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Chick *noggin* is expressed in the organizer and neural plate during axial development, but offers no evidence of involvement in primary axis formation

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ABSTRACT We have cloned and examined the early developmental expression of the chick homolog of *noggin*, a gene originally isolated in *Xenopus* that can dorsalize gastrular mesoderm and induce anterior neural tissue from gastrular ectoderm when expressed experimentally. Chick *noggin* is expressed at relatively low levels, but at sites equivalent to those seen in amphibian development, namely Hensen's node and the endo- and mesodermal head process. There is also diffuse expression in the early CNS, centered on the ventral midline, and later hindbrain-associated expression. Since the earlier of these expression sites are consistent with endogenous organizer functions suggested by the properties of the protein in *Xenopus* experiments, we have used recombinant mammalian Noggin protein secreted by CHO cells in tests for developmental disturbance on the early gastrula-staged chick blastoderm. Comparable tests sensitively detect effects, on chick, of various other secreted proteins that simulate or replicate early developmental signals in *Xenopus*. We have been unable to observe such effects with a range of Noggin concentrations including those that dramatically dorsalize *Xenopus* ventral marginal zones. To illustrate effects observed in such tests with secreted proteins active on early stages, we show results with the known *Xenopus* ventralizer Bone Morphogenetic Protein 4 (BMP-4).

KEY WORDS: *noggin, chick, dorsalization, neural induction, BMP-4*

Introduction

Noggin, a gene cloned recently in *Xenopus* and mammals (Smith and Harland, 1992; Lamb *et al.*, 1993; Smith *et al.*, 1993), encodes a secreted protein of a novel family. The original expression cloning strategy used was designed to select *Xenopus* RNAs, expressed during early development, that cause blastomeres into which they are injected to develop with properties of the 'organizer'. The natural organizer of the amphibian embryo exists first as a signaling centre in dorsal vegetal material (Nieuwkoop, 1969; Gimlich, 1985), and then as the dorsal anterior mesoderm (dorsal blastoporal lip) induced by proximity to that center (Spemann, 1938). This latter region in turn emits dorsalizing and neuralizing signals into its mesodermal and ectodermal surroundings during gastrulation (Cooke and Gimlich, 1983; Smith and Slack 1983). Since Noggin protein is secreted, and the protein or RNA is able to simulate the two major signaling functions expected of the organizer in appropriate *Xenopus* functional assays, it is a candidate for an intercellular signal functioning in this way *in vivo*. It can dorsalize ventral mesoderm during gastrula stages (Smith *et al.*, 1993), and induce neural tissue directly in ectoderm at similar stages (Lamb *et al.*, 1993), though the protein concentration reported to be required for the latter function is perhaps higher than might be expected for a naturally functioning ligand. Recombinant mammalian Noggin protein is active in these *Xenopus* assays, suggesting strong conservation of steric specificity as has been observed for various early developmental signals.

At the time of discovery of the organizer properties of the amphibian dorsal lip (Spemann and Mangold, 1924), Spemann was inclined to believe that the same signals might be responsible both for organizing pattern within mesoderm and for inducing the neural rudiment in adjacent or overlying ectoderm (see Hamburger, 1988). But his students and other workers have mainly propagated the contrary view that mesodermal patterning and neural induction, while overlapping in time, are quite separate processes. It has recently been realized again that this need not be so. At least the initial signals that locate and pattern the nervous system may spread while its presumptive territory is

Abbreviations used in this paper: BMP-4, bone morphogenetic protein 4.

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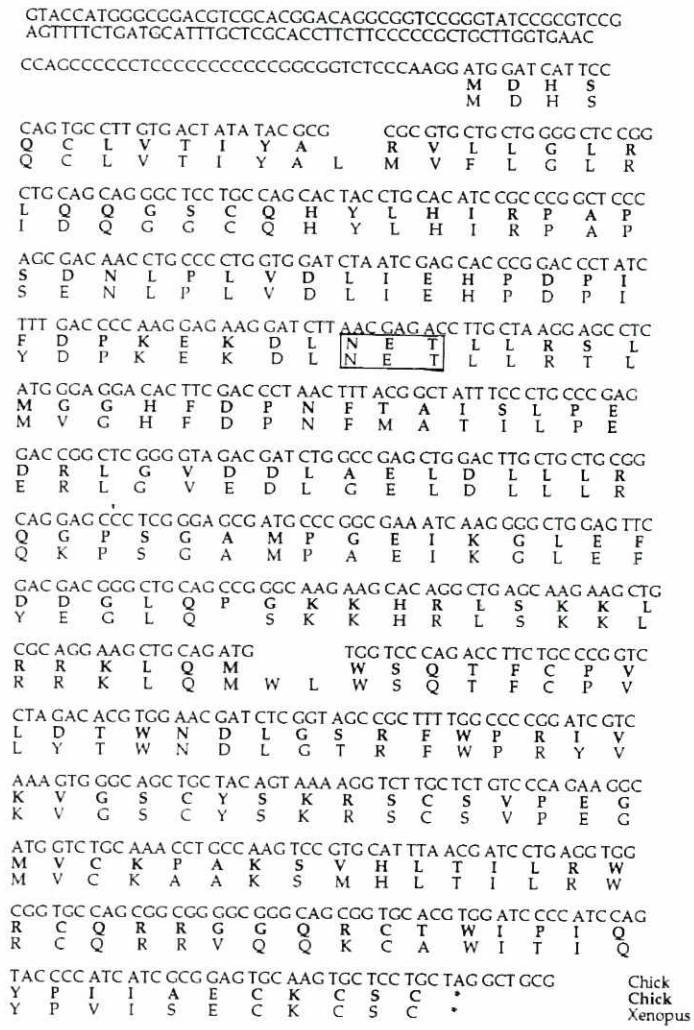


Fig. 1. The sequence of chick noggin. *DNA sequence (top) and optimal alignment of chick (middle) and Xenopus (bottom) amino acid sequences. Boxed amino acids indicate possible glycosylation sites.*

within the same cell layer as the organizer, i.e. before gastrulation (Ruiz i Altaba, 1990; Doniach *et al.*, 1992; Keller *et al.*, 1992). Also, by onset of gastrulation, most of the future ectoderm has already been left aside as a distinct cell lineage from mesoderm, so that one signal coming from the axial mesoderm could in principle be involved both in specifying the future differentiations of surrounding mesoderm *and* in neuralizing dorsal ectoderm. *Noggin* has thus been a candidate gene for a major signal emanating from the organizer (Harland, 1994). In addition to the need for such function ultimately to be demonstrated in a phenotype of homozygous mutant animals, presumably mice or zebrafish, great evolutionary interest attaches to the degree of conservation in expression and function for such developmental genes across vertebrate types.

Here we describe the cloning of chick *noggin* and its expression pattern during gastrulation and early axial development (up to 15 somites), as seen by *in situ* hybridization using digoxygenin-labeled riboprobes. While showing some common features with what is described for *Xenopus* (Smith and Harland, 1992), including dynamic expression within the mesodermal organizer, this chick pattern suggests at least some quantitative differences in deployment of the gene among different vertebrates. *Noggin* is also known to have specific expressions in later vertebrate developmental stages, and since these might correspond with functions essential for normal phenotype, the gene could be functionally redundant with respect to others in the earliest parts of its expression pattern (Brookfield, 1992).

We have used recombinant mammalian Noggin protein, expressed by transfected cells and active in the *Xenopus* gastrula-stage assays, in two different tests for signaling functions at equivalent stages of bird development. The chick blastoderm, incubated off its vitelline membrane in simple media at stages before node regression, is equivalent to the ligand-receptive *Xenopus* blastular and gastrular animal cap and marginal zone (Cooke and Smith, 1989). It is highly sensitive to several proteins that are, or that mimic, early developmental signals in the *Xenopus* system (Cooke and Wong, 1991; Cooke *et al.*, 1994; Streit *et al.*, 1995; Connolly *et al.*, in preparation). When replaced on membranes and cultured onwards with the ring culture method (New, 1955), blastoderms pre-incubated with such active proteins exhibit systematic perturbation or even obliteration of axial development, in ways that may be equivalent to results of global ectopic overexpression of the developmental signals. We have been unable to observe any such perturbations of body-pattern development by Noggin protein in two different tests. It could be that the relative degrees of involvement of a gene such as noggin, in early and then later developmental functions, differ among embryo types, and that to perturb the relevant developmental steps, abnormalities of expression for noggin and for one or more other genes might be simultaneously required. Our results are not relevant to possible noggin functions at later stages of refining the axial pattern.

Results

Cloning, sequence and structure

Using the *Xenopus noggin* gene, an avian homolog was isolated from a stage 11 chick embryo cDNA library. Sequence analysis reveals that this gene encodes a protein of 220 amino acids with a predicted Mr of 26.4 kDa (Fig. 1), which has 91% amino acid similarity with the *Xenopus* gene when conservative substitutions are taken into account. Like the *Xenopus* homolog, it has a signal peptide suggesting that it is a secreted protein, as well as a single potential site for N-linked glycosylation. The only significant difference is that the chicken gene has two fewer amino acid residues. Southern blot analysis of chicken genomic DNA failed to reveal the presence of additional noggin-related sequences (data not shown).

Examination of expression during embryogenesis by northern blotting revealed that, as in *Xenopus*, two major noggin transcripts are seen, but these are larger than their amphibian counterparts at 5.3 and 2.9 kb (data not shown).

Expression pattern

Figures 2 and 3 show representative appearances of early chick *noggin* expression in whole-mount preparations and in sections of such preparations. Much of the expression pattern is at intensities close to the threshold of *in situ* signal detection, even though probe

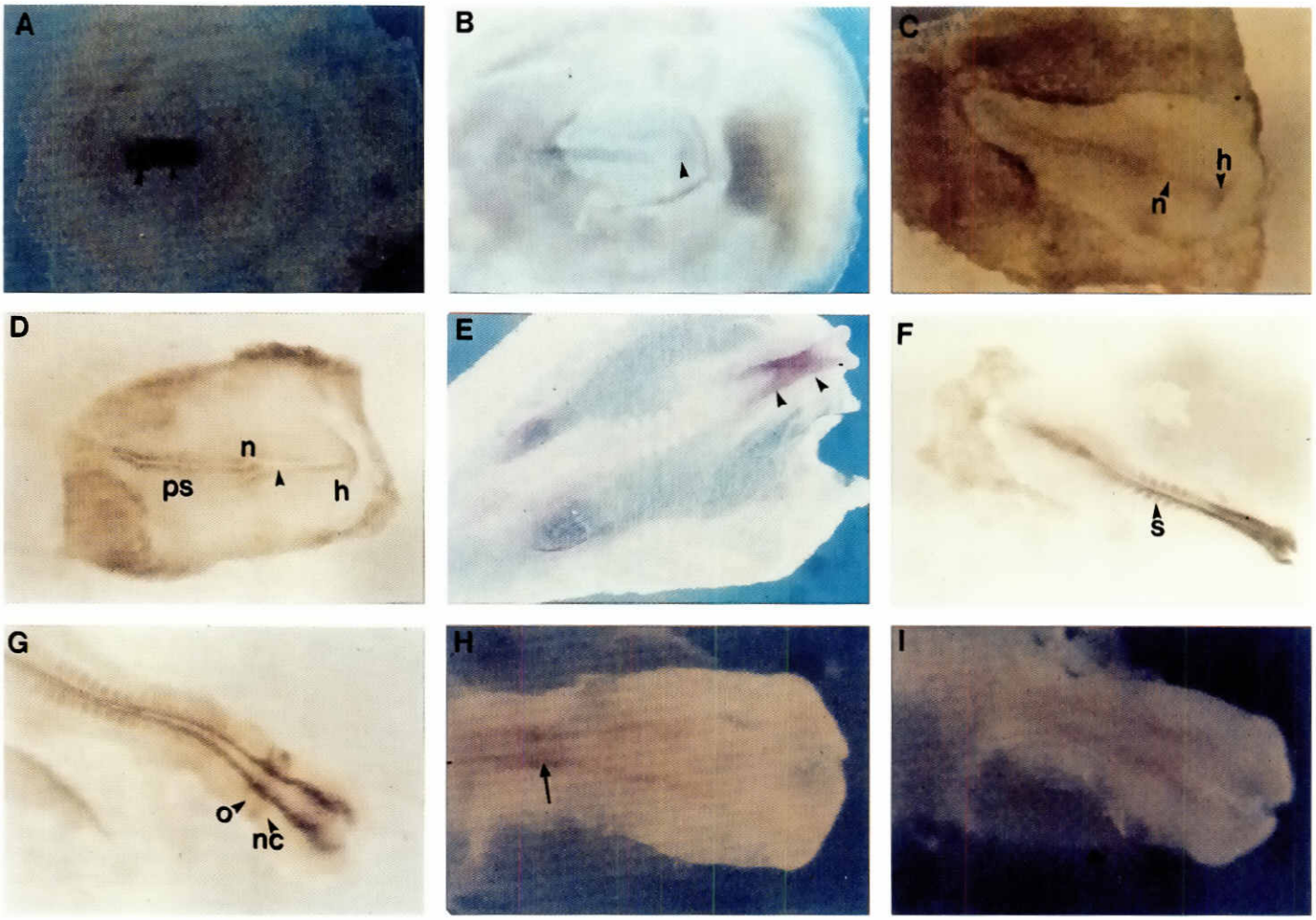


Fig. 2. Whole-mount *in situ* hybridizations to noggin mRNA. (A) *Stage 3. Expression in the half-length primitive streak (arrowheads).* **(B)** *Stage 5. Expression in the pre-chordal head process (arrowhead).* **(C)** *Stage 6. Expression in pre-chordal mesoderm (h) and notochord (n).* **(D)** *Stage 7. Expression continues in anterior head process and in node and streak flanks (ps), but is absent from most of the emerging notochord (arrowhead).* **(E)** *Stage 9. Neural expression concentrated in the mid-to-hindbrain region (arrowheads).* **(F)** *Stage 9-. Mesodermal expression in the dorsal tissue-layer of somites (s), as well as continuing in the streak remnant.* **(G and H)** *15+ somites. Hindbrain expression (arrow) maintained in neural crest outflow (nc) from rhombomeres in region of otic vesicle (o).* **(I)** *Sense control-probed 15 somite specimen.*

sizes and other conditions are comparable to those producing strong signals for a variety of other early expressed genes in this laboratory. In using the Digoxygenin *in situ* technique with such gene expression levels, among synchronously staged specimens going through the entire protocol side by side, some may show a particular part of the pattern while others appear negative. Thus we only consider as part of the expression pattern a positive appearance, frequently seen in a particular structure following antisense probe but never observed with the sense control probe. Even so, as seen in Figure 3 particularly, careful inspection of control-probed specimens from each processed batch is necessary to identify the regions of significant noggin RNA expression in early chick. Radiolabeled probe used on sections might improve this detection (though not necessarily so), but use of the DIG method on cryosections is not in our experience advantageous on detection levels, since our embedding method gives no obvious loss of signal relative to that seen in whole-mounts (see Materials and Methods).

Expression is first detected in the anterior part of the primitive streak during stage 3 (Hamburger and Hamilton, 1951) (Fig. 2A). During the full-length streak stage 4, upregulation occurs in the anterior of the node just before emergence of the first, fan-shaped part of the mesodermal head-process. This structure, and the immediately following anterior part of the notochord rudiment, are positive on their emergence from the regressing node (Figs. 2B,C, 3A-C). The fan-shaped part remains positive until stages with many somites segmented, by which time it has become the flattened prechordal mesoderm underlying the forebrain (Figs. 2C, 3H). It is impossible to see directly whether the gene is expressed in the very anteriormost midline mesoderm, the thin pseudo-epithelial layer underlying the anteriormost neural wall and referred to as prechordal plate (Seifert *et al.*, 1993). This seems probable however, since at stages 7-9 there is expression in the anteriormost midline roof of the foregut endoderm, where this is most closely associated with the prechordal mesoderm beneath the forebrain region (Fig. 3G). Prechordal plate is not clearly separable from this

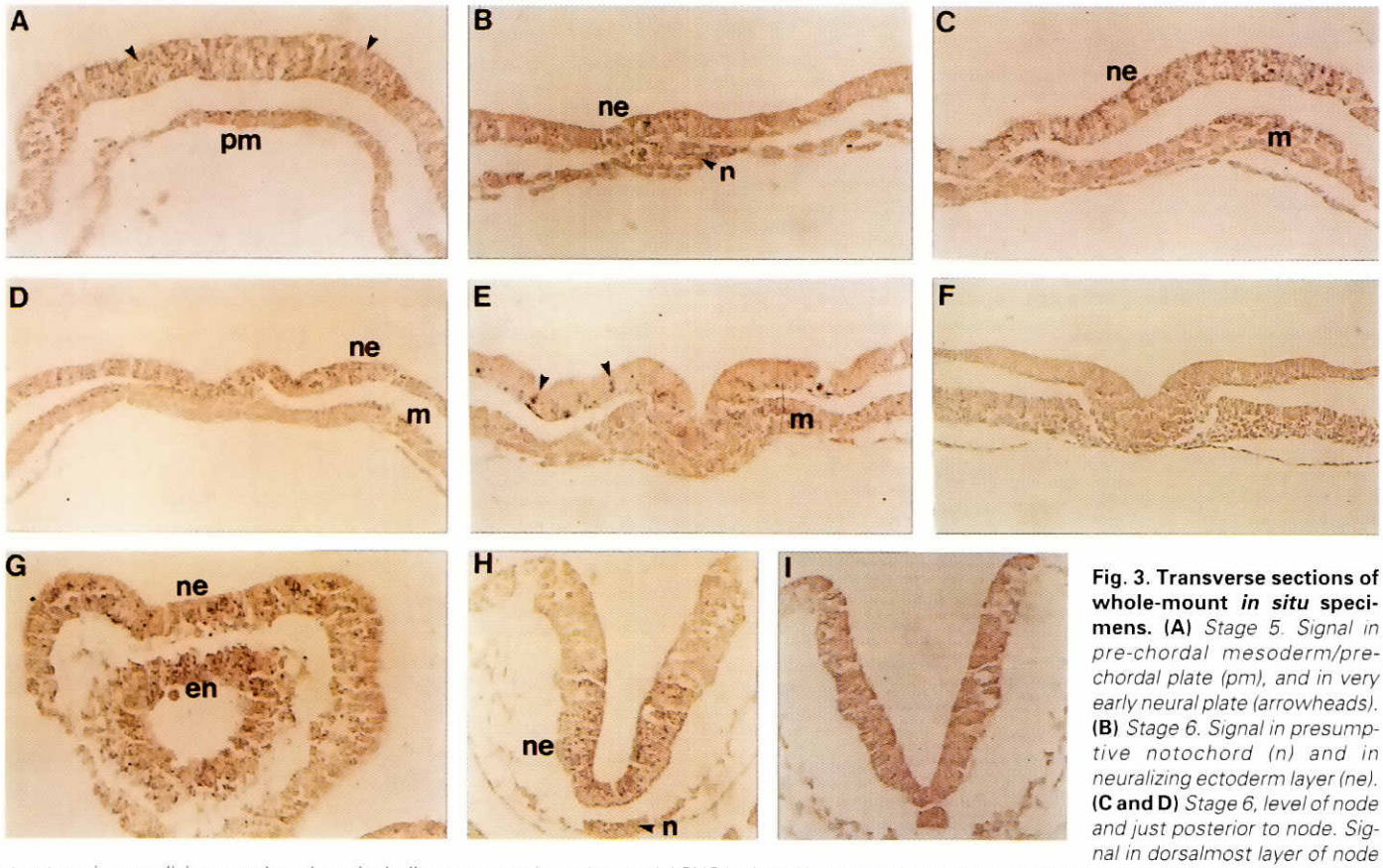


Fig. 3. Transverse sections of whole-mount *in situ* specimens. (A) *Stage 5. Signal in pre-chordal mesoderm/pre-chordal plate (pm), and in very early neural plate (arrowheads).* **(B)** *Stage 6. Signal in presumptive notochord (n) and in neuralizing ectoderm layer (ne).* **(C and D)** *Stage 6, level of node and just posterior to node. Signal in dorsalmost layer of node structure, in neuralizing ectoderm layer including presumptive spinocaudal CNS (ne), and in a layer of paraxial mesoderm (m) fated to form relatively dorsal parts of somite structure.* **(E)** *Stage 6 anterior streak. Diffuse signal in nascent mesoderm, and in epiblast that is probably presumptive spinocaudal CNS (arrowheads).* **(F)** *Sense control-probed specimen, stage 8+ streak region (cp. whole-mount streak expression in 2F).* **(G)** *Grazing section through anterior extreme of stage 7+. Expression in anterior neural fold (ne), and throughout foregut endoderm (en) but concentrated in dorsal midline area of junction with pre-chordal mesoderm (pre-chordal plate, Seifert et al., 1993).* **(H)** *Relatively anterior level, stage 7+. Signal in presumptive notochord and ventral half of neural cross-section.* **(I)** *Sense control - probed specimen; section comparable to* **(H)**.

endoderm during these stages. For some time during stages 6 and 7 around the time of segmentation of the first somite, notochordal cells after emergence from the node region no longer express the gene (Fig. 2D). Expression continues however in the dorsal portion of the node, including the epiblastic layer that is fated to form midline neural tissue (Fig. 3B-D). At all subsequent stages examined in this study, many specimens show an apparent caudo-rostral gradient of increasing notochord expression over levels of the anteriormost few somites and into the head, as well as the constant prechordal expression. The precise temporal pattern of expression in tissue at each level of the notochord is thus hard to resolve. Such tissue at anterior levels may emerge from the node expressing *noggin*, rapidly downregulate the gene, and later re-express at progressively lower levels according to its precise position. Alternatively, after the very anteriormost levels have emerged (around stage 6), subsequent notochordal tissue may only turn on various levels of expression for the first time, well after its emergence from the node.

A more diffuse expression remains along the streak itself, during stages of node regression. At headfold stages this takes the form of expression in the epiblastic ridges of the shortening streak, and includes the posteriorly extending, presumptive spinocaudal neuralized region (Figs. 2B-D, 3D,E). In the later embryo it continues in the thickened, gutter-shaped tissue mass, the remnant of the streak, that is producing both neural tube and segmental plate posteriorly (Fig. 2F). Sections of stage 7-8 headfold embryos consistently show longitudinal bands of light expression extending anteriorly from immediately post-nodal levels, in those cells of the paraxial mesoderm further from the notochord (Fig. 3C,D), that will later be in dorsalmost somite (Fig. 2F) .

Noggin expression in the neuralized area and neural plate itself is detectable from around stage 5. Initially this is diffuse and at low level (Fig. 3A,C,D), but in most headfold staged specimens becomes concentrated in the ventral part of the cross-section with a diffuse gradation into more dorsal parts (Fig. 3H). In somewhat more advanced embryos, expression begins to be concentrated in the hindbrain region (Fig. 2E), though diffuse expression spreads throughout the neural tube cross-section. Expression therefore spreads widely into neural territories other than the mid-ventral ones that would be direct descendants of the noggin-expressing cells of the superficial part of Hensen's node (Selleck and Stern, 1991).

In embryos of 15-19 somites, upregulation in the hindbrain region continues (Fig. 2G,H), with signal visible in the crest

outflows around the ear vesicle. A distinct patch of expression also appears in intermediate mesoderm adjacent to the coelom and medial to the cardinal vein (not shown). This is in the position of pronephric differentiation, so that expression of the gene in subsequent nephric development should be looked for.

Tests of noggin function

At stage 3, with its well-formed streak but before the start of node regression with its accompanying convergent extension in ingressed mesoderm and in neurectoderm (Schoenwolf and Yuan, 1995), the bird blastoderm may be taken as equivalent in organization to the *Xenopus* early gastrula before its equivalent movements. The epiblast is known to be highly competent to ectopic neural inducing grafts (Storey *et al.*, 1992), while certain regions at least of the mesoderm are competent to dorsalization, as in the formation of host-derived somite tissue next to such grafts (Hornbruch *et al.*, 1979). Such competences to grafts are also present in the *Xenopus* model, where additionally, Noggin protein is able to cause direct neural induction in ectoderm and dorsalization in mesoderm (Lamb *et al.*, 1993; Smith *et al.*, 1993). Noggin will not by itself induce mesoderm from ectoderm in *Xenopus*, an interaction that occurs earlier in normal development, and that is believed to occur before and during the first appearance of the primitive streak in the chick (Eyal-Giladi, 1984). Streak formation has occurred before the first detection of *noggin* in chick, at least by *in situ* hybridization. We therefore used the streak stages 3+ or 4- in assays for possible early developmental functions of the gene in mesoderm dorsalization and/or neural induction, rather than in mesoderm formation itself.

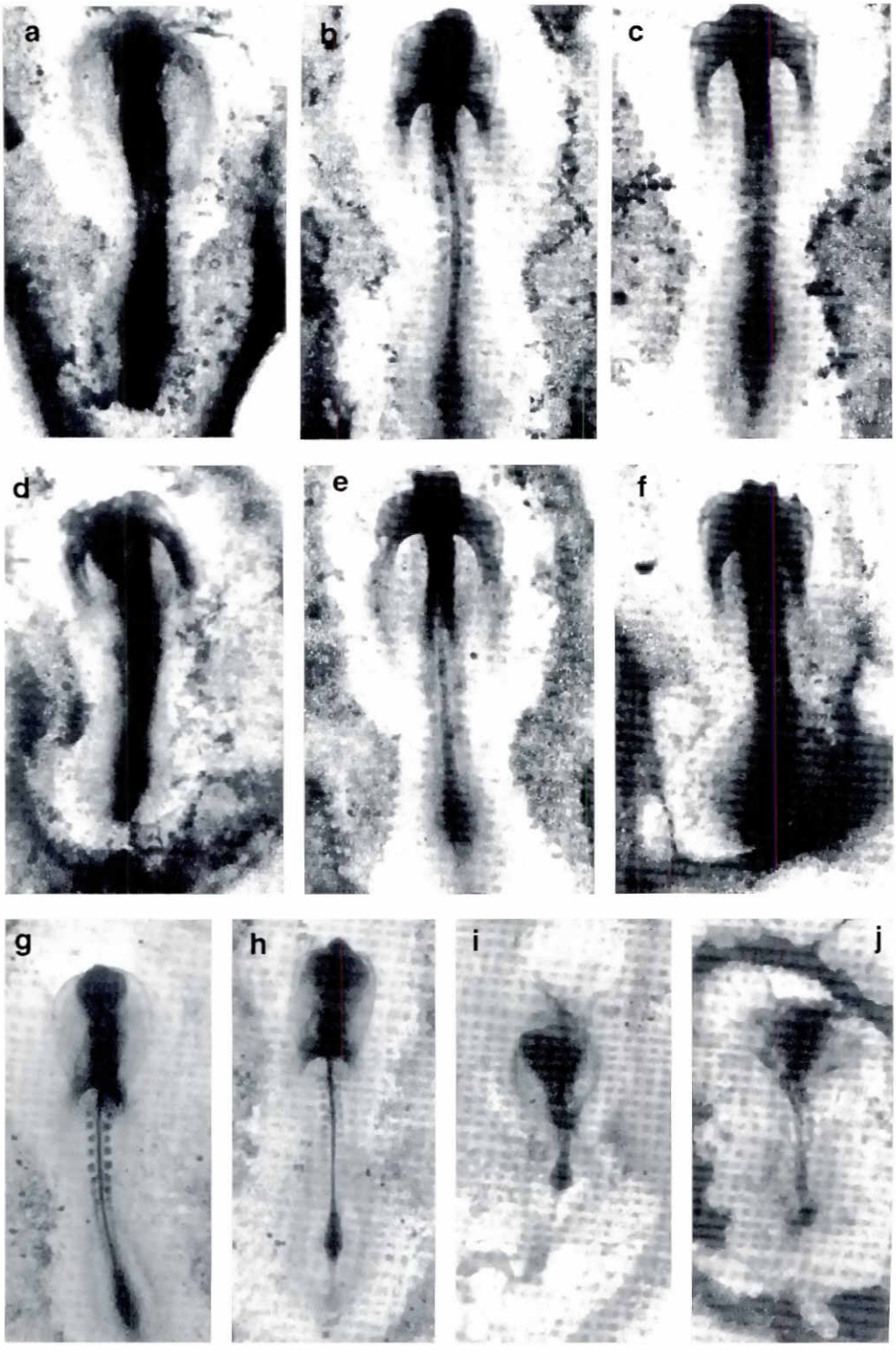
First we assayed the effect of widespread exposure of epiblast, and especially mesoderm, to the protein. Blastoderms were incubated in shallow dishes for 2 h, with either epiblast or hypoblast side uppermost, and with or without slitting of hypoblast in the streak region, in the presence of secreted mammalian Noggin protein. We used concentrations of the expressing CHO supernatant preparations from 1 to 10 times those which caused vigorous dorsalization of *Xenopus* ventral marginal zone explants in parallel assays. Control blastoderms were incubated with the equivalent addition of the cell culture supernatant concentrate from control CHO cells. Blastoderms were inspected after replacement on vitelline membranes in ring culture and further incubation for 18 h. By this time, control examples treated in this way during stage 3 have typically reached stage 8+ to 9 (5-9 somites). Such embryos are qualitatively normal examples of the body-pattern for their stage, though development has been delayed by some hours, and cross-sections (thus cell numbers) in nervous systems and somites are typically smaller than those of similar staged embryos from continuously incubated eggs. In five experiments involving over 30 control and 30 experimental blastoderms, we were unable to detect any effects upon the rate of development, form or size of any of these structures due to exposure to the Noggin supernatant. Figure 4 shows typical specimens.

Next, we asked whether incubation of excised stage 4 Hensen's nodes in Noggin supernatant could potentiate, or otherwise change, their capacity to organize second axial patterns when grafted into peripheral (presumptive extra-embryonic) positions near the *area opaca* of stage 3+ host blastoderms. In these experiments the hosts, in ring culture, had no direct exposure to free Noggin protein. Nodes grafted into such sites characteristically cause new axial patterns by a) inducing new neural plates in overlying epiblast and b) sustained convergent extension and self-differentiation into notochord and floorplate, with variable graft/host contributions to a set of somites after dorsalization of any contacting host-derived mesenchymal mesoderm (Storey *et al.*, 1992; Connolly *et al.*, in preparation). This operation is thus quite closely equivalent to the classical one of ventral implantation of a dorsal blastoporal lip graft in the amphibian gastrula. The rationale for the present test is that the graft, of essentially the entire gastrular 'organizer' region, normally expresses *noggin* and can cause neural induction and mesodermal dorsalization in its new (non *noggin* expressing) surroundings. The results of such grafts are individually variable, and this variability might plausibly be related to whether individual grafts sustain the auto-regulatory activations of crucial signaling genes, and/or retain sufficient of such secreted protein signals to be immediately effective after implantation as sources of the signals. Again we failed to find any effect of prior incubation in Noggin protein upon the organizing ability of such grafts, using a range of concentrations up to 25 times those causing strong responses in the *Xenopus* dorsalization assay. Follistatin, another protein with experimental dorsalizing/neuralizing effects in *Xenopus* work, has caused strong systematic effects in this assay (Connolly *et al.*, in preparation).

The normal range of chick axial patterns following Noggin treatment is borne out at histological level, where no readily detectable alteration in relative sizes of cell populations in the major pattern parts is seen (not shown). Figure 4G-J illustrates, for comparison, results of a typical experiment with Bone Morphogenetic Protein 4 (BMP-4), a strong candidate for an endogenous 'ventralizing' principle. The global disruption of axial morphogenesis caused is perhaps not the exact equivalent of what has been observed after global BMP-4 over-expression in *Xenopus* (Fainsod *et al.*, 1995; Schmidt *et al.*, 1995). It is nevertheless dramatic, and consistent with a form of ventralizing role for the protein in avian development. This will be described fully elsewhere.

Discussion

Previous experiments had revealed that the very early, blastular chick blastoderm is highly sensitive in culture to ligands of classes that simulate the earliest inductive steps in *Xenopus* development (Cooke and Wong, 1991; Cooke *et al.*, 1994). Access of these proteins can be *via* the basal as well as apical surfaces of epiblast, due to free access of medium to the early mesenchymal layers. Related work in this laboratory is revealing that the later, gastrular blastoderm, used in tests of the type reported here, is sensitive to other secreted proteins that *Xenopus* work has indicated to be involved in gastrular patterning. Of these we illustrate here, for comparison, an effect of BMP-4. In the case of Noggin protein, such tests for gastrular developmental function have shown nothing, across a range of levels of recombinant human protein extending up to many times those that dramatically affect *Xenopus*. This is worth recording, even though it lacks the decisiveness of the (gene targeting or antisense) *loss*-of-function test for the necessity of a gene for each developmental phase. We have also performed extensive antisense experiments, targeting *noggin* transcripts with phosphorothioated DNA oligos in the whole chick blastoderm with methods successful for at least two other genes in this laboratory (Nieto *et al.*, 1994; Isaac and Cooke, 1997). These experiments have been without effect, for the early axial developmental period



accessible to this potential interference method, a fact again worth recording despite the known, idiosyncratic differences among gene sequences in suitability for antisense targeting.

The distribution of *noggin* transcripts during chick early axial and neural stages is consistent with *in vivo* signaling roles that correspond with those postulated for *Xenopus*. Thus in 'dorsalization', somite determination would be induced and stabilised in tissue emerging from the anterior flanks of the streak, by proximity to Hensen's node (Selleck and Stern, 1992), while in primary neural induction, a forebrain state of specification would be induced in a region of epiblast around the node by planar signaling and reinforced by continued signal from the newly emerging head process (Dias and Schoenwolf, 1990). The continued transcription observed within the early CNS rudiment itself is particularly consistent with an early dorso-ventral patterning role there.

We might expect parts of gene transcription patterns corresponding to former functions to remain in organisms, even when co-option of other genes has made these expression components redundant or near-redundant in the course of evolution. This is because of the known small size of the crucial elements in the control regions of many genes, considered as targets for mutational change. For retention of each functional gene, it is simply required that *some* part of its total expression pattern is necessary for completely adequate development (see e.g., Brookfield, 1992). *Follistatin* provides a recent example of discrepancy between a gene's early expression pattern and experimental over-expression properties as investigated in one embryo (*Xenopus*, Hemmati-Brivanlou *et al.*, 1994), and its endogenous developmental role when tested by gene targeting in another vertebrate type

Fig. 4. Development in ring culture after blastoderm exposure to Noggin protein or to BMP-4. (a-f) *Specimens from one experiment, after 18 h of culture following exposure for 2 h at stage 3+ to 4- to control cell supernatant concentrate* **(a-c)**, *or to an equivalent dilution of Noggin-secreting cell supernatant concentrate* **(d-f)** *(see Materials and Methods). The range of developments, typical for this culture period after off-membrane incubation at the stated stages, is not detectably altered by exposure to this and other Noggin concentrations spanning the range that induces strong dorsalization of Xenopus ventral marginal zones.* **(g-j)** *Blastoderms from one experiment after 24 h of culture, following incubation for 2 h at stage 3+ to 4- in control medium with carrier BSA* **(g,h)** *or the same medium plus 50 ng/ml of BMP-4 protein* **(i,j)**. *Control embryos are typical for this stage of culture after the off-membrane procedure, while BMP-4 incubated ones show a global disruption of dorsal-axial development to be described in detail elsewhere (in preparation). Such concentrations of BMP-4 protein ventralize the character of mesoderm induction and can prevent neuralization in Xenopus gastrulae.*

(Matzuk *et al.*, 1995). Such discrepancies might imply that a gene product, while experimentally effective as a particular signal, is not involved and not even efficacious in the corresponding endogenous developmental step. But in addition, the relative importance of each gene for each embryologically defined step (e.g. dorsalization, neural induction) might vary between vertebrate types. Thus the definitive null mutant phenotype, from targeted mutagenesis in the mouse, might only inform us about each gene's first essential role in *mouse* development. *Noggin* could turn out to be vital for neural induction and/or dorsalization in *Xenopus* but dispensable or even irrelevant for these early steps in one or more other vertebrate types. Reports of the *noggin* null mutant phenotype, at the time of submitting the present paper, suggest that the gene is indeed dispensable for primary axial dorsalization and neural induction in mouse, though important for somewhat later processes (A. McMahon and R. Harland, personal communication).

Despite the above arguments relating to functional redundancy, apparent lack of phenotype for the equivalent of global *noggin over*-expression at early stages in chick is surprising, in view of its experimental properties in *Xenopus* (Lamb *et al.*, 1993; Smith *et al.*, 1993). The latter work suggests that Noggin protein, once freely secreted, is readily and simply available to responsive cells or to interacting ligands in extracellular space. It could be that dorsalization processes in amniote vertebrate development are more subtly regulated against perturbation than are those of amphibians.

Materials and Methods

Molecular biology

A 1.8 kb fragment corresponding to the entire *Xenopus noggin* coding region (gift of R. Harland) was labeled by random priming and used to screen 2.5×10^5 recombinant bacteriophage from a stage 11 chick embryo cDNA library constructed in Lambda Zap (Stratagene). Duplicate library lifts were hybridized with a probe concentration of 1×10^6 cpm/ml, and filters subsequently washed to a maximum stringency of 0.5xSSC, 0.1% SDS at 55°C. Six strongly duplicating positive clones were detected of which three survived isolation. Restriction mapping revealed these to represent the same gene, and the longest, containing a 2.1 kb insert, was sequenced using sequenase version 2.0 (US Biochemicals) according to manufacturer's instructions.

For Southern blot analysis, chicken genomic DNA was digested with EcoR1, BamH and Hind III, resolved on a 0.7% agarose gel and transferred to Hybond N according to manufacturer's instructions. Blots were probed with the entire coding region of the isolated chicken gene, at stringencies ranging from 4xSSC, 0.1% SDS at 50°C, to 0.1 SSC, 0.1% SDS at 65°C.

For Northern blotting, RNA was isolated from a range of freshly dissected embryos (HH stages 3+ to 12) using Triazol reagent (Gibco), and resolved on a 0.8% agarose/formaldehyde denaturing gel. After transfer to Hybond N= according to manufacturer's instructions, the blot was hybridized with a radiolabeled antisense probe comprising the entire coding region of the isolated chicken gene. Filters were examined at stringencies ranging from 0.5xSSC, 0.1% SDS to 0.1xSSC, 0.1% SDS at 80°C.

In situ hybridization and histology

Free-range fertile chicken eggs from local sources were incubated at 38°C to the stages required for *in situ* analysis and culture experiments. Whole-mount *in situ* hybridization was performed as described by Nieto *et al.* (1995), using sense (control) and antisense Digoxygenin-Utp-labeled riboprobes. Probes derived from both the entire coding region, and a 1 kb fragment at its 3' end, were used and gave identical patterns, though the larger (2.1 kb) fragment gave greater background coloration with both sense and antisense probes. Specimens were examined and photographed whole after extensive washing in saline, then some were re-fixed in 2% formalin, 2% glutaraldehyde in phosphate buffer for 30 min, dehydrated in two 10-minute changes each of absolute methanol and absolute iso-propanol, cleared in tetrahydronaphthalene (20 min) and embedded via 2 changes of fibrewax (58°C) for transverse sectioning at 8 μ. Sections were lightly counterstained with eosin and viewed using the bright field condenser diaphragm to optimize signal against structural background.

Cell culture

Culture supernatant from CHO cells expressing Noggin protein (gift of R. Harland and T. Lamb) was prepared by 2-3 days' culture of near-confluent cells in low-serum medium, without the methotrexate which is normally included to maintain the transfected state in these cells. Such medium was then concentrated in two steps using centricon tubes to retain proteins with Mr>10 kDa (Amicon Ltd), to achieve approx. 50-fold concentration. Such stored concentrates were added back to the short-term chick embryo culture media in biological tests, to give concentrations of the secreted protein that are expressed in terms of the original unconcentrated supernatant. Thus vigorous elongation (convergent extension) occurred in stage 10 - cultured *Xenopus* ventral marginal zone explants in 1.5, 2 or 5-fold dilutions of original supernatant.

Embryo manipulations

Stage 3 blastoderms (Hamburger and Hamilton, 1951) were explanted into ring culture (New, 1955), then removed from the vitelline membranes and incubated at 38°C for 2 h in a 2-3 mm deep layer of 50% Hank's BSS with 0.1 millimolar $CaMg^{++}$: 50% air-buffered Liebovitz TCM+glutamine (see Nieto *et al.*, 1994) with 1 mg/ml BSA. In some experiments, slits were made in the hypoblasts of such embryos before the incubations. Experimentals received the active Noggin cell-culture supernatant concentrate, while controls received control CHO cell supernant concentrate. Following such incubation, blastoderms were returned to the vitelline membranes and cultured onwards. In other experiments, similar blastoderms were exposed instead to 50-100 ng/ml BMP-4 protein (Genetics Institute, Boston, USA), using 5 mg/ml BSA in the off-membrane medium (to prevent absorption of the active protein to plastic surfaces) for controls and experimentals.

In other experiments, Hensen's nodes dissected from stage 4 donor embryos were incubated for 2 h in the same Hank's/Liebovitz medium with addition of experimental Noggin or control supernatant concentrates, before implantation into the area pellucida/area opaca margin at antero-lateral positions in stage 3- 3+ hosts in ring culture.

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