

Preventing the loss of competence for neural induction: HGF/SF, L5 and Sox-2

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SUMMARY

The response to neural induction depends on the presence of inducing signals and on the state of competence of the responding tissue. The epiblast of the chick embryo loses its ability to respond to neural induction by the organizer (Hensen's node) between stages 4 and 4⁺. We find that the pattern of expression of the L5²²⁰ antigen closely mirrors the changes in competence of the epiblast in time and in space. For the first time, we describe an experiment that can extend the period of neural competence: when L5²²⁰ expression is maintained beyond its normal time by implanting HGF/SF secreting cells, the competence to respond to Hensen's node grafts is retained. The host epiblast forms a non-regionalized neural tube, which expresses the pan-neural marker SOX-2 (a Sry-related transcription factor) but not any region-specific markers

for the forebrain, hindbrain or spinal cord. Although HGF/SF secreting cells can mimic signals from Hensen's node that maintain L5 expression, they cannot rescue the ability of the node to induce anterior structures (which is normally lost after stage 4). The ectoderm may acquire stable neural characteristics during neural induction by going through a hierarchy of states: competence, neuralization and regionalization. Our findings allow us to start to define these different states at a molecular level, and show that the competence to respond to neural induction is not entirely autonomous to the responding cells, but can be regulated by extracellular signalling molecules.

Key words: neural induction, competence, chick embryo, Hensen's node, neural induction, HGF/SF, *Sox-2*

INTRODUCTION

Inductive interactions during development are governed not only by the properties of the inducing cells, but also largely by the responsive capacity, or **competence**, of the tissue receiving the inducing signals (Holtfreter, 1933, 1938; Waddington, 1940; for review see Gurdon, 1987). Of all the inductive interactions that occur during early development, neural induction is the archetypal form, and is often called 'primary embryonic induction' since its discovery by Spemann and Mangold (1924). Despite the obvious importance of neural competence, it has been studied surprisingly little, largely due to the lack of good molecular markers until very recently.

Early studies based on morphology alone (Waddington, 1936, 1940; Woodside, 1937; Gallera and Ivanov, 1964; Gallera, 1970, 1971), and more recent studies using molecular markers (Dias and Schoenwolf, 1990; Storey et al., 1992, 1995) have established the time-course of changes in both the inducing ability of the organizer (Hensen's node) and the responsiveness of the ectoderm (competence) in the chick embryo. They showed that neural inducing ability is lost gradually, starting at the full primitive streak stage (stage 4).

As the head process emerges (stage 5), the inducing capacity of the node diminishes to about half, and only posterior nervous system can be generated. By stage 7, the node can no longer induce neural structures from the host, and can only self-differentiate. The loss of competence of the responding epiblast is much more abrupt. While a complete nervous system can be generated from peripheral regions of stage 4 host embryos by a grafted node, the same regions in stage 4⁺ embryos are completely unresponsive.

The pattern of expression of the carbohydrate epitope L5 in the chick embryo has suggested its possible involvement in neural competence (Roberts et al., 1991; Streit et al., 1995). Here, we describe that a second molecule, the SRY-related transcription factor SOX-2 (Kamachi et al., 1995; Uwanogho et al., 1995; Collignon et al., 1996) has a similar but not identical pattern of expression. We have therefore compared these patterns with regions in the chick embryo that are competent to respond to neural induction, and find that only L5-positive regions can be neuralized. We then show that when L5 is maintained beyond its normal time of expression, neural tissue can be induced in a host that would otherwise be too old to do so. The induced neural tube lacks regional character. HGF/SF can

mimic signals from Hensen's node to maintain L5, but these signals do not appear to be sufficient to complete neural induction without additional signals from the node. We propose that neural induction can be subdivided into at least three distinct events, all of them under the control of signals external to the responding cells: competence, neuralization and regionalization.

MATERIALS AND METHODS

Fertile hens' eggs (white Leghorn; Spafas, Mass.) and quails' eggs (Karasoulas, CA) were incubated at 38°C for 8-30 hours to give embryos between stages 3⁺ and 8 (Hamburger and Hamilton, 1951).

Cells and transfection methods

In a previous study (Stern et al. 1990), HGF/SF was delivered to a localized region of the chick embryo either using grafts of producing cells or by implanting ion exchange beads soaked in the factor. It was found that while the beads were effective, the cells supplied the factor for a longer period and therefore produced more reliable results. In the present study, therefore, we have used MRC5 cells (a human cell line that produces HGF/SF naturally; Stoker et al. 1987) as well as COS cells transfected with a chick expression plasmid.

MRC5 cells were obtained from ATCC (#CCL-171) and grown in DMEM, containing non-essential amino acids and 10% newborn calf serum (Hyclone, Utah). COS-1 cells were a kind gift from Barbara Han, Columbia University and were grown in DMEM, 10% new born calf serum. MDCK cells for assays of scattering activity were obtained from ATCC (CCL-341, NB2-2).

COS cells were transfected with full length chick *HGF/SF* (Théry et al., 1995) in pcDNA1 under the control of the cytomegalovirus promoter using lipofectin (Gibco, BRL). In these experiments, COS cells transfected with a plasmid encoding bacterial β -galactosidase in the same vector were used as controls.

Scatter factor assay

To assay for the presence of HGF/SF in cell culture supernatants we performed scattering assays with MDCK cells as described previously (Stoker et al., 1987).

Dissection and grafting techniques

Host embryos were explanted and maintained ventral side up in New culture (New, 1955), modified as described by Stern and Ireland (1981). Quail donor embryos were submerged in Pannett-Compton (1924) saline, and Hensen's node excised using mounted insect pins by cutting along the contours of the node. Grafts were then placed into the inner margin of the area opaca of a chick host as described previ-

ously (Storey et al., 1992) at the same level as the node. In most cases each embryo received two grafts, one on the left and another on the right area opaca.

One day prior to grafting, cultured cells were trypsinized and set up as hanging drop cultures, to form a pellet suitable for grafting. 18-24 hours later, pellets of 400-600 cells were grafted into the inner margin of the area opaca of a host embryo in New culture.

To test the effects of maintaining L5²²⁰ expression on neural competence ('competence rescue experiments'), MRC5 cells (experimental) or COS cells (controls) were grafted into the area opaca of stage 4 host embryos, which were then grown in New culture until they had reached at least stage 5. Then a graft of stage 3⁺/4 quail Hensen's node (experimental) or posterior primitive streak (control) was placed next to the grafted cells, and the embryos maintained in culture for another 16-24 hours.



Fig. 1. Expression of L5 and *Sox-2* mRNA and protein in early chick development. (A-D) L5 immunoreactivity. (E-H) In situ hybridization to reveal *Sox-2* mRNA distribution. (I-L) Immunodetection of SOX-2 protein. At stage 3⁺-4, L5 and *Sox-2* mRNA (A,E) are both expressed as a broad domain encompassing most of the area pellucida epiblast and extending to the inner part of the area opaca. At this stage, in contrast to *Sox-2*, L5 is not expressed in the most posterior part of the embryonic or extraembryonic regions (pao), but the two patterns overlap anteriorly (aao). SOX-2 protein (I) can only be detected at high levels in the central part of the area pellucida. At stage 5-6, L5 (B), *Sox-2* mRNA (F) and protein (J) all become progressively restricted to the future neural plate. At stages 8-9 (C,G,K) and 11-13 (D,H,L) all three expression patterns are confined to the entire central nervous system.

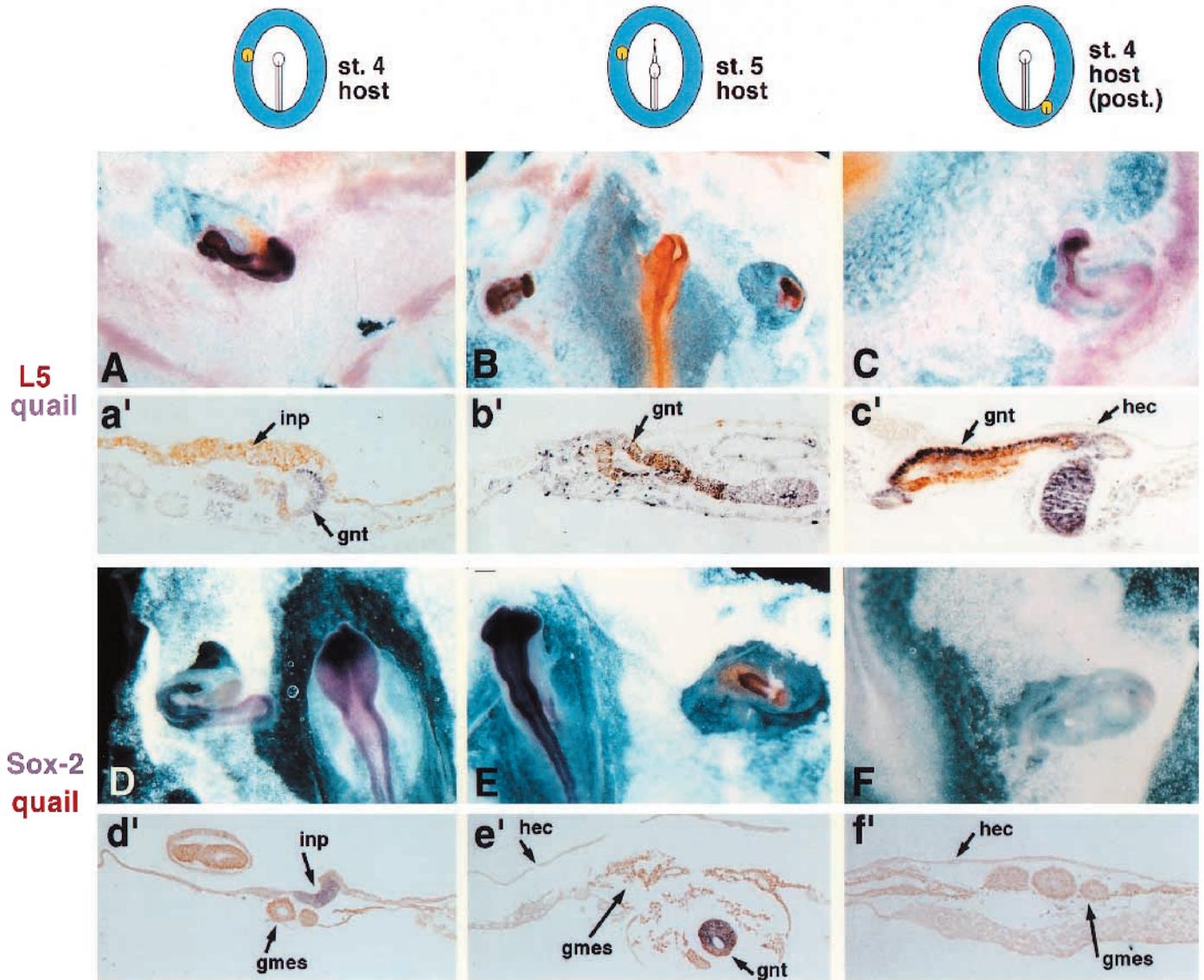


Fig. 2. Regions competent to respond to neural induction. The grafted quail node can only induce the host (chick) epiblast to form a neural plate (inp) in the anterior part of the area opaca of a stage 3⁺-4 host (A,a', D,d'). This region in stage 5 embryos (B,b', E,e') is not competent to respond and only supports self-differentiation of the graft itself (gnt). The same result is obtained when a node is grafted into the posterior area opaca of a stage 3⁺-4 host (C,c', F,f'). Note that in the top two rows, the brown colour represents L5, visualized by immunoperoxidase and the blue signal corresponds to quail cells seen with the QCPN antibody and alkaline phosphatase detection. In the lower two rows, the brown colour is immunoperoxidase staining of quail cells, and the blue represents the localization of *Sox-2* transcripts. inp, induced neural plate. gnt, graft-derived neural tube. hec, host-derived ectoderm. gmes, graft-derived mesoderm. A-F are whole mounts, a',-f' are sections through the same embryos.

Immunocytochemistry

Whole-mount immunostaining with L5 monoclonal antibody was performed as previously described (Streit et al., 1995). Whole-mount immunostaining with 3A10 antibody (Yamada et al., 1991; a kind gift of Susan Morton) was performed according to Storey et al. (1992).

SOX-2 protein was detected by whole-mount immunostaining with polyclonal antibodies directed against mouse SOX-2 (Kamachi et al., 1995). Embryos were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 15 minutes on ice, washed 3 times in PBS for 10 minutes and incubated for 3 hours at 4°C in PBS with 50 mM NH₄Cl to block aldehyde groups. After rinsing in PBS containing 10% sheep serum and 1% Triton X-100, they were incubated overnight at 4°C in the same buffer containing 0.1% H₂O₂. The

embryos were then washed 3 times for 10 minutes in PBS, 1% Triton X-100, followed by a 15-minute wash in the same buffer containing 2 mg/ml BSA (PBTB) and then blocked in PBTB with 10% sheep serum for 1 hour. Polyclonal antibody was then added to a final concentration of 1:500 and the embryos incubated overnight at 4°C. After five 1-hour washes in PBTB at room temperature, the embryos were incubated in HRP-conjugated goat anti-rabbit antibody overnight at 4°C. Embryos were then washed in PBTB four times for 1 hour, followed by two 30-minute washes in 100 mM Tris-HCl pH 7.4 and a 1-hour incubation in 0.5 mg/ml diaminobenzidine in the same buffer. The colour reaction was started by addition of H₂O₂ to a final concentration of 0.003%. The reaction was stopped in tap water and the embryos were refixed in 4% formaldehyde in PBS.

To visualize quail tissue we used the monoclonal mouse IgG QCPN developed by Dr B. M. Carlson and obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205 and the Department of Biological Sciences, University of Iowa, Iowa City 52242, under contract N01-HD-2-3144 from the NICHD. Following L5 staining, embryos were postfixed in 4% formaldehyde in PBS for 30 minutes, washed 3 times in PBS, 0.1% Tween 20 and incubated at 65°C for 30-60 minutes to inactivate endogenous alkaline phosphatase activity. Embryos were then rinsed twice with PBS containing 0.1% Triton X-100, incubated in PBS containing 1% BSA, 1% normal goat serum and 1% Triton X-100 (blocking buffer) for 1 hour at room temperature, followed by a 36-hour incubation with QCPN (1:5 in blocking buffer) at 4°C. Embryos were then washed extensively in PBS, 0.1% Triton X-100 and incubated overnight in anti-mouse IgG coupled to alkaline phosphatase (absorbed against rat Ig's; Jackson). After washing in PBS containing 0.1% Triton X-100 the embryos were washed twice for 10 minutes in 100 mM Tris-HCl containing 1 mM MgCl₂, 500 mM NaCl and the colour reaction was developed using BCIP and NBT as substrates in the same buffer.

Following whole-mount in situ hybridization, embryos were postfixed in 4% formaldehyde in PBS for 30 minutes at room temperature and immediately processed for QCPN staining as described above, except that anti-mouse IgG-HRP (Jackson) was used as secondary antibody.

Whole-mount in situ hybridization

In situ hybridization of chick embryos was performed as described by Théry et al. (1995). The hybridization and posthybridization washing temperatures were selected according to the length of the riboprobe as follows: *Tailless* (Yu et al., 1994 for identifying forebrain; 1.2 kb, 68°C), *Krox-20* (Chavrier et al., 1988; Wilkinson et al., 1989; kind gift from Dr D. Wilkinson, for identifying hindbrain; 180 bp, 63°C), *Hoxb9* (kind gift from Dr R. Krumlauf, for identifying posterior nervous system; 1.5 kb, 68°C), *Sox-2* (Kamachi et al., 1995; Uwanogho et al., 1995; a 1.6 kb probe spanning 3' UTR and the 3' end of the coding region excluding the HMG-box, and 780 bp probe for 3' UTR, 68°C), *gooseoid* (Izpisua-Belmonte et al., 1993; for identifying the organizer and prechordal region; 1.4 kb; 68°C), *brachyury* (kind gift from Dr J. C. Smith, for identifying mesoderm; 350 bp; 65°C).

After in situ hybridization, embryos were fixed in 4% formaldehyde, dehydrated in methanol for 5 minutes, and isopropanol for 10 minutes, and then cleared in tetrahydronaphthalene before embedding in paraffin wax for sectioning.

Immunoprecipitation

For immunoprecipitation, the anterior, inner third of the area opaca and the whole of the area pellucida of stage 3⁺-4 embryos were collected separately in Tyrode's saline. Tissue obtained from 5 embryos for each sample was incubated in methionine-free minimal essential medium (Gibco BRL) containing 2 mM glutamine and 10 mM HEPES for 1 hour in a CO₂ incubator at 37°C. Then, HGF/SF (40 ng/ml) or prothrombin (100 ng/ml; Sigma) and [³⁵S]methionine (100 µCi/ml, 800 Ci/mmol; Amersham) were added and the explants incubated for a further 5 hours. Culture supernatants were centrifuged for 30 minutes at 10,000 g at 4°C and then processed for immunoprecipitation as described below. The explants were washed twice with calcium- and magnesium-free PBS and lysed in ice-cold 20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 0.5% NP40 (pH 7.4), containing the protease inhibitors iodoacetamide, phenylmethylsulfonyl fluoride and soybean trypsin inhibitor (1 mM each) and extracted by shaking for 45 minutes at 4°C.

Immunoprecipitation with L5 monoclonal antibody (1 µg/sample) from culture supernatants and cell lysates was performed as described previously (Streit et al., 1990). Precipitates were separated by SDS-

PAGE under non-reducing conditions on 6% gels, which were then processed for autoradiography.

Immunoblotting

For immunoblotting, embryos of different stages were dissected in calcium- and magnesium-free Tyrode's saline containing protease inhibitors (see above), collected in a minimal volume of saline and immediately frozen on dry ice. HGF/SF treated explants were obtained as described above for immunoprecipitation except that they were not radioactively labelled. The tissue was homogenized in sample buffer for SDS-PAGE without reducing agents, the samples boiled for 10 minutes and centrifuged at 10,000 g for 5 minutes. The supernatant was separated by SDS-PAGE on 6% gels and transferred to nitrocellulose BA85 (Schleicher & Schüll). Blots were probed with L5 monoclonal antibody; HRP-coupled secondary antibodies and the ECL-system (Amersham) were used for detection.

RESULTS

Expression of L5 and Sox-2

First, we compared the expression of the antigen L5 (Streit et al., 1990, 1995, 1996) with that of *Sox-2* mRNA and protein (Kamachi et al., 1995; Uwanogho et al., 1995) during early chick development by whole-mount in situ hybridization with DIG-labelled riboprobes and immunostaining with antibodies specific for L5 and SOX-2. The three patterns of expression are roughly similar. At stages 3⁺-4, L5 is expressed in a broad domain centred around the node and extending to the lateral and anterior part of the area opaca (aao in Fig. 1A), while the posterior part (pao in Fig. 1A) lacks expression. *Sox-2* mRNA is detected at low level in the entire area pellucida and in a thin ring of area opaca that includes anterior and posterior regions (aao and pao in Fig. 1E). SOX-2 protein can only be detected at high levels in the area pellucida, centred on the node (Fig. 1I). By stages 6-7 (Fig. 1B,F,J) all of them have become confined to an identical region, corresponding to the future neural plate. This is more evident at later stages (from stage 8-9; Fig. 1C,D,G,H,K,L), where L5 and *Sox-2* mRNA and protein are all restricted to the entire neural tube.

The early patterns of expression of both molecules are reminiscent of the regions of the embryo that are competent to respond to neural induction as described in the literature (Waddington, 1936; 1940; Woodside, 1937; Gallera and Ivanov, 1964; Gallera, 1970, 1971; Dias and Schoenwolf, 1990; Storey et al., 1992; Streit et al., 1995). However, given the slight differences, we can investigate directly whether competence is mirrored more closely by L5 or by *Sox-2*.

Competence to respond to neural induction is confined to L5-expressing regions

To assess which of the two molecules most closely reflects the changes in competence of the epiblast, a node was grafted into regions of the area opaca expressing both markers, neither marker or *Sox-2* only (Fig. 2). The regions chosen were, respectively: the area opaca of a stage 4 host at the level of the node, the same region at stage 5-6, and the posterior region of the area opaca at stage 4. We used quail/chick chimaeras to distinguish graft from host. The induction of a neural plate in the host was assessed both by morphology in sections and by the expression of L5 and *Sox-2*, the only available pan-neural markers at these stages of development.

Grafts of Hensen's node into a region expressing both L5 and *Sox-2* induce an ectopic neural plate in the host, which expresses both L5 (6/6 embryos; Fig. 2A,a'; see also Roberts et al., 1991) and *Sox-2* (10/12; Fig. 2D,d'). In contrast, when grafted into a region that expresses neither marker, the host does not form a neural plate and expression of L5 ($n=8$; Fig. 2B,b') and *Sox-2* ($n=13$; Fig. 2E,e') is confined to graft-derived quail cells. Finally, when a node is grafted into the posterior region of the area opaca, which expresses only *Sox-2*, there is no induction of a neural plate from the host and both markers are again only expressed in graft-derived quail tissue ($n=14$; Fig. 2C,c'; F,f'). In conclusion, L5 expression reflects the competence of the epiblast to respond to a graft of Hensen's node more closely than does *Sox-2*: only L5-positive areas can respond to neural induction.

HGF/SF maintains L5 but not *Sox-2* expression in vivo

We previously reported that the expression of L5 can be maintained and upregulated in vitro in the presence of HGF/SF (Streit et al., 1995), a factor expressed in Hensen's node at the time of neural induction. We therefore tested whether this factor can upregulate the expression of L5 and/or *Sox-2* in vivo, using grafts of HGF/SF-secreting MRC5 cells or transfected COS cells into the inner margin of the area opaca of a stage 3⁺-4 host embryo. MRC5 cells maintain L5 (14/15; Fig. 3A,E) but not *Sox-2* (0/19; Fig. 3D) in this region. HGF/SF-transfected COS cells grafted into a host embryo also generate ectopic L5 expression (9/10; Fig. 3B); however, this is less widespread, being confined to the epiblast immediately adjacent to the grafted cell pellet. This difference correlates with the level of expression of HGF/SF in both cell types as determined in the standard MDCK scattering assay: transfected COS cell supernatant was 16-32 times less active than MRC5-conditioned medium (not shown). Thus, HGF/SF can maintain L5 but not *Sox-2* expression.

If MRC5 cells are grafted into the lateral area opaca of a stage 4⁺-6 embryo or into the posterior area opaca of a stage 4 embryo (both regions are L5 negative), the epiblast cannot be induced to express L5 (stage 4⁺-6: 1/14 (not shown); posterior area opaca: 0/10; Fig. 3C). Therefore, HGF/SF secreting cells can maintain and upregulate L5, but only in regions that already express it.

Rescue of the competence of the epiblast

The results described above implicate L5, but not *Sox-2*, in neural competence. If so, we would predict that maintenance of L5 expression for a longer period than in normal development should also retain the competence of the epiblast. Since cells secreting HGF/SF can be used to prolong L5 expression, we designed the following experiment (Fig. 4A). MRC5 (or control cells) were transplanted into the area opaca of a host embryo at stage 4 to maintain L5 ectopically. The embryos were then grown until they had reached stages 5-6, by which time the area opaca has normally lost both L5 expression and neural competence. Then, a node (experimental) or posterior primitive streak (control) from a quail donor was transplanted next to the cell pellet and the embryos incubated until they had reached stages 10-15. After fixation, the morphology of the secondary axis was assessed and the embryos scored for induction of a nervous system from host cells, and for its regional characteristics. We

used immunostaining with a quail-specific antibody (QCPN), *Sox-2* (as a pan-neural marker) and the early-expressed region-specific neural markers *Tailless* (forebrain), 3A10 (diencephalon, hindbrain and anterior spinal cord), *Krox-20* (rhombomeres 3 and 5) and *Hoxb-9* (posterior neural tube). In total, 69 experimental and 60 control grafts were scored.

In 68% (47/69) of experimental grafts (initial graft of MRC5 cells followed by a node), a very elongated secondary axis developed containing neural structures, notochord and somites as judged by morphology in whole mounts. Of the remaining cases, 19% showed very modest elongation of the graft and 13% remained undifferentiated. When the contribution of the host to the ectopic axial structures was assessed, we found that the notochord and somites were always derived from the grafted quail node, as expected. However, an ectopic, **host**-derived neural tube was observed in 91% of cases (29/31; Fig. 4B-E), always located above the quail tissue and not above the grafted MRC5 cells. There was no apparent correlation between the extent of self-differentiation of the graft and the presence of the host neural plate. None of the regional markers tested (*Tailless*, 0/8; *Krox-20*, 0/13; 3A10, 0/6; *Hoxb-9*, 0/21) was expressed in host tissue (not shown). In contrast, *Sox-2* expression in host cells was found in 85% (18/21) of cases (Fig. 4B-E).

Three types of control experiments were performed: (a) a quail node grafted alone into a stage 5-6 host ($n=19$); (b) control cells followed by a node ($n=16$); (c) MRC5 cells followed by a graft of **posterior** primitive streak (which lacks inducing activity; $n=25$). Each experimental embryo contained one of these controls on the contralateral side. Only in 2/60 of the first two types of controls had the graft elongated considerably; in 22% the graft showed modest elongation but never morphologically distinct features such as somites, notochord or neural tube and 74% remained undifferentiated. Cells derived from grafts of posterior streak always dispersed and the quail tissue integrated into the host. None of the control grafts analyzed had a host-derived neural plate above the transplant, or ectopic expression of any of the pan-neural or regional markers (Fig. 4F-G).

In another set of experiments, embryos that had received a graft of HGF/SF secreting cells at stage 3⁺-4 were cultured to stage 6-7, before a node was grafted next to the cell pellet. We observed a host-derived neural plate expressing *Sox-2* above the graft in about 60% of the cases (data not shown); however, we did not find any evidence of a neural tube. This is most probably due to the fact that these embryos cannot be cultured for long enough for folding to occur.

These results show that the loss of competence of the epiblast that occurs between stages 4 and 4⁺ can be rescued by a graft of HGF/SF-secreting MRC5 cells, which maintains L5 (but not *Sox-2*) expression. This demonstrates that the period of neural competence of cells outside the prospective neural plate can be prolonged experimentally.

HGF/SF-secreting cells act on the host epiblast, not the grafted node

Could the results of the competence rescue experiment described above be explained by a direct action of HGF/SF on the grafted node? To test this, we grafted a stage 4 quail Hensen's node together with a pellet of MRC5 cells into a stage 5 host chick embryo. None of the grafts gave rise to host-derived neural structures expressing *Sox-2* (0/11; not shown).

The effects of HGF/SF-secreting cells are not due to prior induction of mesoderm

When MRC5 cells are transplanted into the area pellucida of early primitive streak stage embryos, they occasionally cause the appearance of a second primitive streak or mesodermal condensations (Stern et al., 1990). This raises the question whether the maintenance of L5 expression and of competence seen after grafting MRC5 cells is due to prior induction of mesoderm, which then induces neural tissue. To test this, we analyzed embryos grafted with pellets of MRC5 cells for expression of *gooseoid* (a marker for the organizer and prechordal region) and of *brachyury* (a general mesodermal marker). We did not find expression of either marker (Fig. 3F,G; $n=12$).

Two distinct L5 glycoproteins define competence and early neural differentiation

To investigate whether the early expression of L5 (related to neural competence) and the later (neural specific) phase represent different molecules, we performed immunoblots at different stages (Fig. 5A). We find that early embryos (stages 2-4) express a protein of approximately $220 \times 10^3 M_r$ which is undetectable at later stages. Embryos at stage 4 begin to express a high relative molecular mass doublet of greater than 450×10^3 , which becomes stronger after stage 6. Hereafter, we will refer to these two glycoproteins as L5²²⁰ and L5⁴⁵⁰, respectively. The expression of L5²²⁰ corresponds to the period of neural competence, while L5⁴⁵⁰ correlates with the neural-specific phase.

HGF/SF upregulates the competence-related L5²²⁰, but not the neural-specific L5⁴⁵⁰

Since two distinct L5 glycoproteins are associated with competence (L5²²⁰) and neural differentiation (L5⁴⁵⁰), we investigated which of these is a target of HGF/SF by immunoprecipitation of metabolically labelled explants (Fig. 5B) and by immunoblots (Fig. 5C). We find that only the lower molecular mass glycoprotein L5²²⁰, which can be detected in the culture supernatant, is upregulated by HGF/SF in both area opaca and area pellucida explants (Fig. 5B, arrow). This upregulation is dependent on the concentration of HGF/SF (Fig. 5C). The area opaca never expresses the larger, neural-

specific doublet L5⁴⁵⁰, even after HGF/SF treatment. This finding supports the conclusion that HGF/SF modulates competence, rather than being an inducer of some 'early neural' state.

Distinct signals are required for the maintenance of L5 and Sox-2

Previous experiments showed that the inducing capacity of Hensen's node starts to decline after stage 4 (Gallera, 1970; Dias and Schoenwolf, 1990; Storey et al., 1992). To investigate whether the ability of the node to induce and/or maintain expression of L5 or *Sox-2* correlates with its neural inducing ability, we performed the following experiments. Nodes from quail donors of different stages (3⁺-8) were transplanted into the area opaca of host embryos of a constant stage (3⁺/4) and then stained for L5 or *Sox-2*. We find that stage 3⁺-4 Hensen's nodes can generate ectopic expression of both L5 (6/6) and *Sox-2* (10/12) (Fig. 2A,a'; D,d'). By stage 4⁺-5 the ability of Hensen's node to elicit *Sox-2* expression in host tissue has been lost (0/6; Fig. 6A,B), whereas L5 expression can still be generated by some stage 4⁺-7 nodes (14/24 [58%]; Fig. 6C,D). Thus, the ability of the node to generate L5 is retained for as long as it possesses the ability to induce any type of neural tissue in the host (Storey et al., 1992), whereas *Sox-2* can only be induced by nodes capable of inducing a full range of both anterior and

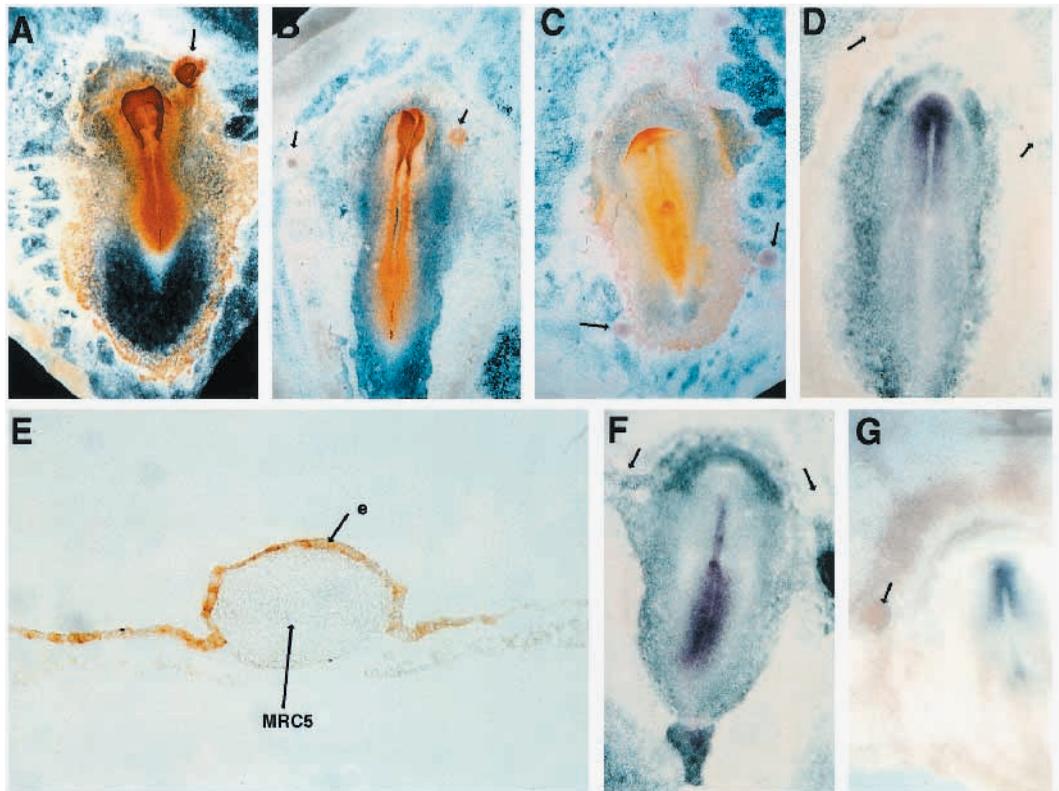


Fig. 3. MRC5 cells and COS cells transfected with *HGF/SF* maintain L5 but not *Sox-2* expression, and do not induce mesoderm. When MRC5 cells (A,E) or *HGF/SF*-transfected COS cells (B) are implanted into the anterior-lateral area opaca of stage 3⁺-4 hosts, L5 expression is prolonged to at least stage 8. The position of the grafts is indicated by arrows in the whole mounts. In B, the left graft corresponds to control cells transfected with *lacZ* and the right to *HGF/SF* transfected cells; only the right implant maintains L5. When MRC5 cells (arrows) are implanted posteriorly (C), no maintenance of L5 is seen. Anterior-lateral grafts of MRC5 cells (arrows) into the same stage embryos do not induce *Sox-2* (D) or the mesodermal markers *brachyury* (F) or *gooseoid* (G). E is a section through the same embryo as in A, showing the grafted cells (MRC5) and the L5-expressing epiblast above them (e).

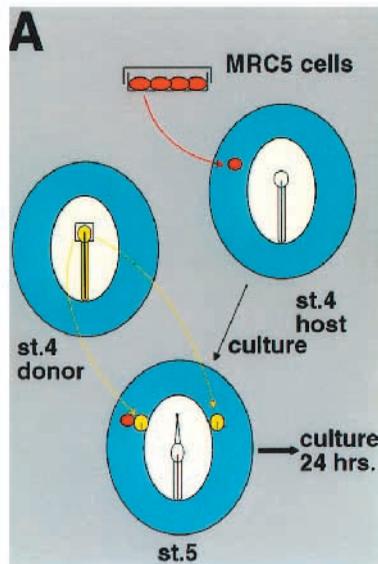
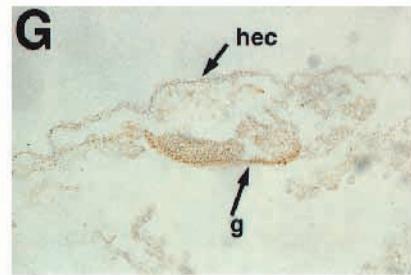
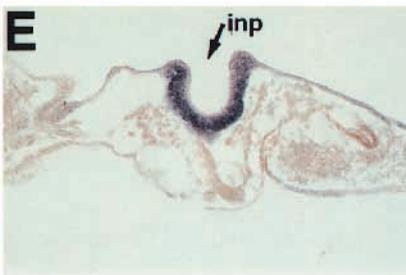


Fig. 4. Ectopic maintenance of L5 rescues the loss of competence to respond to neural inducing signals from a node graft. (A) Diagram explaining the experimental design. When MRC5 cells are grafted into the anterior area opaca of a stage 4 embryo, followed at stage 5 by a quail node, the host embryo can respond to the node graft by generating an ectopic neural plate/tube that expresses *Sox-2* (left arrow in B; C-E). This is not seen when a node is grafted at stage 5 without prior implantation of MRC5 cells (right arrow in B; G) or when this is preceded by a graft of control cells (F). In all cases, brown immunoperoxidase product reveals the grafted quail cells and the blue signal corresponds to *Sox-2* mRNA.



posterior structures (up to stage 4). This suggests that different signals are required for the maintenance of L5 and *Sox-2*.

To distinguish whether *Sox-2* induction requires a signal that is lost from the node after stage 4 or whether there is a quantitative decrease in the strength of the signal, we grafted two nodes from stage 5 quails (to compensate for a quantitative loss) into the area opaca of a stage 3⁺-4 host embryo. We find that such grafts cannot induce *Sox-2* in host tissue (0/10). We therefore conclude that the signal(s) controlling *Sox-2* expression disappear from the node after stage 4⁺.

HGF/SF secreting cells cannot rescue the ability of stage 5 Hensen's node to induce anterior nervous system

We previously reported that *HGF/SF* mRNA is expressed in stage 3⁺-4⁺ Hensen's node in the chick embryo and disappears thereafter from the node (Streit et al., 1995). This coincides with the time when the inducing ability of Hensen's node starts to decline and only posterior nervous system can be generated.

We therefore investigated whether the ability of the node to induce anterior neural structures can be rescued by the presence of cells secreting HGF/SF. Quail stage 5 Hensen's node was transplanted into a host chick embryo at stage 3⁺-4 together with a pellet of MRC5 cells; embryos were kept in New culture until they had reached stage 11-15 and then processed for in situ hybridization with the forebrain marker *Talless*, the hindbrain marker *Krox-20* or the pan-neural marker *Sox-2*. We did not detect expression of any of the markers (0/26; not shown) in host tissue. These data indicate that disappearance of *HGF/SF* from stage 5 Hensen's node does not account for the inability of the node to induce anterior nervous system.

Taken together, our results implicate L5²²⁰ and HGF/SF in defining the competence of the epiblast to respond to neural inducing signals from the node, and show that competence is not just cell autonomous but can be regulated by external signals. *Sox-2* expression in the nervous system appears to be further downstream in the response to an inducing stimulus.

DISCUSSION

The early expression patterns of L5 and *Sox-2* raised the question of whether either or both of these markers could correlate with the ability of the epiblast ('competence') to respond to neural inducing signals. Expression of both begins in a broad domain that resembles the region generally believed to be competent (Gallera, 1970; Hara, 1978), and then becomes confined to the forming neural plate and neural tube. However, the patterns are not identical, and the differences allowed us to discriminate which of the two is a better marker: L5 expression closely mirrors the competent regions in both time and space, while *Sox-2* is also expressed initially in cells that lack the capacity to respond to a graft of Hensen's node.

In normal development, L5 expression and competence are both lost abruptly from the area opaca between stages 4 and 4⁺. A pellet of HGF/SF-secreting MRC5 cells or of transfected COS cells can maintain and enhance L5 expression beyond this stage. Does this also rescue neural competence? When Hensen's node is grafted into a region of a stage 5 embryo where L5 expression has been prolonged experimentally, neural structures develop from the host, which would otherwise be too old to respond ('competence rescue' experiment). This is not due either to an effect of the MRC5 cells on the grafted node or to prior induction of mesoderm. These experiments demonstrate that the competence to respond to neural inducing signals is not cell autonomous and that it can be manipulated by signals external to the responding cells.

MRC5 cells alone do not induce a neural plate

When MRC5 cells are transplanted into an embryo, they sometimes cause differentiation of a neural plate-like thickening of the ectoderm (Stern et al., 1990). Could this mean that MRC5 cells themselves are inducing a neural plate, which is subsequently stabilized by the grafted node in our competence rescue experiments? Rather than the typical columnar morphology of neural plate cells (Woodside, 1937; Bellairs, 1959; Messier, 1969; Gallera, 1970; Álvarez and Schoenwolf, 1991; Schoenwolf, 1985, 1992, 1994), these thickenings resemble the earliest response to a node graft (formation of cuboidal epithelium; Fig. 3E), which occurs just 6 hours after grafting (Gallera and Nicolet, 1969). These cuboidal thickenings never develop further and fail both to elongate and to fold into a tube (Stern et al., 1990). The responses to MRC5 cells followed by a graft of a node are quite different: not only does the epithelium become truly columnar (Fig. 4E), but it always forms a tube that elongates along one axis and in 85% of cases expresses the pan-neural marker, *Sox-2* (Fig. 4).

After MRC5 cell grafts, we observed such thickenings in just 15% of cases. In contrast, when the MRC5 cells are followed by a node, full neural structures are seen in 91% of cases. Therefore, the thickening of the epiblast seen after grafts of MRC5 cells alone cannot explain the behaviour after transplanting MRC5 cells plus a node.

Finally, the epiblast overlying grafts of MRC5 cells always expresses L5, regardless of whether or not a thickening is present. In conclusion, the transition of the epiblast to a cuboidal epithelium is **preceded** by maintenance and upregu-

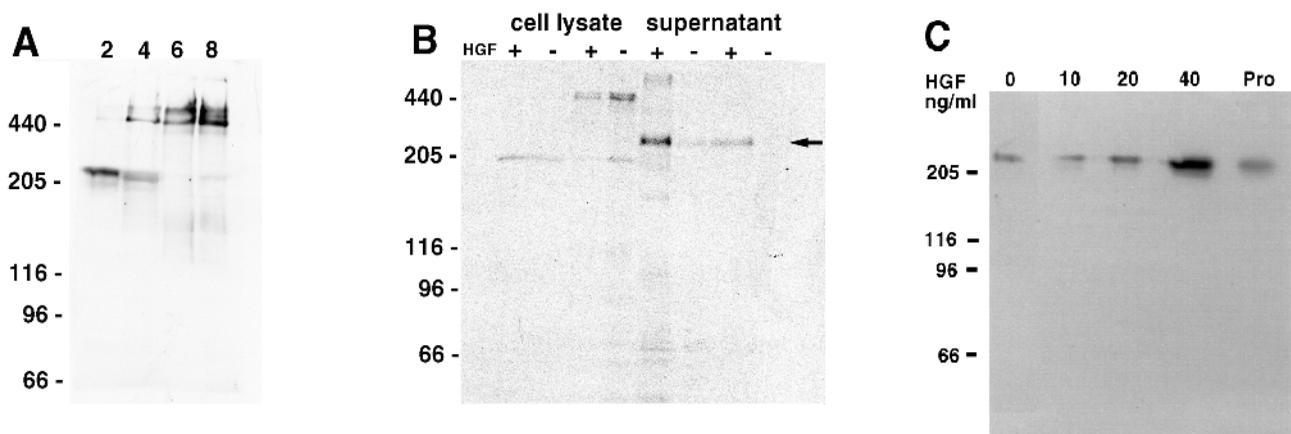


Fig. 5. Analysis of L5-glycoproteins. (A) Immunoblot of protein extracts of embryos at stages 2-8, probed with L5 antibody. Each lane (containing the equivalent of two embryos) is labelled with the stage, and molecular mass markers are shown on the left. Note that a lower molecular mass band of approximately 220×10^3 is present only at stages 2-4, while a higher molecular mass doublet (above 450×10^3) appears after stage 4. Thus, expression of L5²²⁰ corresponds to the period of neural competence and L5⁴⁵⁰ appears at the time of neural induction. (B) Immunoprecipitation of biosynthetically labelled explants. Explants of area opaca (lanes 1-2 and 5-6) and area pellucida (lanes 3-4 and 7-8) from stage 3⁺-4 chick embryos (5 each) were treated with HGF/SF (+) or prothrombin (-) in the presence of [³⁵S]methionine. After 5 hours, cell culture supernatants were collected, the cells lysed and both immunoprecipitated with 1 μ g L5 antibody. The immunoprecipitates were then separated by non-reducing SDS-PAGE and autoradiographed. L5²²⁰ (arrow) is present in the culture supernatant (lanes 5-8) and is strongly upregulated in HGF/SF treated explants from both regions. The expression of L5⁴⁵⁰ in the area pellucida (present in the cell lysates) is not affected. The area opaca explants do not contain the 450×10^3 M_r doublet, again suggesting that L5⁴⁵⁰ marks induced neural tissue. (C) Immunoblot of area opaca explants treated with different concentrations of HGF/SF, probed with L5 antibody. Area opaca explants from five stage 3⁺-4 chick embryos were cultured in the absence (0) or presence of prothrombin (100 ng/ml; Pro) or HGF/SF at 10 ng/ml, 20 ng/ml and 40 ng/ml (lanes 10, 20 and 40) for 5 hours. Culture supernatants were collected, precipitated with acetone and the pellets dissolved in sample buffer for SDS-PAGE without reducing agents. Samples were separated by SDS-PAGE on a 6% gel, blotted and the blots probed with L5 antibody. L5²²⁰ is upregulated by HGF/SF in a concentration dependent manner. Molecular mass markers are indicated on the left.

lation of L5 expression, which reflects an elevated competence of the tissue to respond to neural inducing signals.

Reciprocal interactions between the induced neuroepithelium and the inducing node

The finding that nodes placed adjacent to a prior graft of MRC5 cells elongate more than do nodes grafted alone or grafted simultaneously with the MRC5 cells suggests that the induced neuroepithelium somehow acts back on the node to stimulate its development. This agrees with other findings (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994; Münsterberg and Lassar, 1995) suggesting that signals from the neuraxis promote somite development.

The role of HGF/SF in neural induction and competence

In situ hybridization to detect *HGF/SF* showed that this factor is no longer expressed in Hensen's node after stage 5. However, nodes as old as stage 7 can still maintain the expression of L5 in the epiblast. It is possible that the node can secrete a factor other than HGF/SF that is also capable of maintaining the expression of L5. However, HGF/SF protein may still be secreted by the node for some hours after the disappearance of transcripts; in fact, the time difference between stages 5 and 7 in the chick is just 1-4 hours (Hamburger and Hamilton, 1951).

In vitro, epiblast explants treated with HGF/SF maintain and upregulate the expression of L5²²⁰ in 100% of cases and additionally, in 50% of cases, they continue to differentiate into cells with mature neuronal traits, such as neurofilament proteins and the growth of long neurites (Streit et al., 1995). Could HGF/SF be a neural inducer? This seems unlikely because grafts of secreting cells never lead to neural differentiation in vivo unless a node is also transplanted. This suggests that additional factors are present in the cultures (which also contain serum) that cooperate with HGF/SF in giving rise to neurons in a proportion of the cases. Finally, HGF/SF only maintains and upregulates the expression of the early L5²²⁰ glycoprotein, and not the neural-specific L5⁴⁵⁰ molecule. These experiments provide direct support to the idea that HGF/SF maintains the competence of the epiblast to respond to other, neural inducing signals.

The role of L5 in neural induction and competence

Our experiments establish L5²²⁰ as a marker for the competence of the epiblast to respond to signals from Hensen's node: its expression in the embryo mirrors competence very closely in time and space, and ectopic maintenance of its expression, in the absence of *Sox-2*, is able to rescue the loss of competence that normally occurs at stage 4⁺. In addition, L5 may be involved directly in the response to Hensen's node derived signals, because anti-L5 antibody inhibits this response (Roberts et al., 1991). This 'loss-of-function' type experiment complements the present 'gain-of-function' approach, suggesting that L5 is involved in the response and that ectopic maintenance of its expression also maintains competence.

It is also interesting that only those regions that already express L5 can be made to maintain this expression by grafts of either MRC5 cells or a node. One might therefore speculate that maintenance of L5 expression is under positive feedback control and requires active enhancement through the participation of L5-positive molecules.

A hierarchy of steps in response to signals from Hensen's node

Waddington and Needham (1936) and subsequently Nieuwkoop and colleagues (Nieuwkoop et al., 1952; Nieuwkoop and Nigtevecht, 1954) suggested that at least two distinct steps make up the responses of the ectoderm to the organizer in generating a nervous system. The first, which Waddington termed 'evocation' and Nieuwkoop 'activation' consists of a diversion of the fates of the ectodermal cells from epidermal to neural. The second step, called 'individuation' by the former and 'transformation' by the latter, is the process by which the early neural primordium acquires stable anteroposterior characteristics. The main difference between the proposals of the two authors is that Nieuwkoop suggested that the earliest inducing signals give rise to anterior neural structures, while the later 'transforming' principles gradually posteriorize these to generate the remaining regions of the CNS. Recently, FGFs and Wnts have been implicated in this process in *Xenopus* (Cox and Hemmati-Briivanlou, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995; McGrew et al., 1995; Launay et al., 1996). Waddington's 'evocation', however, implies that early signals produce a neural primordium without regional character of any sort, and later signals then impart this.

Our experiments begin to place three distinct groups of signals into a molecular cascade involved in the responses of cells to signals emanating from the organizer. We have called them **competence**, **neuralization** and **regionalization**. We can now use L5²²⁰ as a marker for cells that are competent to respond to signals that generate a neural plate, and have shown that sources of HGF/SF (MRC5 cells, transfected COS cells and the node itself) can maintain and upregulate its expression (Streit et al., 1995 and the present experiments). *Sox-2*, in contrast, appears to lie further downstream. It can be induced by the node but not by HGF/SF. Since HGF/SF can maintain both L5²²⁰ and competence but cannot induce further neural differentiation in the absence of a node, this factor appears to lie upstream of the signals emanating from the node that are required for later steps in neural development. Moreover, the experiment in which we combined an older node with MRC5 cells, which failed to rescue its ability to induce anterior structures, suggests that MRC5 cells act by maintaining competence but do not affect neuralizing or regionalizing signals.

When does anteroposterior regionalization occur?

Two unrelated observations suggest that regionalizing signals can at least continue until rather late stages of development, long after the node has lost its inducing activity. First, when metencephalic neuroepithelium is transplanted to the forebrain at stage 10, cells surrounding the graft acquire *En-2* expression and cerebellar phenotype (Itasaki et al., 1991; Martínez et al., 1991; Bally-Cuif et al., 1992; Nakamura et al., 1994; Crossley et al., 1996). Second, transplantations of somitic mesoderm can respecify the Hox-code of neighbouring hindbrain neuroepithelium (Itasaki et al., 1996), as can relocations of the hindbrain neuroepithelium itself (Grapin-Botton et al., 1995; Itasaki et al., 1996).

Interestingly, the neural plate that develops in our 'competence rescue' experiment expresses the pan-neural marker *Sox-2*, but does not express any of the early regional markers tested, which cover the forebrain (*Tailless*), diencephalon (3A10),

hindbrain (3A10 and *Krox-20*) and posterior spinal cord (*Hoxb-9*). Thus, our experiments allow us to separate neuralization from regionalization experimentally: these neural plates are neuralized, but fail to become regionalized along the anteroposterior axis, even though these embryos were sometimes allowed to develop to stage 15, long after the time (stages 8-11) when all these markers start to be expressed normally. There are two possible explanations for this failure of regionalization. First, it is possible that the acquisition of positional identity starts much later than neuralization. But then, why does the node and its derivatives fail to impart this information in our experiment, if it can do it in normal embryos within the same time course? Second, it is possible that regionalization, although it remains plastic for a long time (as discussed above), actually must **begin** very early, before stage 5.

Comparison with neural induction studies in amphibians

Our results in which a graft of a node at stage 5, preceded by MRC5 cells ('competence rescue' experiment) gives rise to a neural plate that does not express any regional marker supports the concepts of Waddington (Waddington and Needham, 1936; Waddington, 1940) rather than the activation-transformation hypothesis of Nieuwkoop (Nieuwkoop et al., 1952; Nieuwkoop and Nigtevecht, 1954). To our knowledge, this is the first known instance of a nervous system being induced that lacks all regional characteristics; Hamburger (1988; page 171) stated: "... *no experiment on the induction of the nervous system has produced a clear case in which a generalized, not region-specific, but otherwise well-developed nervous system was differentiated.*"

Interestingly, the three main molecules thus far reported to have neural inducing activity in *Xenopus* (Noggin, Lamb et al., 1993; Follistatin, Hemmati-Brivanlou et al., 1994; Chordin, Sasai et al., 1995) all induce expression of anterior but not posterior neural markers, apparently supporting Nieuwkoop's ideas. Moreover, prospective anterior nervous system can be made to express more posterior markers by combination with a posterior explant or treatment with FGFs or Wnts (Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995; McGrew et al., 1995). Is there a significant difference in the mechanism of neural induction in amphibians and amniotes, or are the differences due to the assays that can be used in the two vertebrate classes?

That at least some of the signals for early neural development are conserved between different vertebrate classes has been illustrated most clearly by numerous interspecific grafting experiments (Waddington and Waterman, 1933; Waddington, 1934, 1936; Oppenheimer, 1936; Kintner and Dodd, 1991; Blum et al., 1992; Streit et al., 1994; Hatta and Takahashi, 1996). However, it is interesting that chick Hensen's nodes older than stage 6-7, which have very reduced, if any, neural inducing activity in a chick host, can nevertheless

still induce a *Xenopus* animal cap (Kintner and Dodd, 1991) and zebrafish ectoderm (Hatta and Takahashi, 1996). This might suggest that it is easier to neuralize the ectoderm of lower vertebrates than chick epiblast. One possible explanation is that *Xenopus* animal caps excised from stage 10-11 embryos have already been exposed to early neuralizing signals, but still require further signals before they undergo neural differentiation and expression of regional markers. This is also supported by observations that cell dissociation can neuralize amphibian ectoderm (Godsave and Slack, 1991) but not chick epiblast (unpublished observations), and Noggin and Chordin appear to lack direct neuralizing activity in chick (unpublished observations).

Neural competence is not entirely cell autonomous

In *Xenopus*, four types of molecules have been implicated in the establishment or maintenance of neural competence: G-proteins, Protein Kinase α , *Xotch* and *ASH-3* (Pituello et al., 1991; Otte and Moon, 1992; Otte et al., 1992; Coffman et al., 1993; Turner and Weintraub, 1994). However, all of the results presented by these authors can be interpreted equally well in terms of these molecules acting downstream of neural inducing signals, because dorsoventral differences in the ectoderm could result from exposure to early inducing signals. None of these studies has tested neural competence directly by prolonging the time during which cells can respond to neural induction. In the case of G₀-like protein (Pituello et al., 1991), the claim is based entirely on an expression pattern comparable to that described here for *Sox-2*, yet we demonstrate that *Sox-2* is not involved in neural competence. For these reasons, we believe our study on L5 to be the first demonstration that the period of neural competence of cells outside the prospective neural plate can be prolonged experimentally.

Interestingly, all of the molecules whose manipulation has resulted in alterations in competence in *Xenopus* are intracel-

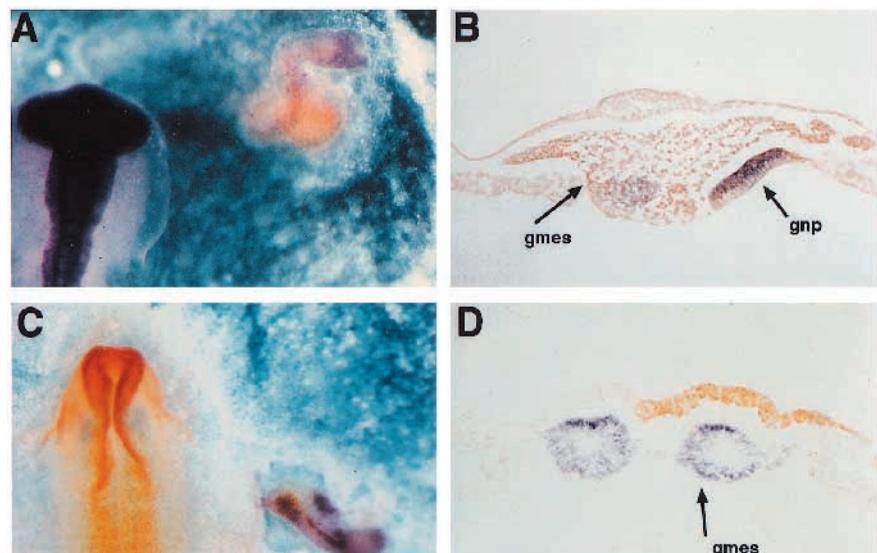


Fig. 6. Older nodes can still maintain L5, but not *Sox-2* expression. (A,B) When a stage 5 quail (brown) node is grafted into the area opaca of a stage 4 host, *Sox-2* (blue) is not induced in the host. (C,D) When a stage 5-6 quail (blue) node is grafted into the area opaca of a stage 4 host, L5 expression (brown) is maintained in the host. gmp, graft-derived neural plate. gmes, graft-derived mesoderm.

lular, while HGF/SF is a secreted factor. This argues against the idea, derived mainly from experiments such as aging animal caps in isolation, that competence is entirely cell autonomous (Holtfreter, 1938; Gurdon, 1987; Servetnick and Grainger, 1991).

Conclusion: a hierarchy of decisions during neural induction

The experiments described in this paper firmly establish $L5^{220}$ as a marker for cells competent to respond to neural inducing signals from Hensen's node in the chick embryo. They also allow us to begin to place these signals into a hierarchical, molecular cascade in which $L5^{220}$ and competence lie upstream of neuralization and of the mechanisms localizing *Sox-2* to the nervous system, and all of the above appear to be independent of anteroposterior regionalizing signals. This distinction between different classes of signals should help in the design of screening strategies for the molecules involved.

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