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The induction of anterior and posterior neural genes in *Xenopus laevis*

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Summary

We have investigated the interactions between mesoderm and ectoderm that result in the formation of a regionally differentiated nervous system in *Xenopus* embryos. We have used genes expressed at different positions along the neural tube as regional markers of neural induction in both whole, and in experimentally manipulated embryos. By comparing transcription from the anterior marker, XIF3, with that from the posterior marker, XlHbox6, and the general neural marker XIF6, we have shown that the normal induction process requires interactions between ectoderm and mesoderm that persist through gastrulation into the late neurula stages.

We have found that competence of the ectoderm to respond to induction is lost at the same early neurula stage for all three marker genes. Using rhodamine dextran-labelled mesoderm, we have established that the duration of contact between ectoderm and mesoderm required for gene activation in conjugates is the same for each of the markers. We have, however, identified regions of the mesoderm that can induce different combinations of neural marker gene expression. The anterior mesoderm induces expression of the anterior marker, XIF3, and the later migrating posterior mesoderm induces the ectoderm overlying it to express the posterior marker XlHbox6.

It has been proposed that neural inducing signals reach the ectoderm by two different routes: from mesoderm lying directly beneath the ectoderm or along the plane of the ectoderm. We have assessed the contribution of each route in respect of our three neural markers and find that a signal passing directly from mesoderm to ectoderm fully accounts for neural gene expression. We were unable to detect an inducing signal that passes along the plane of the ectoderm.

Key words: *Xenopus*, neural induction, neural genes.

Introduction

A long-standing problem in development is how different regions of the nervous system arise after induction by mesoderm (Hamburger, 1988). Until recently, neural induction has been analyzed very largely by histological analysis (Witkowski, 1985). The advent of molecular markers of neural induction has made it possible to assay the results of experiments earlier and more quantitatively than by histology (Gurdon, 1987). The molecular markers used so far to analyze neural induction have been genes expressed throughout the nervous system (e.g. N-CAM) (Jacobson and Rutishauser, 1986; Kintner and Melton, 1987), or at one end of the axis, but outside the nervous system, such as cement gland genes (Jamrich and Sato, 1989; Sive *et al.* 1989). In this paper, we describe for the first time the combined use of two regional markers within the nervous system, namely genes expressed in the anterior (XIF3) (Sharpe *et al.* 1989), or posterior (XlHbox6) (Sharpe *et al.* 1987), parts of the embryonic neural tube.

Theories of how neural induction leads to regional gene expression fall into two categories. The conventional view is that regional differences along the mesoderm, instruct ectoderm to express posterior or anterior genes as the two tissues come into contact during gastrulation (recently reviewed by Saxen, 1989). The other class of explanation is that all regions of the mesoderm emit the same kind of inducer at a constant rate and that regional gene expression is achieved either by differences in the properties of the responding ectoderm, or by the ectoderm receiving different amounts of inducer as a function of mesoderm migration during gastrulation. We have used the ratio of expression of anterior and posterior neural genes to examine which of these classes of mechanism best explains neural gene activation. We conclude that in our experimental system, regional differences in the underlying mesoderm are the major factors initiating the differential expression of our neural marker genes.

We also extend previous results concerning the source of neural inducing signals (Dixon and Kintner, 1989) by asking whether the inducing signal passes more easily from underlying mesoderm to ectoderm rather than along the plane of the ectoderm. We find that the neural marker genes studied here are activated strongly

by underlying mesoderm, which we therefore suggest is the primary route followed by normal inducer substances.

Materials and methods

Embryo manipulations

Embryos were dejellied in cysteine–HCl and raised in 1/10×MBS as previously described (Gurdon, 1977). Manipulations were performed in 1×MBS using sharpened watchmakers forceps. Dissected pieces of embryos and conjugates were cultured in 1×MBS in plastic Petri dishes containing a layer of 2% agarose in 1×MBS. Ectodermal fragments were removed and cultured under glass coverslides as described by Grainger and Gurdon (1989). Conjugates of mesoderm and stage 10 ectoderm were constructed as described by Sharpe *et al.* (1987).

In order to identify mesodermal cells, embryos were injected with about 500 ng of rhodamine-labelled dextran in 10 nl at the 2-cell stage. Conjugates of labelled mesoderm and unlabelled ectoderm were taken apart under a dissecting microscope and the mesoderm removed. Remaining mesodermal cells were located under a fluorescence microscope, removed, and the ectoderm again checked by fluorescence until free of mesodermal cells.

Molecular techniques

Total nucleic acid was isolated from embryos and embryonic fragments (Mohun *et al.* 1984) and expression of the neural markers determined by RNase protection assay as described by Krieg and Melton (1987) or by Northern analysis of RNA separated on formaldehyde–agarose gels (Hopwood *et al.* 1989). The protection assay probes were the ones previously described (Sharpe *et al.* 1989; Sharpe, 1988), except for XlHbox6 which used clone pG1S (kindly provided by Dr E de Robertis, UCLA), linearised at the 3′ *Sma*I site to produce an antisense RNA probe with T7 RNA polymerase. The protected band is 229 bases long. Northern blots were screened with the DNA inserts from the transcription clones that had been labelled to a high specific activity by random priming (Feinberg and Vogelstein, 1984).

Results

Markers of neural induction

We have previously described three molecular markers; their main features are summarized in Table 1 and Fig. 1. Transcripts from XlHbox6, a homeobox-containing gene, are found predominantly in the spinal cord but not in the hindbrain or anterior neural structures. The anterior boundary of XlHbox6 expression has not been determined more precisely. We have also noted low levels of XlHbox6 expression in lateral mesoderm from tailbud embryos (Sharpe *et al.* 1987). XIF3 expression at the tailbud stage is mainly in the motor neurones of the hindbrain and in some cranial nerves as shown by *in situ* hybridisation (Sharpe *et al.* 1989). RNase protection assays using regions dissected from tailbud embryos have shown that XIF6, the neurofilament-M homologue, is expressed in neural tissue along the whole length of the embryo (Sharpe, 1988). XlHbox6 and XIF6 are activated only after induction by mesoderm. The first XIF3 transcripts are partly maternal and partly zygotic, and are formed in the absence of neural induction, but at least 90% of XIF3 transcripts present in the neurula are formed in response to induction (Sharpe *et al.* 1989). With the

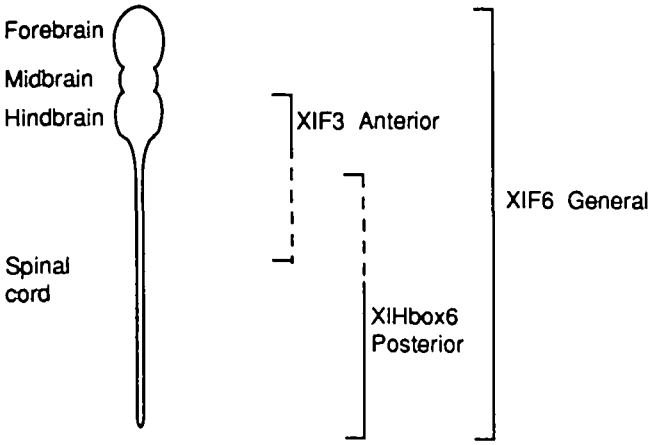

Fig. 1. Sites of expression of the three neural markers. Transcripts from XIF3, the anterior marker, are found abundantly in some cranial nerves and in the motor neuron column of the hindbrain (solid line). Fewer transcripts are found in the neural tube, in the midbrain and then for approximately two-thirds the length of the embryo (dashed line). Embryos that have been dorsalized and anteriorized by treatment with lithium ions (Kao and Elinson, 1988) express increased levels of XIF3 (Sharpe *et al.* 1989). XlHbox6 transcripts (the posterior marker) are found predominantly in the spinal cord. The anterior boundary of expression has not been accurately determined (dashed line). XlHbox6 transcripts are not found in the brain (Sharpe *et al.* 1987). Lithium-treated embryos have decreased levels of XlHbox6 expression. XIF6 is the neurofilament-M gene. RNase protection assays show that XIF6 is expressed both in brain and spinal cord regions (Sharpe, 1988). The exact locations of XIF6 expression have not been determined, but, in *Xenopus*, NF-M protein is found in most neurones (Szaro and Gainer, 1988). For these reasons, we consider that XIF6 is a general neural marker at the tailbud stage. XIF6 expression is only slightly reduced in lithium-treated embryos.

Table 1.

Marker	Identity	Expression in tailbud embryos	
		Region	Tissues
XlHbox6	Homeobox-containing gene	Posterior	Spinal cord; (lateral plate mesoderm)
XIF3	Intermediate filament type III (peripherin)	Anterior	Hindbrain; cranial nerves
XIF6	Intermediate filament type IV (NF-M)	General	Most neurones

possible exception of XlHbox6, these three neural genes represent the result, and not the cause, of neural differentiation.

The timing of neural induction in normal development

We needed first to establish the stages in development when the induction processes take place that cause cells to express the three genes we are studying. The profile of transcript accumulation for each gene is summarized in Fig. 2. It shows that all three genes are strongly expressed by the tailbud stage. In the following experiments, we have grown dissected parts of embryos to the tailbud stage using the three genes as markers of neural induction. To determine when the inductive events that lead to this activation take place, neurectoderm was separated from mesoderm at various stages and cultured until stage 28. The number of marker transcripts was measured by RNase protection and quantified by comparison with whole embryos at the same developmental stage. If neural induction was only partially complete, the level of marker transcripts in the isolated and subsequently cultured tissue would be only a fraction of that in whole embryos. In this way, we could assess the time of commitment of the ectoderm to become neural tissue.

We were able to obtain additional information about the regional commitment of the ectoderm to become neural tissue in the same experiments by cutting the neurectoderm into anterior, posterior and ectomesodermal fragments and culturing these separately to stage 28 before analysis. We consider neural gene expression in the region of the ectomesodermal junction, close to the dorsal lip, to be difficult to interpret, since, in addition to neural markers, the cardiac actin gene (Mohun *et al.* 1984) was expressed in this fragment (data not shown) indicating the presence of mesodermal cells. We could not therefore separate neurectoderm from mesoderm in this region where a distinction between the two cell types is imperceptible. The anterior and posterior regions showed no cardiac gene expression, and were therefore considered to be free of mesodermal cells.

The results are summarized in Fig. 3. We see that neural induction continues through neurulation up to stage 18. The posterior marker, XlHbox6, shows a

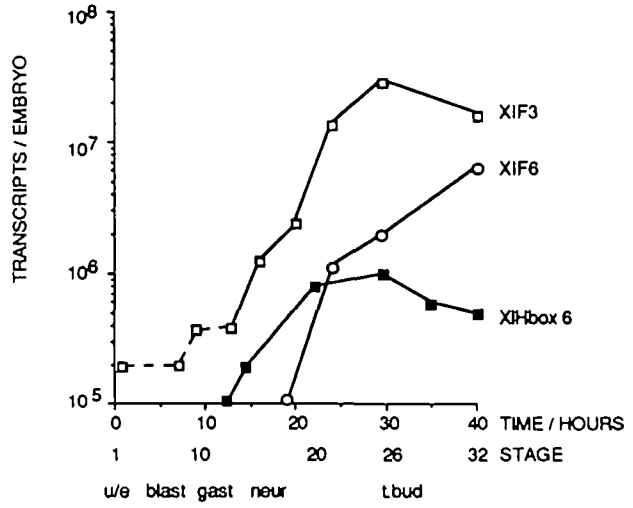

Fig. 2. The accumulation of neural marker transcripts during normal development. The early accumulation of XIF3 transcripts (dashed line) is independent of neural induction (Sharpe *et al.* 1989). XlHbox6 transcripts are first detected at stage 13 and their numbers peak in the tailbud embryo. XIF6 transcripts accumulate steadily from the late neurula stage. In subsequent experiments, isolated fragments have been allowed to grow to stage 26–28 in order to detect marker transcripts at a level of greater than 10^6 per embryo. u/e, unfertilized egg; blast, blastula; gast, gastrula; neur, neurula; t.bud, tailbud stages. Stage numbering is according to Nieuwkoop and Faber (1967).

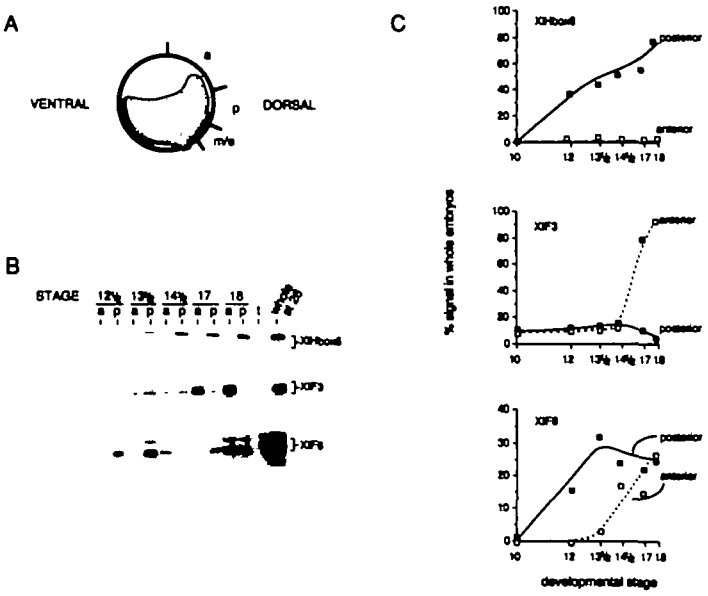

Fig. 3. The timing of neural induction in normal development. (A) The prospective neurectoderm was removed from the dorsal side of the embryo and divided into anterior (a), posterior (p), and ectomesodermal (m/e) fragments. These were cultured in isolation to the tailbud stage. Since the ectomesodermal piece expressed a mesodermal marker when cultured to the tailbud stage, it was not considered further. (B) Neurectodermal regions dissected from late gastrula (stage 12½) to late neurula (stage 18) stages were grown to stage 26 and the levels of marker transcripts determined by RNase protection assay. Each lane represents the analysis of five fragments divided between the three probes for individual assay. RNA from five whole tailbud embryos was similarly divided to provide a control for complete induction. (C) The transcript levels of all three marker genes in the representative experiment shown were quantified by densitometry and the patterns of expression compared. Dashed line, anterior fragment; solid line, posterior fragment. Note the enlarged scale on the y-axis of the XIF6 graph. When removed at stage 18 the combined expression of XIF6 in anterior and posterior fragments grown to the tailbud stage is greater than 50% of that found in whole embryos. By discarding the ectomesodermal fragment, which represents about one-third of the prospective neural tube, expression from the neural markers, in particular XlHbox6 and XIF6, reaches a plateau at less than 100% of the level seen in control embryos.

simple pattern of expression; the posterior fragment shows a progressive accumulation in the number of XlHbox6 transcripts; it is not expressed at all in the anterior fragment. In contrast, XIF3, the anterior marker, is expressed equally in anterior and posterior regions isolated from stages 12½ to 14½. However, this level of expression is not significantly greater than the pre-induction level (Sharpe *et al.* 1989). After isolations at stages 17 and 18, we see a large increase in the number of XIF3 transcripts in the anterior, but none in the posterior, region. When isolated at stage 18, the level of XIF3 transcripts in the anterior region is equal to that in the whole embryo. Finally, XIF6, the general neural marker, gave results that looked like the sum of the anterior and posterior markers. That is, the posterior neurectoderm first becomes committed to express XIF6 at the same time as the posterior marker, XlHbox6. Subsequently, the anterior neurectoderm becomes committed to express both XIF6 and the anterior marker, XIF3, at the same stage of development.

The results can be summarised as follows. The posterior neurectoderm is first committed, during the late gastrula stages, to express the posterior marker XlHbox6, but never, as a result of induction, to express the anterior marker, XIF3. Similarly, the anterior neurectoderm becomes committed during the mid-neurula stages to express the anterior, but not the posterior marker. The entire neurectoderm shows a progressive posterior to anterior commitment to express the general neural marker, XIF6. In this way the neurectoderm is committed to the full expression of all three neural marker genes by stage 18.

Loss of ability of the ectoderm to respond to neural induction

One of the properties of ectoderm that might affect its response to induction is its competence, that is its ability to respond to an inducing signal. It is known that mesoderm retains an ability to induce neural tissue from the early gastrula until the late neurula stages (Jones and Woodland, 1989). There could be a change in the ability of the ectoderm to respond to mesodermal induction during this time. For example, if the anterior ectoderm were to lose competence to respond to an inducer several hours before the posterior ectoderm, it would be exposed to the inducer for a shorter time, and would therefore receive a smaller amount of inducer. Conversely, the posterior ectoderm would experience a longer exposure and a larger amount of inducer, and could therefore respond differently, even though all regions of mesoderm might be emitting the same inducer at a constant rate.

To test this idea, we need to know whether the stage at which ectoderm loses competence is the same for anterior and posterior marker genes. For this and subsequent experiments, we have used conjugates of ectoderm and mesoderm, rather than whole embryos. We constructed sandwiches of dorsal mesoderm surrounded by two pieces of stage 10 ectoderm (Fig. 4), since we find these conjugates undergo substantial neural induction (Sharpe *et al.* 1987).

We combined ectoderm of different ages with stage 12½–13 inducing mesoderm. To avoid previous contact with mesoderm, we cultured stage 10 ectoderm under glass (Grainger and Gurdon, 1989) to stop it rolling up, until it had reached the desired developmental stage. We found that competence of the ectoderm is lost at the same stage (stage 13–14) for all three markers. To ensure that loss of competence did not simply reflect the adverse effects of culturing ectoderm under glass, we kept younger (stage 9) ectoderm under glass for the same period of time and showed that it was still competent to respond at the end of this time. If loss of competence is due to experimental treatment alone, then the effect would have been seen after the same number of hours under glass in both samples. If, however, the effect is dependent on the stage of development, then loss of competence in the late blastula tissue would have been delayed by two hours compared to the stage 10 ectoderm samples. We find that the latter is the case, indicating that the procedure itself does not unduly perturb the ectoderm (Fig. 5).

We conclude that ectoderm loses its competence to be induced at the same stage for the three neural markers. We therefore believe that the time of loss of competence does not contribute to the differential expression of the neural marker genes.

The duration of contact

An aspect of neural induction that varies along the

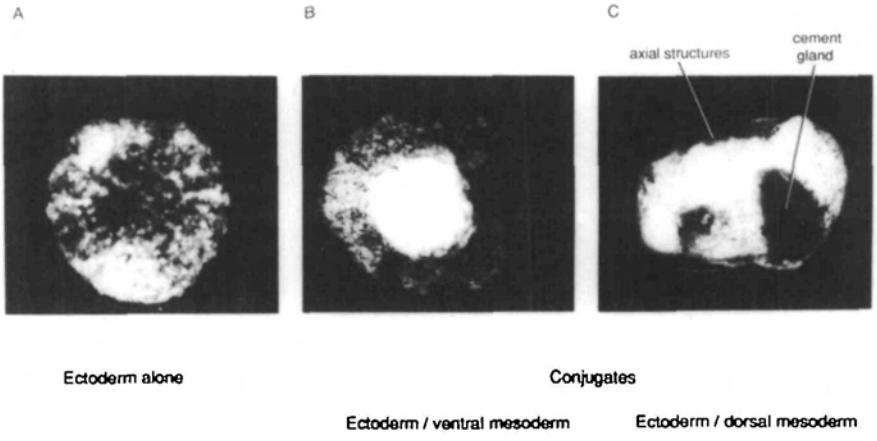

Fig. 4. The appearance of cultured ectoderm and neural conjugates grown to the late tailbud stage. (A) Two pieces of stage 10½ ectoderm placed together develop into an evenly pigmented ball of cells with no external features. (B) Neural conjugates made from stage 11½ ventral mesoderm and stage 10½ ectoderm typically form a smooth external surface and a region of greater pigmentation characteristic of a small cement gland. (C) Neural conjugates of stage 10½ ectoderm and stage 11½ dorsal mesoderm develop well-formed cement glands and externally visible ridges characteristic of axial structures.

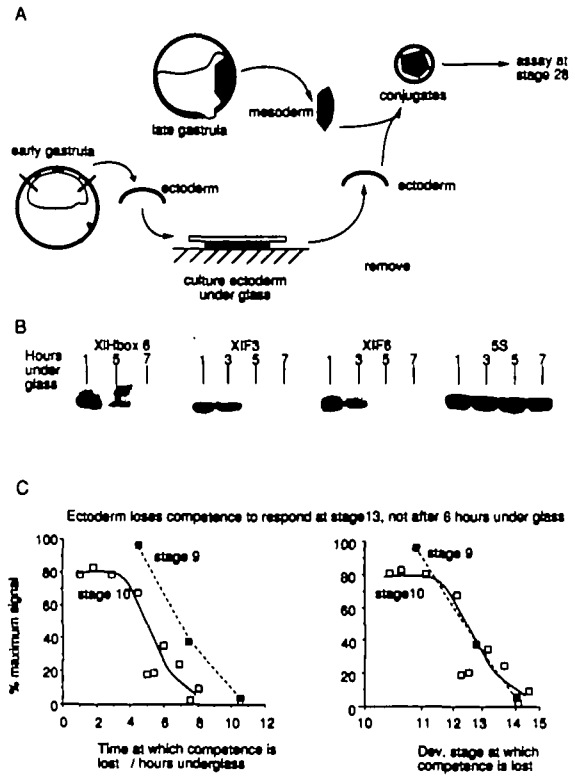

Fig. 5. The loss of competence to respond to neural induction. (A) Experimental design. Ectoderm from early gastrulae was cultured under fragments of glass coverslides on 2% agarose before being removed and combined with mesoderm freshly removed from either mid- or late-gastrula embryos. The conjugates, consisting of one piece of mesoderm surrounded by two pieces of ectoderm were cultured to the tailbud stage before assay. (B) RNase protection assay for each of the three markers and 5S RNA as a control for RNA recovery. At each time point RNA was prepared from five pooled conjugates and divided between the four probes. XIF3 and XIF6 were assayed, in this experiment, by combining the two probes with each RNA sample. The results shown for XlHbox 6 are derived from a separate experiment from those shown for XIF3 and XIF6. For each marker, competence is lost after 5–7 h under glass corresponding to stage 13 of development. In this series of experiments, late gastrula mesoderm was used as inducer. (C) Demonstration that loss of competence is due to the developmental age of the ectoderm rather than the time spent under glass. Ectoderm was removed at stages 9 and 10 and treated as in A. The conjugates were assayed for XIF3 by Northern blotting and quantified by densitometry. Ectoderm removed at stage 9 loses competence two hours later than ectoderm removed at stage 10. However, when the data are plotted against the developmental age of the ectoderm, it is seen that competence is lost at the same stage of development in both sets of samples.

anterior–posterior axis is the duration of contact between ectoderm and migrating mesoderm. In the early gastrula, the mesoderm present at the dorsal lip moves in an anterior direction, thereby coming into contact with progressively more anterior regions of ectoderm (Gerhart and Keller, 1986). Therefore, the anterior ectoderm is exposed to inducer for a shorter time than the posterior dorsal ectoderm.

To define the required contact time for the activation of each marker, conjugates of ectoderm and mesoderm were dismantled after various lengths of time and the ectoderm cultured in isolation to stage 28 before analysis. We could only be sure that all mesodermal cells had been removed from the ectodermal sandwich by combining mesoderm from rhodamine dextran-labelled embryos with unlabelled ectoderm and monitoring the removal of mesodermal cells with a fluorescence microscope (Fig. 6). Labelled control embryos developed normally.

In two series of experiments, we made use of dorsal mesoderm from stage 11½ or from stage 12½–13 embryos. Within each series, we were unable to detect any significant difference in the contact time required to activate the marker genes (Fig. 7). Using stage 11½ mesoderm, this was 8–9 h for XIF3 and XIF6; using stage 12½–13 mesoderm, the time was about 4 h for XIF3 and XlHbox6. These results are consistent with the observations that neural induction continues into the neurula stages of development (Fig. 3). It is interesting that the time of gene activation is related to a particular stage of development, and not to the time that has elapsed since exposure to inducer, in agreement with results for mesoderm-forming induction (Gurdon *et al.* 1987).

We conclude that differences in expression of the regional neural markers cannot, in a simple way, be accounted for in terms of the duration of contact between mesoderm and responding ectoderm.

Regional differences in the inducing ability of the mesoderm

We now describe experiments designed to test the view that there are regional differences in inducing ability within the mesoderm, as originally indicated by Mangold (1933) and recently reviewed by Saxen (1989).

We divided mesoderm, removed at three stages from 11 to 12½, into anterior and posterior regions. It is probable that these pieces also contained some endodermal cells. The mesodermal fragments were made into conjugates with stage 10 ectoderm and cultured to stage 28 before analysis. The results are shown in Fig. 8. It can be seen that, during these three stages, both regions of mesoderm can induce the anterior marker XIF3. However, the ability to induce the posterior marker, XlHbox6, is acquired only at stage 12, and then only in the posterior mesoderm. The most reliable interpretation of these results involves a comparison of different regions of mesoderm at the *same developmental stage*; at all three stages analyzed, the anterior and posterior regions of mesoderm have different inductive effects. Our explanation of this result is that as the mesoderm advances from the dorsal lip forwards to the anterior end of the embryo, it induces the ectoderm cells it is in contact with to express XIF3, the anterior marker. Then the later gastrulating mesoderm will further induce the posterior neurectoderm, which it comes to underlie, to express the posterior marker

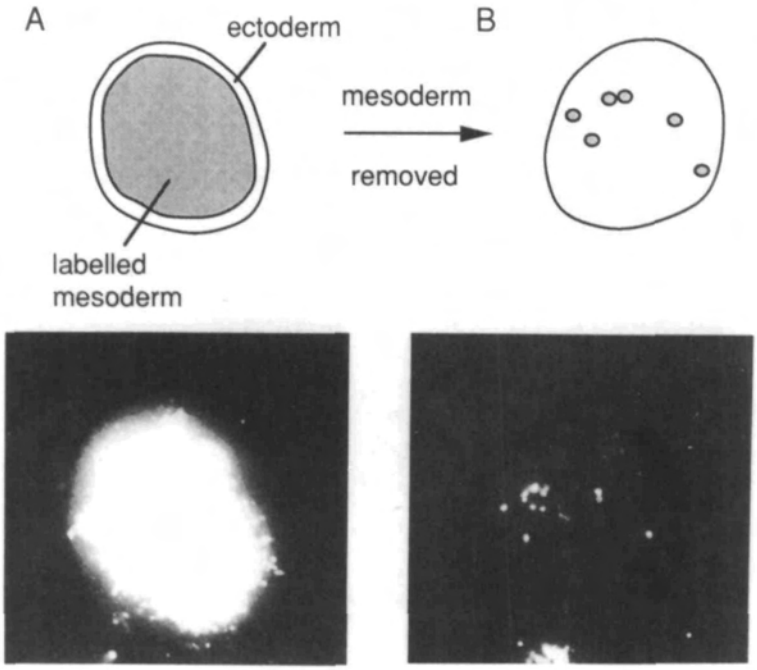

Fig. 6. The removal of rhodamine-labelled mesoderm from neural conjugates. (A) A neural conjugate from which one piece of ectoderm has been removed to expose the labelled mesoderm, viewed using a fluorescence microscope. (B) Mesodermal cells were removed from the ectoderm and the remaining ectoderm checked for contaminating mesodermal cells. The positions of remnant fluorescent cells were noted. Remnant mesoderm cells were subsequently removed until only small amounts of subcellular material remained attached to the ectoderm.

XlHbox6. This concept is similar to that expressed by Nieuwkoop (1955, 1958 and see Discussion). The conclusion is that, during gastrulation, the mesoderm possesses different capabilities for regional neural induction along the anterior–posterior axis of the embryo under the presumptive neurectoderm.

In these conjugate experiments, we have combined stage 11 to 12½ mesoderm with stage 10½ ectoderm. In case the results have been affected by the difference in developmental stage, or by the experimental manipulations involved, we have used another design of experiment that has given similar results. In this case, we removed regions of the mesoderm still attached to their natural adjacent ectoderm at stage 12½; these natural mesoderm:ectoderm isolates were then cultured until stage 28 and analyzed for expression of the neural markers. As shown in Fig. 9, the anterior piece expresses only XIF3 transcripts whilst the posterior piece expresses both XIF3 and XlHbox6 transcripts.

We can conclude from both series of experiments that different regions of late gastrula (stage 12½) mesoderm do indeed have different inductive effects. Both anterior and posterior regions of mesoderm induce the

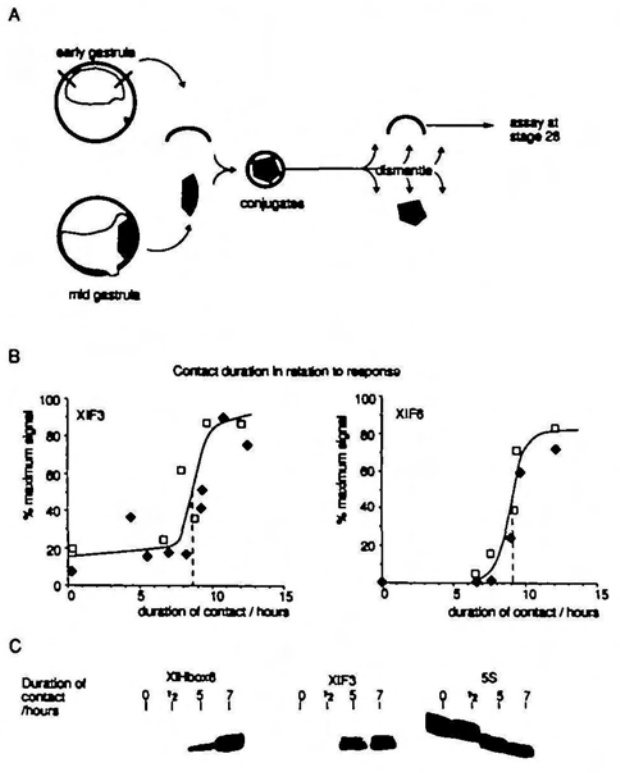

Fig. 7. The duration of contact between ectoderm and mesoderm that is required for neural induction. (A) Experimental design. Conjugates of rhodamine-labelled mesoderm and early gastrula ectoderm were grown for a fixed length of time before being dismantled and the ectodermal fragments grown on, in isolation, to the tailbud stage. The ectodermal fragments were then collected and the levels of marker gene transcripts determined by RNase protection assay. (B) The combined results from two experiments using mid-gastrula mesoderm as the inducer. It can be seen that both XIF3 and XIF6 gene expression is activated in the ectoderm following 7 to 8 h contact between mesoderm and ectoderm. Maximum signal (y-axis) is that obtained from conjugates that were not dismantled. The open and closed squares represent two independent experiments in which the amounts of transcript from each gene was determined for five combined conjugates at each time point. (C) RNase protection assay showing the activation of XIF3 and XlHbox6 genes in ectodermal samples assayed at stage 28. Late gastrula–early neurula mesoderm was used in these conjugates, which results in the shorter duration of contact required for XIF3 expression in this experiment compared to the one shown in B. RNA was collected from five pooled ectodermal samples at each time point. The sample assayed for 5S RNA was diluted 150 fold compared to the other assays.

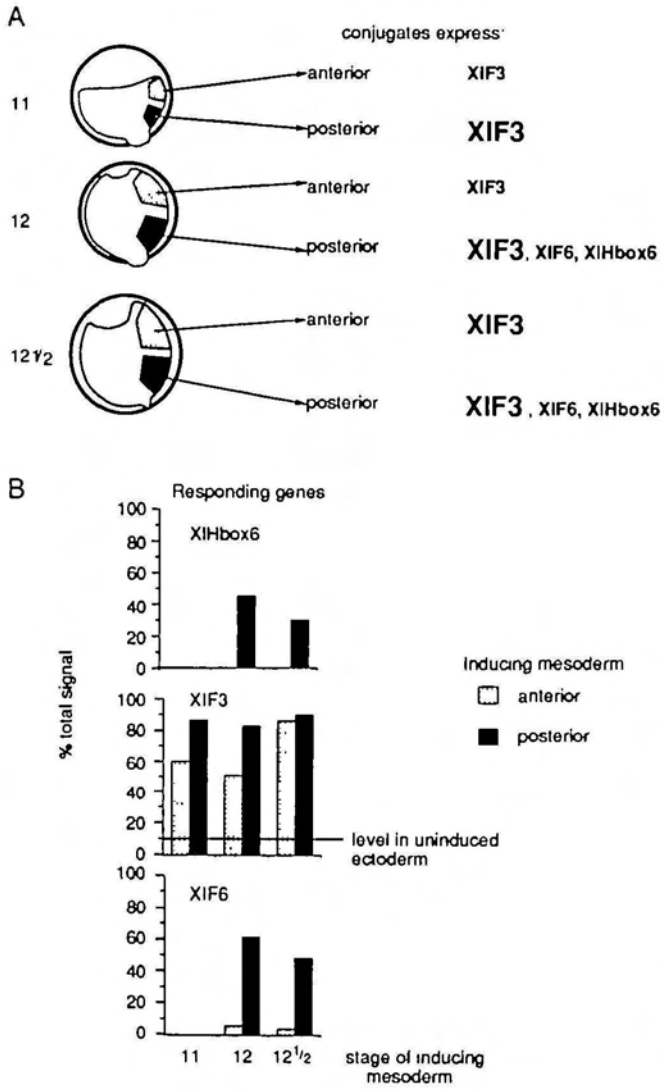

Fig. 8. The ability of different regions of mesoderm to induce the neural marker genes. (A) Summary of the results using mesoderm isolated during gastrulation. Dorsal anterior (stippled) or dorsal posterior (black) mesoderm was used to construct conjugates with early gastrula ectoderm. Levels of transcripts were compared by RNase protection assay with those found in whole embryos. Small print, 20–60 %, and large print >60 % of the transcript level seen in the equivalent number of whole embryos. (B) A quantitative comparison of the different regions. At stage 12½ the mesoderm possesses different inducing capability along the anterior–posterior axis of the embryo. RNase protection assays using RNA pooled from 5 conjugates at each stage, with each region of mesoderm, were quantified by densitometry and compared to the level of transcripts found in whole embryos.

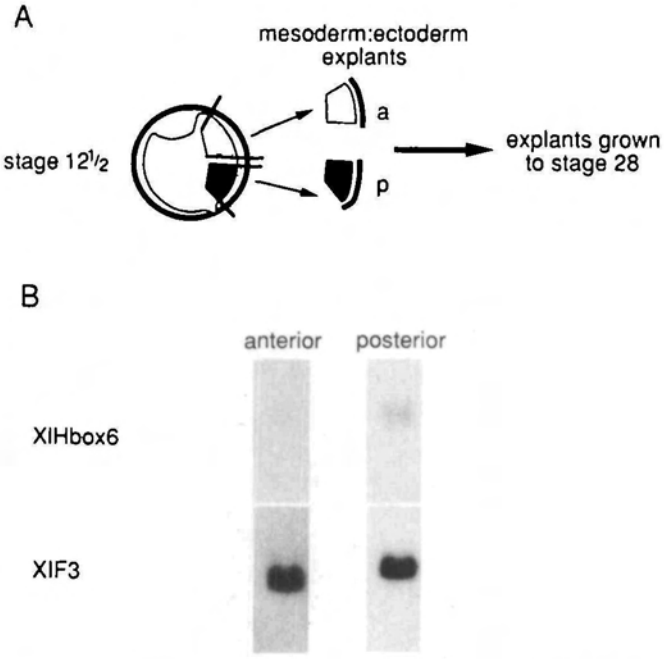

Fig. 9. Expression of the neural markers in pieces of neural plate mesoderm:ectoderm removed at the late gastrula stage. (A) Regions of mesoderm corresponding to those in Fig. 8A (stage 12½) were isolated together with the attached overlying ectoderm. These were then grown in isolation to the tailbud stage before assay by Northern blotting. (a), anterior piece; (p), posterior piece. (B) Northern blots of RNA from five pooled mesoderm:ectodermal pieces from each of the two regions probed sequentially with the two regional markers XlHbox6 and XIF3. Transcripts from the XlHbox6 gene are not detected in the anterior explants but are found in the posterior pieces. XIF3 transcripts, however, are found in both anterior and posterior samples.

anterior marker XIF3, but only the posterior mesoderm induces the posterior marker XlHbox6.

The underlying mesoderm is the source of a normal neural inducer

Having shown that the underlying gastrula mesoderm can cause regional neural gene expression under experimental conditions, we can now ask whether this is the route by which neural induction takes place in normal development.

Dixon and Kintner's (1989) experiments indicate an important function for an inducer that passes along the ectoderm in a posterior to anterior direction in addition to a lesser contribution of inducer from the underlying mesoderm. We have tried to determine by which of these routes inducer is provided to activate our neural markers.

Two kinds of experiment were carried out. In the first, pieces of ectoderm, with naturally attached dorsal lip mesoderm, were removed from early gastrulae and were placed face to face (Fig. 10A) as described by Dixon and Kintner (1989). One set of such conjugates was cut into anterior and posterior halves before commencing culture, and an equivalent set was cut similarly at the end of the culture period when control embryos had reached the tailbud stage. If an inducing influence passes along the ectoderm to cause gene activation in the anterior ectoderm, this should be seen in the conjugates cut after culture, but not in those cut before. Neither neural marker was expressed in anterior fragments, but both were strongly expressed in posterior pieces, whether cut before or after culture.

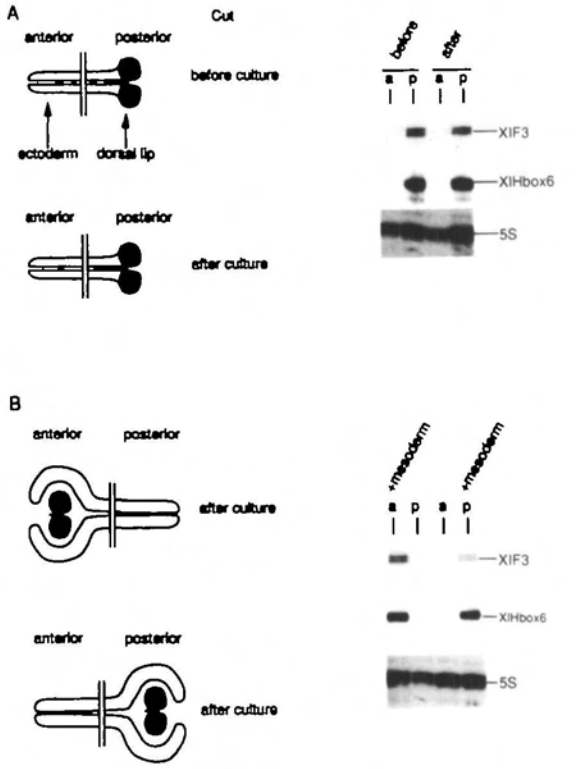

Fig. 10. The major route of neural induction. (A) Pieces of stage 10 embryos removed as described by Dixon and Kintner (1989), were placed in contact and then divided into anterior and posterior fragments either before culture or after culture to the tailbud stage (stage 28). RNase protection assays of 5 pooled fragments from each sample show the levels of the neural markers in the fragments. Note the lack of transcripts in the anterior part of the ectoderm in samples cut both before and after culture. Levels of total RNA were compared by RNase protection assay using a 5S probe with 150-fold diluted samples of RNA. (B) Dorsal lip mesoderm was isolated and placed asymmetrically, between two aligned sheets of ectoderm, either at the anterior or the posterior end of the sheet. After culture the conjugates were divided into mesoderm:ectodermal and ectodermal derivatives and assayed for the neural markers. RNase protection assays of five pooled fragments of each of the samples shows that the neural markers are expressed predominantly in the mesoderm:ectodermal portions of the conjugates. Close examination of the autoradiograms shows predisposition of parts of the ectoderm to express neural markers as previously described (Sharpe *et al.* 1987).

We could not therefore detect an inductive influence passing along the plane of the ectoderm.

As a control we made sandwiches of ectoderm (lacking any naturally associated mesoderm) with dorsal lip mesoderm placed in apposition. Subsequent analysis showed that inductive effects are transmitted effectively to overlying ectoderm in these conjugates (Fig. 10B).

In conclusion, we have found no evidence that either XIF3 or XlHbox6 are induced by an influence that spreads in an anterior direction along the ectoderm. We do however see a strong inductive influence from mesoderm placed directly underneath the ectoderm, suggesting that this may be the normal major route of neural induction in the case of these genes.

Discussion

In this report, we describe experiments designed to analyse the activation of marker genes for different regions of the nervous system. We set out to determine whether or not the localized expression of our anterior (XIF3) and posterior (XlHbox6) genes depends on regional differences within the inducing mesoderm. In the course of this analysis, we have tested and excluded the termination of competence and the duration of contact between mesoderm and ectoderm as contributory factors. We have previously described a predisposition of the dorsal ectoderm to respond to neural induction (Sharpe *et al.* 1987), but have been unable to subdivide this region accurately enough to find out whether the ectoderm itself is predisposed to anterior or posterior neural gene expression.

We have been able to find substantial differences in inducing ability between anterior and posterior axial mesoderm. Our conclusions can be summarized as follows: at stage 12½, the late gastrula stage, the dorsal mesoderm can be dissected into an anterior region that induces anterior neural marker gene expression, and a posterior region that induces both posterior and anterior neural marker gene expression. The underlying mesoderm can therefore be physically divided into two regions with different inducing capacity. It is not possible to determine from our experiments whether the combined anterior and posterior inducing activity of the posterior mesoderm is due to different regions within this area that we have been unable to separate or whether the same mesoderm cells in this position have the capacity to induce the expression of both markers. The regional capacity of the mesoderm to induce first anterior and then both anterior and posterior neural markers is similar to the two-step model of neural induction (Toivonen and Saxen, 1955; Nieuwkoop, 1955, 1958). In this model, ectoderm is first 'activated', a process which in the absence of other interactions will result in the formation of anterior neural tissue; part of it is subsequently 'transformed' by a further induction to form posterior structures. The combined effects of activation and transformation result in the generation of posterior neural tissue. Our conjugation and mesoderm:ectoderm isolation results suggest the presence of anterior and posterior inducing signals within the same region of posterior mesoderm underlying the prospective spinal cord.

Using a gene expressed in the cement gland as a marker for anterior ectodermal differentiation, Sive *et al.* (1989) have recently demonstrated a progressive commitment of ectoderm to a cement gland fate when removed from along the axis at different stages of development. Another signal from the underlying mesoderm then changes this fate from cement gland to neural tissue. In comparison, the anterior neural

marker XIF3 has a different pattern of expression in isolated ectoderm fragments. XIF3 is expressed only in anterior neurectoderm, but unlike the cement gland markers, we have been unable to detect transient expression in the posterior ectoderm between stages 12½ and 18. This result may at first sight seem to contradict the observation that XIF3 is expressed in conjugates containing posterior mesoderm and isolated regions of posterior mesoderm:ectoderm. However, we believe that in normal development the posterior ectoderm is diverted by underlying mesoderm to a posterior fate before it is capable of expressing the anterior marker XIF3. For this reason, posterior ectoderm removed at an early stage during normal development will not have seen inducer for long enough to express XIF3. In both the conjugates and the isolated mesoderm:ectodermal pieces, the ectoderm and mesoderm remain in contact for long enough to allow the expression of XIF3. Nevertheless, our results do give support to the concept of Sive *et al.* (1989), that the first invaginating mesoderm promotes anterior neural differentiation.

Although regional differences in the underlying mesoderm during gastrulation are clearly important for anterior and posterior neural gene expression, our observations do not provide a complete explanation of regional neural induction. One specific problem concerns the ability of the mesoderm to induce XIF6 gene expression. Since the XIF6 message and protein is found throughout the nervous system at the tailbud stage (Sharpe, 1988; Szaro and Gainer, 1988; Godsave *et al.* 1986), we might expect each region of isolated mesoderm to be capable of inducing XIF6. However, we find that at stage 12½ the anterior mesoderm is not a strong inducer of XIF6 gene expression despite the fact that it is a potent inducer of the anterior neural marker XIF3. It therefore seems that, at this stage, differences in the mesoderm can explain differences in the expression of anterior and posterior neural marker genes but not of the general neural marker XIF6. Whilst it seems clear that differences in the inducing ability of the underlying mesoderm are an important factor, this result suggests that they are not entirely responsible for the pattern of neural gene expression seen in the later embryo.

Our emphasis on the importance of regional differences in the inducing ability of the mesoderm led us to ask whether the inducing signals that activate our anterior and posterior marker genes in normal development are transmitted from the underlying mesoderm or in the plane of the ectoderm as proposed by Dixon and Kintner (1989). We have been unable to detect the Dixon and Kintner (1989) effect using our neural markers. We are able to account for between 30 % and 90 % of the normal level of marker transcripts in conjugates where the mesoderm is placed directly under the ectoderm. Given that our conjugates contain only a portion of the inducing mesoderm and furthermore have been subjected to experimental manipulation, we consider that this degree of induction is satisfactory and indicative of the normal major route used by neural inducing signals.

In conclusion, we set out to determine the features of neural induction that contribute to the expression of the regional markers, XlHbox6 and XIF3 at different positions along the neural tube. We find, in agreement with the established view, that differences in the mesoderm make a major contribution to this effect. Moreover, we have been able to quantify other aspects of neural induction such as the duration of mesoderm–ectoderm interaction and the period of ectodermal competence and have shown that these factors are unlikely to be important in regional neural gene expression. In keeping with this view, we have shown that the primary route by which the neural inducing signals take effect is through a direct interaction between mesoderm and overlying ectoderm.

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References

Dixon, J. E. and Kintner, C. R. (1989). Cellular contacts required for neural induction in *Xenopus laevis*: evidence for two signals. *Development* **106**, 749–757.

Feinberg, A. P. and Vogelstein, B. (1984). Addendum: A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **137**, 266–267.

Gerhart, J. and Keller, R. (1986). Region-specific cell activities in amphibian gastrulation. *Ann. Rev. Cell Biol.* **2**, 201–209.

Godsave, S. F., Anderton, B. H. and Wylie, C. C. (1986). The appearance and distribution of intermediate filament proteins during differentiation of the central nervous system, skin and notochord of *Xenopus laevis*. *J. Embryol. exp. Morph.* **97**, 201–223.

Grainger, R. M. and Gurdon, J. B. (1989). Loss of competence in amphibian induction can take place in single non-dividing cells. *Proc. natn. Acad. Sci. U.S.A.* **86**, 1900–1904.

Gurdon, J. B. (1977). Methods for nuclear transplantation in Amphibia. *Meth. Cell Biol.* **16**, 125–139.

Gurdon, J. B. (1987). Embryonic induction – molecular prospects. *Development* **99**, 285–307.

Gurdon, J. B., Fairman, S., Mohun, T. J. and Brennan, S. (1987). Activation of muscle specific actin gene expression in *Xenopus* development by an induction between animal and vegetal cells of a blastula. *Cell* **41**, 913–922.

Hamburger, V. (1988). *The Heritage of Experimental Embryology. Hans Spemann and the Organiser.* Oxford University Press Inc. New York, USA.

Hopwood, N. D., Pluck, A. and Gurdon, J. B. (1989). MyoD expression in the forming somites is an early response to mesoderm induction in *Xenopus* embryos. *EMBO J.* **8**, 3409–3417.

Jacobson, M. and Rutishauser, U. (1986). Induction of neural cell adhesion molecule (N-CAM) in *Xenopus laevis*. *Devl Biol.* **116**, 524–531.

Jamrich, M. and Sato, S. (1989). Differential gene expression in the anterior neural plate during gastrulation of *Xenopus laevis*. *Development* **105**, 779–786.

Jones, E. A. and Woodland, H. R. (1989). Spatial aspects of neural induction in *Xenopus laevis*. *Development* **107**, 785–792.

Kao, K. R. and Elinson, R. P. (1988). The entire mesodermal mantle behaves as a Spemann's organizer in dorsoanterior enhanced *Xenopus laevis* embryos. *Devl Biol.* **116**, 524–531.

Kintner, C. R. and Melton, D. A. (1987). Expression of *Xenopus* N-CAM in ectoderm is an early response to neural induction. *Development* **99**, 311–325.

Krieg, P. A. and Melton, D. A. (1987). *In vitro* RNA synthesis with SP6 RNA polymerase. *Methods In Enzymol.* **155**, 397–415.

MANGOLD, O. (1933). Uber die Induktionsfahigkeit der verschiedenen Bezirke der Neurula von Urodelen. *Naturwissenschaften* **21**, 761–766.

MOHUN, T. J., BRENNAN, S., DATHAN, N., FAIRMAN, S. AND GURDON, J. B. (1984). Cell-type specific activation of actin genes in the early amphibian embryo. *Nature, Lond.* **311**, 716–721.

NIEUWKOOP, P. D. (1955). Independent and dependent development in the formation of the central nervous system in Amphibians. *Expl Cell Res.* **3** (Suppl.) 262–273.

NIEUWKOOP, P. D. (1958). Neural competence of the gastrula ectoderm in *Ambystoma mexicanum*. An attempt at quantitative analysis of morphogenesis. *Acta Embryol. Morphol.* **2**, 13–53.

NIEUWKOOP, P. D. AND FABER, J. (1967). *Normal Table of* Xenopus laevis *(Daudin)*. Amsterdam. North Holland Publishing Co.

SAXEN, L. (1989). Neural induction. *Int. J. Dev. Biol.* **33**, 21–48.

SHARPE, C. R. (1988). Developmental expression of a neurofilament-M and two vimentin-like genes in *Xenopus laevis*. *Development* **103**, 269–277.

SHARPE, C. R., FRITZ, A., DEROBERTIS, E. M. AND GURDON, J. B. (1987). A homeobox containing marker of posterior neural differentiation shows the importance of predetermination in neural induction. *Cell* **50**, 749–758.

SHARPE, C. R., PLUCK, A. AND GURDON, J. B. (1989). XIF3, a *Xenopus* peripherin gene, requires an inductive signal for enhanced expression in anterior neural tissue. *Development* **107**, 701–714.

SIVE, H. L., HATTORI, K. AND WEINTRAUB, H. (1989). Progressive determination during formation of the anteroposterior axis in *Xenopus laevis*. *Cell* **58**, 171–180.

SZARO, B. G. AND GAINER, H. (1988). Identities, antigenic determinants and topographical distributions of neurofilament proteins in the nervous system of adult frogs and tadpoles of *Xenopus laevis*. *J. comp. Neurol.* **33**, 344–358.

TOIVONEN, S. AND SAXEN, L. (1955). The simultaneous inducing action of liver and bone marrow of the guineas pig in implantation and explantation experiments with embryos of *Triturus*. *Expl Cell Res.* **3** (Suppl.) 346–357.

WITKOWSKI, J. (1985). The hunting of the organizer: an episode in biochemical embryology. *Trends Biochem. Sci.* **10**, 379–381.

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