grid of precisely machined holes, which were filled with Nile Blue. Stage 10 embryos were inverted into these wells so that their caps were stained. The size of the holes used were chosen so that about 1/3 of the embryo was immersed in the dye, to cover the prospective neural folds. The dorsal half of these caps was dissected from embryos between stage 10 and 12, for the specification experiments described below. Embryos were microinjected as described previously (Mayor et al., 1993). Nile Blue, in Danilichks buffer (Keller et al., 1985) (5-10 nl), was injected into the blastocoel of embryos in 5% Ficoll in 0.75 NAM.

Ultraviolet irradiation
Fertilised eggs to be irradiated were dejellied immediately after rotation and were exposed to 254 nm ultraviolet light in a silica cell for 2-3 minutes at 40 minutes after fertilisation (Youn and Malacinski, 1981). At stage 10, embryos were sorted into fast and slow developing types. As noted by Cooke (1985), the latter class is highly enriched with embryos with reduced dorsoanterior development.

Expression constructs
Full-length capped transcripts of Xwnt-8 and noggin were prepared by standard methods or provided generously by Dr Vincent Cunliffe. pCSKA-Xwnt-8 and the noggin expression clones were the gift of Dr Richard Harland. Synthetic RNA was injected in 5-10 nl volumes as described previously (Mayor et al., 1993). noggin protein was obtained by injecting oocytes with a plasmid (500 pg per oocyte) that transcribes noggin from the TFIIIA promoter (base pairs 40-355, Pfaff et al., 1990). Oocytes were injected in the superficial layer of the NAM and were cultured at 18°C in 0.8x NAM containing 1 mg/ml of bovine serum albumin for 3 days after injection. The concentration of noggin was expressed in arbitrary units. NCAM was induced in stage 8 caps by 10 u/ml.

Nuclease protection studies
RNAse protection probes were made as described by Kreig and Melton, (1987). Solution hybridisation of five probes to the same sample (at 50°C, for 16 hours in 50% formamide and 0.23 M NaCl) was performed to minimise differences in recovery of the mRNA. The probes used were Xsln bp 189-469, Xsna bp 138-369, NCAM bp 523-
RESULTS

Isolation of Xslu, a cDNA related to Xsna that is expressed specifically in premigratory neural crest

A stage 17 Xenopus cDNA library of Kintner and Melton (1987) was screened using a chicken Slug cDNA as a probe (Nieto et al., 1994). Isolates were screened for sequences that differed from Xsna, by a PCR sequencing protocol using DNA from purified lambda clones and a primer within the zinc finger region. A novel sequence was found in a number of separate isolates. The deduced amino acid sequence of this protein (267 amino-acids) has high similarity to Xsna over the first 40 amino-acids (90%) and the last 123 aminoacids (94%) which contain five zinc fingers (Fig. 1). The long 3′ untranslated sequence, which has no similarity to that of Xsna, has been used as an in situ hybridisation probe and the middle of the coding sequence has been used to prepare RNAse protection probes. Both Xsna and Xslu are significantly more similar to escargot, a recently discovered family member from Drosophila (Whitely et al., 1992) (79 and 80% respectively) in the zinc finger region than to Drosophila snail (66 and 69% respectively) but there are no significant similarities between the non-finger regions of the vertebrate and the invertebrate members of the family. The amino-acid sequence of Slug in Xenopus and chicken is very well conserved (92% identical) compared to conservation of snail between the two species (data not shown).

Whole-mount in situ hybridisation showed Xslu was first expressed on the lateral aspect of stage 12 embryos (Fig. 2A), in a region in which ectodermal Xsna was expressed (Essex et al., 1993; Mayor et al., 1993). RNAse protection assays of Xslu indicated that there was very little, or no mRNA for Xslu present before stage 11.5 (data not shown). At stage 14 (Fig. 2B), the lateral neural folds were strongly labelled and the transverse fold unlabelled while the trunk folds were distinctly but weakly labelled. The less strongly labelled superficial ectoderm contribution to the cranial folds was seen medial to the deep layer (Fig. 2B) as we noted previously with Xsna expression (Essex et al., 1993). By stage 16 (Fig. 2C), Xslu expression was clearly organised into the characteristic premigratory aggregates reported by Sadaghiani and Thiebaud (1987). At stage 18 (Fig. 2D), the neural tube was closed in the trunk and the cranial crest was in condensed masses. Xslu-expressing cells were dispersed over the neural plate in a pattern surrounding the rhombomeres. At stage 22 (Fig. 2E), neural crest derivatives such as the branchial arches and the tissue surrounding the eyes and forebrain expressed Xslu. Further to the posterior, migrating cells expressing Xslu demarcated rhombomeres. After stage 17, there was weak expression in lateral plate mesoderm which increased at stage 26 but was down-regulated in the pronephros region (Fig. 2F) at stage 26.

Expression of Xslu in the deep and superficial layer of the ectoderm was seen in transverse sections through the cephalic crest of stage 18 embryos (Fig. 2G). In the trunk, Xslu expression was in the deep layer of the ectoderm, on the top of the neural folds (Fig. 2H). A parasagittal section of a stage 20 embryo shows Xslu in three premigratory masses in the deep layer of the ectoderm (Fig. 2I). No mesodermal staining was evident in sections.

The origin of the prospective neural fold, relationships with underlying tissues and expression of Xslu in dorsal marginal zone explants

The neural plate border originates from a low arc of tissue just above the marginal zone of early gastrulae, which develops into the characteristically keyhole shaped neural folds as a result of convergence-extension movements within the neural plate. We have determined from where the neural plate border is derived in a stage 10.25 embryo, using dye marking (Fig. 3A). This information facilitates dissection of ectodermal regions that will always contain prospective neural folds and which can be cultured and tested for their capacity to express neural fold markers. The prospective folds at stage 10.25 lie on an arc on the dorsal marginal zone, midway between that described by Keller (1975) and Suzuki and Harada (1988). The angle subtended by the dorsal blastopore lip and the point on the dorsal midline that passes through the neural plate border was about 70-90°.

As the prospective neural plate border was derived from a narrow strip of the dorsal marginal zone, it was of interest to know if the prospective folds were underlain by tissue at this time and if this tissue was the inducer. The first question was investigated by injecting the blastocoel with Nile Blue at various stages between stage 8 and stage 11 and determining whether stage 16 folds were stained. The prospective neural folds were stained blue by the contents of the blastocoel until stage 10.5, but after stage 10.5 the neural folds were unstained. This indicates that after stage 10.5 the prospective folds were underlain with tissue.

To determine whether this underlying tissue was the inducer, we dissected the marginal zone (including tissue underlying the ectoderm) into equal-sized upper and lower strips as shown in Fig. 3B at stage 10.25. These explants were cultured until stage 13 and were examined by in situ hybridisation for Xslu. The upper dorsal marginal zone contains that part of the ectoderm that can form the neural folds and express Xslu (Fig. 3C), and appears almost the same as that found in entire dorsal marginal
zone explants (Fig. 3E). The lower part (principally the involuting marginal zone) did not express Xslu (Fig. 3D) and neither did the remainder of the embryo (Fig. 3F).

**Time of commitment to expression of Xsna and Xslu**

We next investigated when ectodermal Xsna and Xslu could be expressed autonomously, by dissecting explants containing the prospective neural folds at stage 10-12 and culturing them until stage 16 and scoring the appearance of Xsna and Xslu using in situ hybridisation (Fig. 4). To mark the prospective neural plate border, the animal caps of the embryos were stained with Nile Blue so that the same region was used at each time point. This was done by staining the top 1/3 of each embryo, as described above, at stage 10. At each time point, the labelled cap was dissected from the embryo, freed of mesoderm and the dorsal half was used for the specification experiments. Ectoderm could only be cleanly separated from mesoderm up to stage 12. Explants prepared at stage 10 and 11 were unable to express Xsna when cultured to the equivalent of stage 16, whereas explants prepared at stage 12 did express Xsna (Fig. 4A-C). However, this level of expression was evidently established at stage 12 because, if these explants were fixed at stage 12, the level of Xsna expression was the same (Fig. 4D); this indicates that further contact with the inducer is required to raise the level of expression to that seen later in development. Similar experiments were also carried out for Xslu expression. Prospective folds dissected at stages 10, 11 and 12, cultured to stage 16 did not express Xslu (Fig. 4E-G). However, prospective folds, dissected at stage 12 with a small amount of the adjoining section of mesoderm, expressed Xslu strongly at stage 16 (Fig. 4H). This suggests that, although the prospective crest ectoderm is in contact with its inducer from stage 12, only the lower part (principally the involuting marginal zone) expresses Xslu.

**Fig. 2. Expression pattern of Xslu in *Xenopus* embryos obtained using whole mount in situ hybridisation. (A) Stage 12. Xslu on lateral aspect of embryo (c). (b) blastopore. (B) Stage 14, Xslu in prospective cranial neural crest (d, deep layer of ectoderm; s, superficial). Weak expression in trunk crest. (C) Stage 16, Xslu in cranial premigratory crest (Sadaghiani and Thiebaud, 1987). d, deep layer of ectoderm; s, superficial layer. Xslu expression extending over the neural plate. (D) Stage 18. Neural tube now closed with cranial crest masses condensed (m, h and b, mandibular, hyoid and branchial aggregates. Xslu expression over neural plate demarcating rhombomeres. t, trunk neural crest. (E) Stage 22, Xslu in trunk crest (t), branchial arches and, in head surrounding the eyes (e) and forebrain, Xslu seems to demarcate rhombomeres. (F) Stage 25, Xslu in branchial arches (m, mandibular; h, hyoid and b, branchial), in lateral plate mesoderm (lp) with pronephros not expressing (p). (G) Transverse section through cephalic crest of stage 18 embryo. Xslu expression in deep layer (d) and superficial layer (s). np, neural plate; n, notochord; so, somites; a, archenteron. (H) Transverse section through trunk of stage 18 embryo. Xslu expression on top of neural folds in deep layer (d); nt, neural tube. (I) Parasagittal section of stage 20 embryo showing Xslu in three premigratory masses (m, mandibular; h, hyoid and b, branchial).
10.5, the tissue is unable to express Xsna and Xslu autonomously for a surprisingly long time.

**Location of Xslu-inducing activity within the embryo at stage 10.5**

As Xslu is expressed only in a specific part of the ectoderm and not significantly in the mesoderm before stage 19, it is possible to devise a simple assay for induction of Xslu using a conjugate containing any tissue under test and the blastocoel roof of stage 10.25 embryos as the responding tissue. Conjugates prepared with these pieces were cultured to stage 17 in 0.75× NAM, by which time expression would have occurred in control

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**Fig. 3.** Fate map of stage 10.25 embryos and expression of Xslu in dorsal marginal zone explants. (A) Fate map of stage 10.25 embryos. Each spot represents dye marks applied to embryo at this stage. Fate of each spot is ●, neural folds; ○, epidermis; ⊙, eye lens; x, neural plate. a, V, D and b = Animal pole, vegetal pole, dorsal and blastopore respectively. (114 embryos analysed). (B) Dissection of UDMZ (A) and LDMZ (B). Both explants were cut as strips extending round 180° of the embryo as shown; m indicates height of the floor of the blastocoel. C-F were hybridised with Xslu (C) UDMZ (A) (D) LDMZ (B) (E) Complete DMZ (A)+ (B) (F) Remainder of embryo (C).
embryos. The conjugates were then fixed and examined by whole-mount in situ hybridisation using an Xslu antisense probe. The responding tissue alone (animal caps) (Fig. 5A) did not express Xslu but in a conjugate with a very small piece of dorsal marginal zone containing the organizer, Xslu expression was observed (Fig. 5B) while the organiser alone gave no Xslu expression (Fig. 5C). In a similar experiment, the mesoderm of the marginal zone of stage 10.5 embryos was dissected as one piece and then divided into four, the dorsal, ventral and two lateral pieces which were tested in conjugates. Dorsal and lateral mesoderm fragments induced Xslu (Fig. 5D,E) while ventral mesoderm gave a low response (Fig. 5F). The appearance of the conjugates at stage 17 indicates that the dorsal and ventral regions were correctly dissected as, conjugates containing dorsal and lateral explants (Fig. 5D,E) elongated while the ventral explants did not (Fig. 5F). One of the ventral explant conjugates expressed Xslu (Fig. 5F arrow) (32 analyzed), but was evidently incorrectly dissected as it had elongated. These experiments therefore indicate that the capacity to induce Xslu resides in dorsal and lateral mesoderm but not ventral mesoderm.

**Effect of noggin and Xwnt-8 mRNA on Xslu expression**

noggin and Xwnt-8 have reciprocal expression patterns in mesoderm at stage 10, with noggin (Smith and Harland, 1992) in the dorsal quadrant and Xwnt-8 (Christian and Moon, 1993) in the remainder. As the earliest expression of ectodermal Xsna seemed to overlie the junction of these two domains (Essex et al., 1993), we decided to examine the effect of overexpression...
of synthetic RNA of these two genes on the expression of *Xslu* using whole-mount in situ hybridisation, as this marker is specific for prospective neural crest. Embryos obtained from eggs injected in the animal cap with *noggin* RNA, gastrulated slightly abnormally but with no indication of a double axis. At stage 13, we observed *Xslu* expressed in a continuous band around the ventral side of the embryo but not on the developing neural plate (Fig. 6A). At the equivalent of stage 17, this pattern was evidently composed of three parallel bands (Fig. 6B). Injection of large amounts of other mRNAs (such as β-galactosidase and other sequences, data not shown) have no effect on the pattern observed. These bands appear to correspond to the three aggregates of cranial crest (mandibular, hyoid and branchial), which are extended in a ring around the ventral side of the embryo. We reasoned that this pattern indicates that injected *noggin* interacts with a signal on the ventral side and jointly causes ectopic *Xslu* expression on the ventral side while in the neural plate this effect was prohibited. As it was interesting to know if *noggin* was acting after the start of zygotic transcription, we injected embryos with a plasmid construct containing the *Xenopus borealis* actin promoter to drive overexpression of *noggin* starting at stage 10 (Christian and Moon, 1993). This created a pattern of expression of *Xslu* at stage 16 (Fig. 6C) that in the best cases was similar to that obtained with mRNA injections with a band circling the ventral side of the embryo but not on the neural plate. This indicates that the interaction between the ventral signal and *noggin* occurs after zygotic transcription starts.

In contrast, *Xwnt*-8 injected embryos did not express *Xslu* significantly although there were a very small number of embryos with weak expression in the normal position (Fig. 6D). When *Xwnt*-8 and *noggin* RNA were injected together, *Xwnt*-8 suppressed almost all *Xslu* expression (data not shown). The effect of *Xwnt*-8 expression after the start of zygotic transcription using the *Xenopus borealis* actin promoter has not been investigated.

**Xslu can be induced by a combination of noggin and bFGF in naive ectoderm**

The hypothesis, that *Xslu* may be induced directly or indirectly by a pair of factors derived from the dorsal and ventral side of the embryo, was investigated by examining the effect of noggin and bFGF proteins on expression of *Xslu* and markers of neural tissue (NCAM) and mesoderm (*Xbra*) on a cultured explant of naive ectoderm using RNase protection assays (Fig. 7). Animal caps from stage 8 and stage 10.5 embryos were cultured in NAM containing these factors at various concentrations and in combination. *Xslu* expression was induced in gastrula animal caps by a combination of noggin and FGF.
protein (10 u/ml) at modest concentrations (Fig. 7B track i) while the factors alone at the same concentration do not induce Xstu (Fig. 7A and B, c and f).

The presence of FGF with noggin also prevents the induction of NCAM that normally occurs when noggin is administered alone (Fig. 7A and B, a-c) (Lamb et al., 1993). Although Xstu is entirely ectodermal in the embryo, the conditions that induce Xstu also induce the mesodermal marker Xbra in blastula and gastrula caps. Stage 10.5 caps have greatly reduced competence for axial mesoderm formation (Green et al., 1990) and, indeed, we have not observed shape changes in the explants. In blastula animal caps, the pattern of expression of the four markers, with respect to factor concentration, is more complex. At the lower FGF concentrations (Fig. 7A i), Xstu and Xbra are not induced, while Xsna is and, at the highest concentrations of noggin used (Fig. 7A g), NCAM and Xstu are both found in the explants. We have also examined the effect of FGF concentration on Xstu induction in stage 10.5 caps in the presence of a constant amount of noggin (data not shown). Xstu and Xbra are both induced while NCAM is not expressed at 3 u/ml of FGF but at 1 u/ml and below, NCAM is expressed and Xstu and Xbra are not expressed. We are therefore unable to dissociate induction of the ectodermal Xstu from induction of the Xbra-expressing mesoderm.

Xsna, which is expressed in both mesoderm and ectoderm (Sargent and Bennett, 1990), is induced by FGF alone but not by noggin alone. In blastulae, it is induced at a lower concentration of FGF than Xbra.

noggin and FGF administered alone at very high concentrations can induce Xstu in gastrula caps. This probably indicates

Fig. 8. Effect of UV irradiation on Xstu and Xsna. (A) Effect of UV on Xstu distribution at stage 12. Embryos are viewed from dorsal side. (a) Control; (b) regions of cephalic crest slightly closer; (c) cephalic crest regions joined; (d) cephalic fold fused in single dorsal spot; (e) no expression perfectly radially symmetrical embryo. (B) Effect of UV on Xsna distribution at stage 12.5. Note presence of labelled mesoderm underlying ectodermal pattern. Embryos are viewed from dorsal side, arrows indicate ectodermal expression. (a) Control; (b) Xsna in cephalic crest with slightly reduced transverse fold; (c) transverse fold diminished further; (d) cephalic fold fused in single dorsal spot; (e) no expression perfectly radially symmetrical embryo. c, cranial crest; m, mesoderm; y, yolk. (C) Effect of UV on NCAM distribution at stage 17 in a population of selected slow developing embryos. N indicates a normal control. All of this group have shortened axes at the equivalent stage. Most extreme NCAM patterns (x) are extremely short and extend around the blastopore.
that there is a low level of a co-inducer present in stage 10.5 ectoderm.

**Effect of ultraviolet treatment of fertilised eggs on Xsln and Xsna expression**

To investigate the relationship between induction of the neural plate border and the neural plate, we examined expression of *Xenopus* embryos with UV during the first cell cycle reduces the dorsal axis-forming capacity of the embryo producing a range of phenotypes from the extreme radially symmetric embryo ... of UV irradiation during the first cell cycle showed the range of phenotypes described by Malacinski et al. (1977). Using *Xslu* or *Xsna* as a probe, we identified a continuous spectrum of modified folds ranging from normal to almost radially symmetrical embryos with no ectodermal *Xslu* or *Xsna* expression (Fig. 8A,B). There was also mesodermal *Xsna* expression of *Xsna* that tends to obscure the ectodermal pattern (Essex et al., 1993). Between the extreme phenotypes, we found embryos in which the neural folds were not separated into lateral masses but were fused on the dorsal midline. Less affected specimens had the characteristic pair of cranial crest masses separated by varying distances. The frequency of this effect and related effects on NCAM expression in slow developing embryos was enriched for embryos with increased radial symmetry. A population of 90 slow developing embryos was divided into two groups and stained with DIG in situ hybridisation probes for *Xslu* at stage 12.5 or NCAM at stage 17 (Fig. 8C). Almost all of the slow developing group had a shorter neural plate and in the most extreme cases was reduced to a small arc of expression above the blastopore (Fig. 8C) but some NCAM-staining material was always present on the dorsal side. The appearance of *Xslu*-stained embryos fell into three groups (a) those with no *Xslu* expression (35%), (b) those with fused neural folds (15%) and (c) those with apparently normal folds (50%). The most severely affected embryos have greatly reduced NCAM expression but none-the-less slightly dor-salised phenotypes but no *Xslu* expression.

**DISCUSSION**

**Markers of the prospective neural crest and the neural plate border**

The fate map reported above shows the neural plate border originates in a narrow arc above the marginal zone at stage 10+, that extends laterally to about 90° from the dorsal midline. *Xsna* is first expressed at stage 11 in an arc slightly higher than the stage 10+ prospective crest which also extends about 90° from the dorsal midline (Mayor et al., 1993; Essex et al., 1993). *Xsna* expression on the midline of this arc is weaker than in the lateral parts and is down regulated before stage 12.

**Induction of Xsna and Xslu by dorsal mesoderm**

The specificity of *Xsln* for prospective neural crest provides an assay for induction. The capacity to induce *Xslu* is present in dorsal and lateral but not ventral mesoderm, at stage 10.5 when the prospective crest becomes underlain by mesoderm although ectodermal *Xsna* and *Xslu* are not expressed until stage 11 and 12 respectively. Even a very small piece of dorsal
mesoderm (less than 60° arc) has the capacity to induce Xslu and ectodermal Xsna (Mayor and Sargent, 1993). The mesoderm present in the upper dorsal marginal zone at stage 10.25 is sufficient to induce the overlying ectoderm in explants to produce a neural fold pattern comparable to intact embryos. Although these explants are able to express Xslu, the ectodermal component is unable to express Xslu or ectodermal Xsna autonomously until after stage 12.

Xtwi (another marker of the neural plate border; Essex et al., 1993) is induced in early gastrula ectoderm by gastrula dorsal mesoderm (Hopwood et al., 1989). In apparent contradiction of our results, induction of neural-crest-derived melanophores has been demonstrated in microconjugates of ventral ectodermal cells and stage 10.5 lateral (but not dorsal) marginal zone mesoderm of Xenopus embryos (Mittani and Okamoto, 1991). However, the experimental situation may not be comparable as the components of the conjugates were much smaller than ours, were cultured for longer and may have autoneuralised to the extent of providing the dorsal inducer postulated here (Godsave and Slack, 1991).

A model for induction of the neural plate border

The three banded pattern of Xslu expression around the posterior, produced after injection of noggin mRNA suggested that (1) there is probably a factor present throughout the ventral side of the embryo that can interact with ectopically expressed noggin to induce Xslu and (2) that the absence of expression on the neural plate indicates this interaction is prohibited on the neural plate. These observations suggested to us that induction of Xslu requires two elements, one that originates from the dorsal side and another from the ventral side. The dorsal signal could be the secreted protein noggin which has been demonstrated in microconjugates of ventral ectodermal cells and stage 10.5 lateral (but not dorsal) marginal zone mesoderm of Xenopus embryos (Mittani and Okamoto, 1991). However, the experimental situation may not be comparable as the components of the conjugates were much smaller than ours, were cultured for longer and may have autoneuralised to the extent of providing the dorsal inducer postulated here (Godsave and Slack, 1991).

Independent evidence that two factors jointly induce prospective crest comes from experiments in which we show that soluble noggin protein and bFGF administered to blastula or gastrula blastocoel roof induces Xslu expression while the separate factors have no effect. The two factors induce Xslu but not NCAM, seemingly reflecting the clear, binary differentiation seen in vivo between prospective neural crest and neural plate (Essex et al., 1993). As Xbga is also expressed in these explants, they must also contain mesoderm which may be the inducer of Xslu. Competence for axial mesoderm formation in blastocoel roof is lost by stage 10.5 (Green et al., 1992) and we have not observed shape changes in these explants that might indicate that axial mesoderm is present. Using a microculture system, melanophores were induced in animal caps treated with soluble FGF alone from stage 9 (but not at 8), attaining peak competence autonomously at stage 10.5 (Kengaku and Okamoto, 1993). As suggested above, unknown aspects of microculture may conceal the role of the dorsal inducer. The capacity of FGF as a co-inducer of Xslu in vitro has not been demonstrated in vivo. Furthermore, the effect of the FGF receptor dominant negative interference construct suggests that FGF is not required for expression of branchial arches or melanophores (Amaya et al., 1991) and may therefore be acting indirectly on an unknown ventral inducer.

The presence of noggin (Smith and Harland, 1992) and FGF (Isacs et al., 1992) throughout the dorsal region and the lack of expression of ectodermal Xsna and Xslu in the neural plate suggests a prohibition for expression within the neural plate. Independent support for this has been adduced from studies in which the overexpression of certain neural plate genes (a dominant negative form of XOTCH, Coffman et al., 1993; or Xash-3, Turner and Weintraub, 1994) leads to the suppression of the prospective neural crest. This suggests a refinement of the model by which we propose that one signal is ubiquitous but is unable to interact with noggin in the prospective neural plate region because of a prohibitory signal (Fig. 9C).

The effect on dorsal development of precleavage irradiation of fertilised eggs, which tends to prevent development of the notochord and neural plate (Malacinski, et al., 1977; Youn and Malacinski, 1981) can be explained in terms of the absence of the prohibitory signal. After moderate irradiation, a proportion of the embryos have cranial crest domains that are fused on the anterior/posterior orientation but which are now extended to the dorsal side and another from the ventral side. These observations suggested to us that induction of Xslu requires two elements, one that originates from the dorsal side and another from the ventral side. The dorsal signal could be the secreted protein noggin which has been demonstrated in microconjugates of ventral ectodermal cells and stage 10.5 lateral (but not dorsal) marginal zone mesoderm of Xenopus embryos (Mittani and Okamoto, 1991). However, the experimental situation may not be comparable as the components of the conjugates were much smaller than ours, were cultured for longer and may have autoneuralised to the extent of providing the dorsal inducer postulated here (Godsave and Slack, 1991).

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