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The L5 epitope: an early marker for neural induction in the chick embryo and its involvement in inductive interactions

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Summary

The pattern of expression of the carbohydrate epitope L5 was studied during early development of the chick neuroepithelium. Immunoreactivity first appears during gastrulation, at mid-primitive streak stage, and persists until at least 3.5 days of development. The epitope is expressed on all the components of the developing nervous system, both central and peripheral. In immunoblots, the antibody recognises a major component of about M_r 500 000 and several more minor components of lower molecular mass.

If a Hensen's node from a donor embryo is transplanted into the *area opaca* of a host embryo, L5 immunoreactivity appears in the epiblast surrounding the graft. If hybridoma cells secreting the antibody are grafted together with Hensen's node into a host chick embryo, the induction of a supernumerary nervous system is inhibited. We suggest that the L5 epitope is an early and general marker for neural induction and that it may be involved directly in inductive interactions.

Key words: neural induction, chick embryo, glycoproteins, carbohydrate, neuroepithelium.

Introduction

Neural induction is the process during which the axial mesoderm interacts with the overlying ectoderm to instruct it to become nervous system. In the absence of such an inductive interaction, uninduced epiblast only gives rise to epidermis (reviewed by Nieuwkoop *et al.* 1985). It was first discovered by Spemann and Mangold (1924) in amphibian embryos; they demonstrated that the tissue responsible for induction (the 'organizer') is the dorsal lip of the blastopore. In amniotes, the equivalent of the dorsal lip is Hensen's node (see Nieuwkoop *et al.* 1985). In both cases, if organizer tissue is transplanted to regions which do not give rise to neural plate in the normal fate map, the adjacent ectoderm develops into a supernumerary neural plate.

This process has received less attention than another early embryonic induction, that of the mesoderm (reviewed by Smith *et al.* 1988; Smith, 1989). One reason for the dearth of information about neural induction, particularly in amniotes, is the lack of molecular markers specific for the early changes in this process. Such markers are essential if an *in vitro* assay for induction is to be developed.

In amphibians, three genes have been used as early and general neural markers: N-CAM mRNA (Kintner and Melton, 1987), the homeobox gene *XIF3* (Sharpe, 1988) and *Xotch* (Coffman *et al.* 1990). No general markers specific for neuroepithelium have been described in amniotes, except for $pp60^{c\text{-}src}$ in the chick (Manness *et al.* 1986), which is not expressed exclusively in the neuroepithelium and various neurofilament-associated proteins (e.g. Furley *et al.* 1990), which are not expressed in glial cells or some neural crest derivatives.

Several carbohydrate antigens have been described as being expressed in a region-specific manner during early development of diverse embryos such as *Drosophila* and grasshopper (Snow *et al.* 1987), mouse (e.g. Solter and Knowles, 1978; Kannagi *et al.* 1983; Dodd and Jessell, 1985; Dodd *et al.* 1988; Fenderson *et al.* 1988) and chick (Canning and Stern, 1988; Thorpe *et al.* 1988; Loveless *et al.* 1990). In particular, it was recently suggested that the HNK-1/L2 carbohydrate may be involved in induction of the chick mesoderm (Canning and Stern, 1988). Since some of these carbohydrate antigens are expressed on molecules with a role in cell recognition, we decided to investigate the expression of one such carbohydrate epitope, described by Streit *et al.* (1990) and known as L5. In adult mouse brain, this epitope is carried by cell recognition molecules such as L1 (Ng-CAM) and Thy-1 and on a high M_r (500×10^3)

proteoglycan containing chondroitin sulphate (Streit *et al.* 1990).

We report that the L5 monoclonal antibody (Streit *et al.* 1990) can be used as a general, early neural marker in the chick embryo. Immunoreactivity is induced in the epiblast after a graft of Hensen's node and the antibody interferes with the inductive response to a graft of Hensen's node. We therefore suggest that the L5 epitope or its carrying molecules may be involved directly in neural induction.

Materials and methods

Antibodies

Monoclonal antibody L5 was obtained as decribed previously (Streit *et al.* 1990). Briefly, mouse myeloma clone Ag8–653 was fused with splenocytes from female Lou×Sprague Dawley F_1 hybrid rats (4–6 weeks old) that had been immunized (50 μg/injection) with 'rest L2' (Kruse *et al.* 1984; consisting of mouse brain L2/HNK-1-carrying molecules after removal of L1 antigen and N-CAM by immunoaffinity chromatography). Fusions were screened by the immunospot test, and positive supernatants were selected for cell surface immunoreactivity in monolayer cultures of cerebellar cells from early post-natal mice by indirect immunofluorescence. The monoclonal antibody L5 was determined to belong to the immunoglobulin-M subclass by gel filtration, SDS–PAGE and ELISA using subclass-specific antibodies.

Immunocytochemistry

Fertile hens' eggs were obtained locally (Coppocks Farm, Carterton) and incubated at 38 °C to obtain embryos between stage 3 and 25 (Hamburger and Hamilton, 1951). Embryos were explanted in Tyrode's saline and fixed immediately in buffered formol saline (pH 7.0) for 30 min, after which they were washed in phosphate-buffered saline (PBS; pH 7.4). They were then transferred to 5 % sucrose in PBS for a few hours, then into 20 % sucrose in PBS at 4 °C overnight, and then embedded in 7.5 % gelatin (Sigma, 300 Bloom) in 15 % sucrose/PBS at 37 °C. After allowing the gelatin moulds to set at room temperature, the embedded embryos were quickly frozen in isopentane cooled with solid CO_2 and sectioned serially on a cryostat at a thickness of 10 μm. The sections were transferred to gelatinized glass microscope slides.

Slides were stained for indirect immunocytochemistry using either peroxidase- or fluorescein-labelled second antibodies. Sections were immersed in PBS at 38 °C to remove the gelatin and then blocked in 2 % bovine serum albumin (BSA) (Sigma, Fraction V) for 1 h at room temperature in a humidified chamber. They were then incubated for 1 h at room temperature in primary antibody, undiluted supernatant from L5 hybridoma cells (Streit *et al.* 1990), to which BSA had been added to a final concentration of 0.5 %. At the end of this incubation, the sections were washed three times in PBS before incubation in an appropriate secondary antibody. For immunoperoxidase, a peroxidase-conjugated goat anti-rat IgM (Cappel, IgG fraction, μ-chain specific) (1:100 or 1:200 in 0.5 % BSA/PBS). For immunofluorescence, a two-layer system of goat anti-rat IgM conjugated to fluorescein isothiocyanate (FITC) (Cappel, μ-chain specific) was used. In some experiments, a three-layer system was used: the secondary antibody was a rabbit anti-rat IgM (Calbiochem), and this was followed by FITC-conjugated goat anti-rabbit IgG (Cappel, specific for Fc-fragment of γ-chain). In all cases, incubation in the secondary reagents was for 1 h at room temperature.

Slides stained by immunoperoxidase were developed in 500 μg ml^{-1} 3,3'-diaminobenzidine tetrahydrochloride (Aldrich) in 0.1 M Tris buffer (pH 7.4) to which H_2O_2 was added to a final concentration of 0.3 %. They were then washed in tap water and mounted in Aquamount (Gurr). Slides for immunofluorescence were washed again in PBS and then mounted in Gelvatol (14 % polyvinyl alcohol 20/30 [Fisons] containing 8.5 mg ml^{-1} diazobicyclo-octane [DABCO, Aldrich], 30 % glycerol and 350 μg ml^{-1} sodium azide as preservative in a PBS base, pH 6.8). Sections were viewed and photographed on an Olympus Vanox T microscope.

SDS–PAGE and immunoblotting

Samples of neuroepithelium and presumptive epidermis were dissected from stage 6–7 embryos in Tyrode's saline containing a mixture of protease inhibitors (1 mM phenylmethylsulphonylfluoride, 1 mM *N*-ethylmaleimide, 0.5 μg ml^{-1} leupeptin, 0.1 μg ml^{-1} aprotinin, 100 μg ml^{-1} *N*-tosyl-L-phenylalanine chloromethyl ketone, 50 μg ml^{-1} *N*,α-p-tosyl-L-lysine chloromethyl ketone) and immediately frozen on dry ice. After thawing they were homogenized in 50–100 μl of sample buffer without reducing agents (2 % SDS, 50 mM Tris pH 6.8, 10 % glycerol, 0.1 % bromophenol blue) before running at 100 μg protein per lane on a 3–15 % gradient SDS–polyacrylamide gel (Laemmli, 1970). A lane of prestained molecular mass markers (Sigma) and a lane of ^{14}C-labelled, prestained molecular mass markers (Rainbow Markers, Amersham) were also included in each gel. The protein samples were then transferred onto Immobilon P membrane (Millipore) using the semidry discontinuous buffer system as described by Streit *et al.* (1990) and in the LKB Bromma Multiphor II Nova Blot instruction manual; transfer was for 1 h at 250 mA.

In some experiments, the tissue was digested in 20 mM sodium phosphate pH 7.2, 10 mM EDTA, 40 mM *n*-octylglucoside containing 6 units of *N*-glycosidase-F (peptide-*N*-glycosidase-F; E.C. 3.2.2.18; Boehringer-Mannheim) for every 100 μg of tissue digested, at 37 °C for 18 h before immunoblotting as above. Control tissue was treated in the same manner in buffer solution minus the enzyme.

The membranes were then blocked overnight at 4 °C in 3 % BSA (Sigma) and 3 % milk powder (Marvel) in PBS before incubation in the primary antibody (undiluted L5 supernatant) at 4 °C overnight. After washing in PBS the blot was incubated for 2 h at room temperature in rabbit anti-rat IgM (Calbiochem; 1:200). They were then washed again and incubated in ^{125}I-protein G (Amersham; 0.1 μCi ml^{-1}) for 2 h at room temperature, then washed, air dried and exposed at −70 °C with intensifying screens on Kodak XR5 X-ray film for 1–7 days.

Grafting and perturbation experiments

In preliminary experiments to assess whether L5-related molecules play a role in neural induction, nine stage-4 embryos in New (1955) culture were incubated with monovalent fragments of the anti-L5 antibody. Eight control embryos were incubated in the presence of another IgM, antibody 4B5 (Norris, 1989). It was our impression that anti-L5 antibody fragments caused malformations of the neuroepithelium, but these results were obtained in only five out of nine embryos analysed. For this reason, we designed an experiment in which the hybridoma cells producing the anti-L5 antibody were grafted into a host embryo (Fig. 1). To investigate the effects of this antibody on neural induction, we

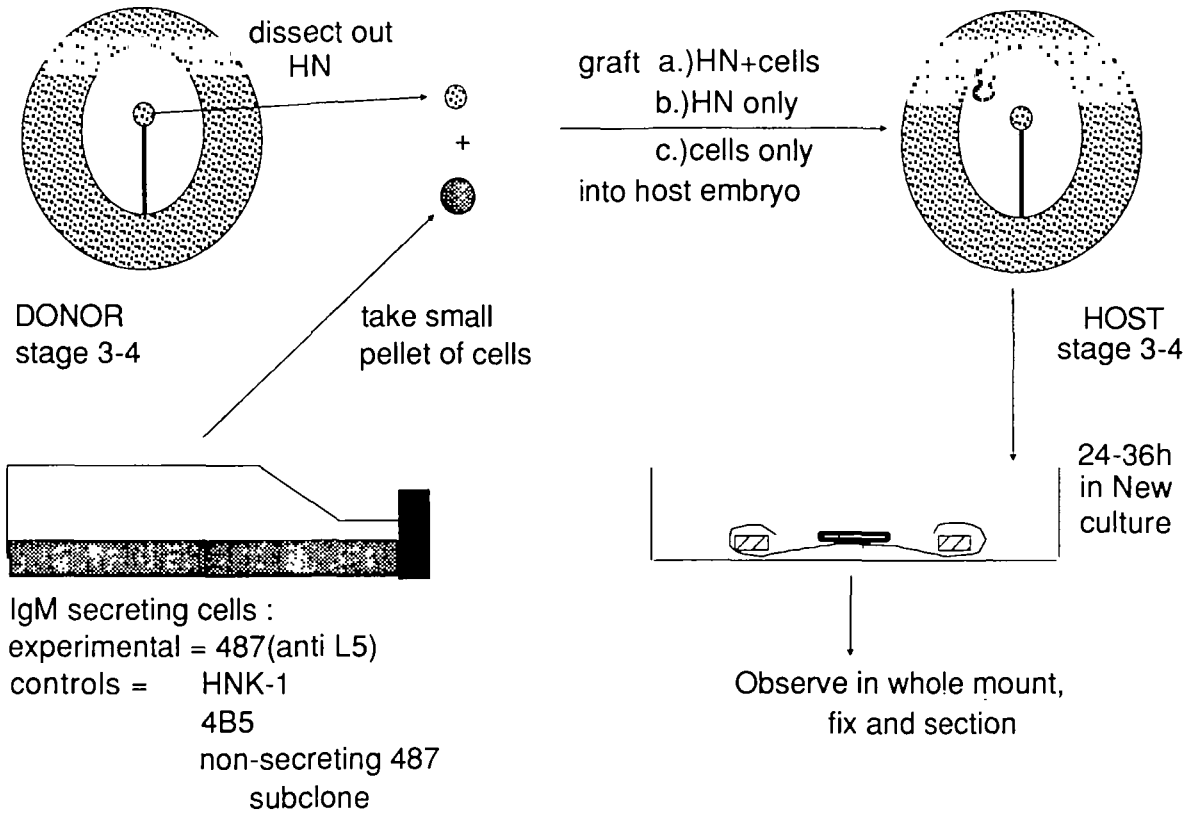


Fig. 1. Experiment to test if blocking the L5 epitope with a monoclonal antibody has a functional effect upon induction by HN.

introduced the hybridoma cells together with a graft of Hensen's node from a donor embryo. As controls, we used either: two other IgM-secreting hybridoma cell lines, whose antibodies recognise cell surface antigens that are also present at the same stages of development in all cells (4B5; Norris, 1989) or in subsets of cells (HNK1; Canning and Stern, 1988). Other control embryos were grafted with a subcloned line of the L5-hybridoma which did not secrete IgM. This was obtained by subcloning the L5-hybridoma and screening each well for Ig production.

Hybridoma cells to be grafted were prepared by brief centrifugation (1000 revs per min in a bench centrifuge). The pellet of cells was then removed from the bottom of an Eppendorf tube with fine steel needles and placed on ice until the donors were ready for grafting. Host stage 3–4 embryos were prepared for New (1955) culture. A Hensen's node from a stage 4 chick donor embryo was grafted under the hypoblast of each host, at the rostral end of the embryo, either alone or with a small piece of the cell pellet (500–5000 cells; see Stern *et al.* 1990) of one of the hybridoma lines, prepared as described above.

Grafted embryos were incubated at 38°C overnight, fixed in buffered formol saline and stored in 70% ethanol before paraffin wax embedding and sectioning at 10 μm. After dewaxing, they were stained with Harris's haematoxylin and mounted in DePeX. A few embryos grafted with hybridoma cells were processed for cryostat sectioning and stained with FITC-labelled goat anti-rat IgM to visualize the grafted hybridoma cells.

In another series of embryos, we investigated whether a graft of Hensen's node into the *area opaca* of a host embryo induces the appearance of immunoreactivity with anti-L5 antibody. Both donor and host embryos were at stage-4 at the time of grafting. After incubation at 37°C overnight, the embryos were fixed in buffered formol saline and sectioned in a cryostat for immunocytochemistry using anti-L5 antibody as described above.

To test whether the neural plates visible in the *area opaca* of grafted embryos was of donor or host origin, the grafted Hensen's node was labelled with the carbocyanine dye DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine perchlorate; Molecular Probes, Inc.). One part of a stock solution of DiI (0.5% in absolute ethanol) was warmed to 45°C and mixed with nine parts of a 0.3 M sucrose solution in distilled water. This was applied to the region of Hensen's node of a donor embryo with air pressure until the whole region was seen to be evenly labelled. Hensen's node was then excised with tungsten needles and grafted into the *area opaca* of an unlabelled host chick embryo as described above. After New (1955) culture of the grafted embryos, the embryos were fixed in 4% formaldehyde in PBS, and photographed under epi-fluorescence optics as whole mounts. After this, a photo-oxidation procedure was used to allow the label to be visible after histology (Stern, 1990). Embryos were washed in 0.1 M Tris–HCl (pH 7.4), placed in a cavity slide containing 500 μg ml^{-1} DAB in the same buffer and covered with a coverslip. The assembly was placed on the microscope under a 10× or 20× microscope objective and illuminated for epifluorescence appropriate for DiI excitation (547 nm; peak emission, 571 nm), and exposed to light for about 1.5 h or until all the fluorescence had disappeared; the microscope was focussed every 10–15 min during the incubation. After incubation, the embryos were washed in PBS, incubated in sucrose and embedded in gelatin as described above. Finally,

the specimens were sectioned in a cryostat and stained with anti-L5 antibody as described above. After mounting in Gelvatol, sections were photographed using epi-fluorescence to visualise L5 and bright field to visualise the DiI-labelled cells.

Results

Immunocytochemistry

Immunoreactivity with monoclonal antibody against L5 in early chick embryos is first detected towards the middle of gastrulation (stage 3), when the central epiblast of the *area pellucida* displays patchy staining (Fig. 2A,B). By stage 4 (definitive primitive streak stage), the epiblast in the vicinity of Hensen's node and the surrounding region (presumptive neural plate) stain very intensely (Fig. 2C,D). There is a sharp boundary between the strongly staining central region and weakly staining more peripheral epiblast (presumptive epidermis); the *area opaca* epiblast is L5-negative. At the light microscope level, immunoreactivity appears to be associated with cell surfaces (Fig. 2F).

At stages 6–7, the elevating neural plate shows very intense staining, and immunoreactivity in the *area pellucida* epiblast decreases towards more peripheral regions of the embryo (Fig. 2E). An anteroposterior gradient of immunoreactivity is also seen at this stage, decreasing posteriorly. By stage 8, immunoreactivity seems to be restricted to the closing neural tube and is still present as a gradient, decreasing from anterior to posterior ends of the neuroepithelium.

From stage 10–11, the entire nervous system is strongly stained (Fig. 3). An anteroposterior gradient in staining intensity is still seen at this stage.

At stages 13–16, strong immunoreactivity is still seen in the neuroepithelium, but some staining is also seen in association with the developing optic, otic and olfactory placodes. The neural crest is also immunoreactive (Fig. 3D–F) as are the developing dorsal root ganglia derived from it (Fig. 3F). At this stage, some regions of the embryo display staining of the endoderm in the midline (Fig. 3B).

Strong staining is still apparent at 3.5 days of incubation. There is still strong staining of the entire nervous system and sensory organs, particularly in the telencephalon, the hindbrain and the posterior part of the tectum. Expression still decreases in a rostral-to-caudal direction along the neural tube. The dorsal root ganglia stain strongly, as do cranial ganglia V, VII and IX. The lens and retina, the semi-circular canals and acoustic ganglion also stain. By this later stage of development other tissues, such as portions of the endoderm of the pharyngeal arches (Fig. 4A–C), the gut and developing pancreas (Fig. 4D) also display L5 immunoreactivity, as do some mesodermal derivatives, especially sclerotome cells surrounding the notochord (Fig. 4E) and some mesonephric tubules (Fig. 4F). In the pharyngeal endoderm, staining appears to be restricted to the posterior and dorsal aspects, in association with the primordium of the developing pharyngeal tonsil (Fig. 4A,B).

Fig. 2. Pattern of L5 immunoreactivity in early stages of chick development. (A) Transverse section of a stage 3 embryo, with the epiblast showing patchy immunoreactivity with the anti-L5 antibody. (B) Transverse section through the epiblast at stage 4, just anterior to Hensen's node. The micrograph shows the edge of the presumptive neural plate region (c.f. Fig. 9). A sharp boundary is seen between expressing and non-expressing regions. Lateral (right) to this boundary, small, isolated groups of cells express immunoreactivity. Within the boundary (left), all cells appear to express. (C) Sagittal section through the posterior (left) end of the presumptive neural plate of a stage 4 embryo. (D) Sagittal section through a stage 4 embryo. L5 immunoreactivity is highest in the presumptive head region (arrow) and decreases anteriorly (left) and posteriorly (right), towards more peripheral regions of the embryo. (E) Transverse section through the early neural plate at stage 6. The immunoperoxidase reaction product is localized to the neuroectoderm layer, except for a few cells more laterally. (F) High power of E, showing the neural plate region. Immunoreactivity appears to be localized to the neuroectodermal cell surfaces. A–D, immunofluorescence; E–F, immunoperoxidase. Scale bars: A–C, 100 μm; D, 200 μm; E, 250 μm; F, 50 μm.

Identification of molecules recognised by anti-L5 antibody

To identify the molecule(s) carrying the L5 epitope at early stages of neural development, immunoblots of proteins extracted from the neural plates and from the remaining non-neural tissue (stages 6–8) were probed with anti-L5 antibody (Fig. 5). The most major component in neural tissue is a doublet, of M_r about 500×10^3; a series of bands in the $M_r 200\times10^3$ range are also seen. Occasionally, a band of about 25×10^3 appears. None of these bands is seen in immunoblots of non-neural tissue.

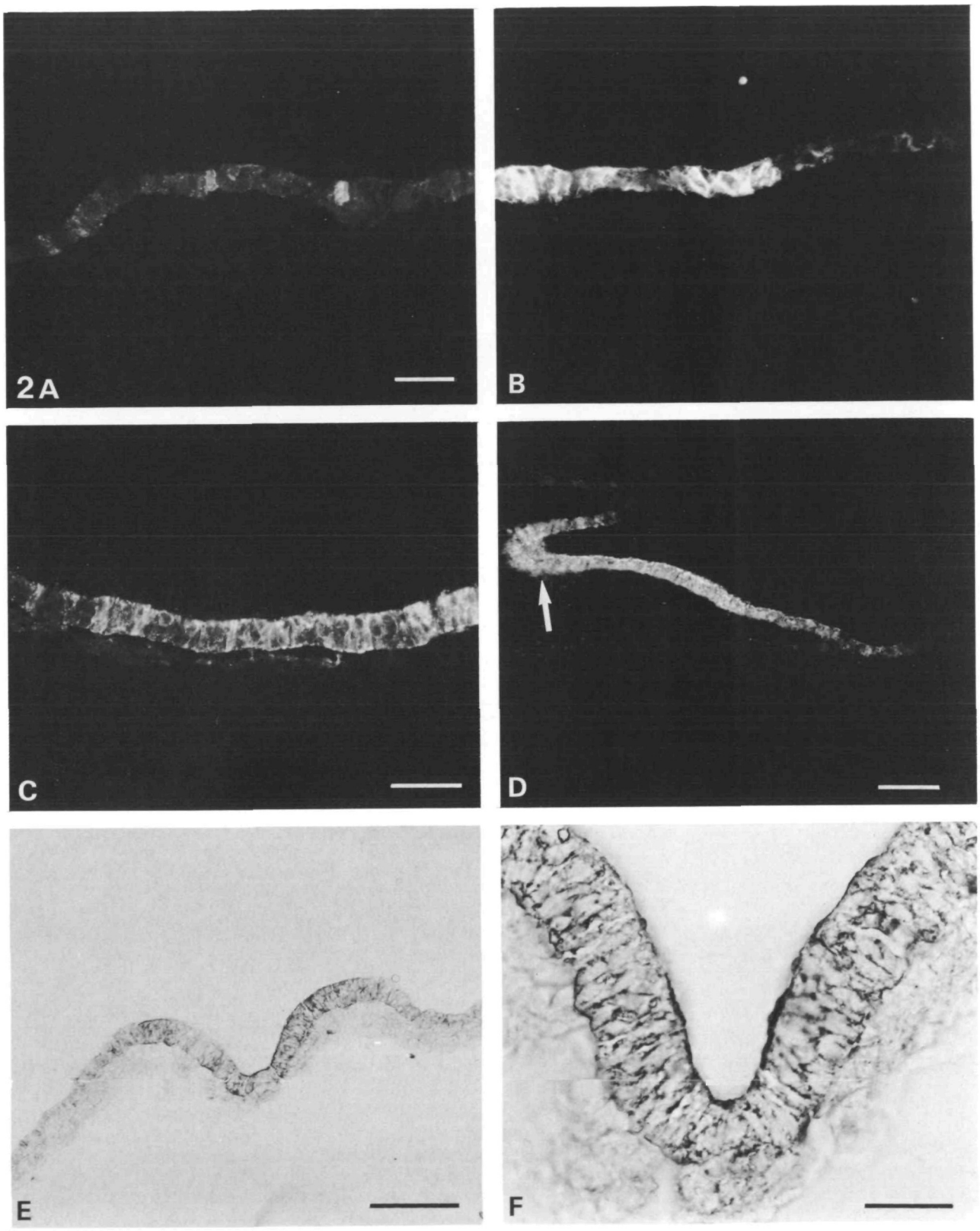
If the neural tissue is first digested with the enzyme *N*-glycosidase F, which cleaves off N-linked oligosaccharides, no bands are seen (Fig. 5B), confirming the findings of Streit *et al.* (1990) that the antibody requires an N-linked carbohydrate for recognition.

Grafts of Hensen's node and L5 immunoreactivity

Embryos in which a donor Hensen's node had been transplanted into the *area opaca* were fixed, sectioned and stained with anti-L5 antibody to test whether immunoreactivity is now expressed in normally immunonegative regions that are undergoing neural induction. Some of the grafted Hensen's nodes were first labelled with DiI to determine whether L5-positive cells were of graft or host origin. After culture of the embryos, strong immunoreactivity was seen in the vicinity of the graft and in the induced neural plate (Fig. 6). Although some immunoreactivity was seen in tissues derived from the graft, the induced, host-derived neural plate was strongly L5-positive (Fig. 6).

Perturbation experiments

To investigate whether the antigens recognised by the



anti-L5 antibody are involved directly in neural induction, a donor Hensen's node was implanted into a host embryo together with hybridoma cells producing anti-L5. As controls, we used either grafts of other IgM-producing hybridoma cells (4B5, against a ubiquitous integral membrane protein; Norris, 1989, or HNK-1, against the HNK-1/L2 carbohydrate, which is expressed in membrane glycoconjugates of other tissues at this stage; Canning and Stern, 1988), or a non-Ig-secreting subclone of the anti-L5 hybridoma.

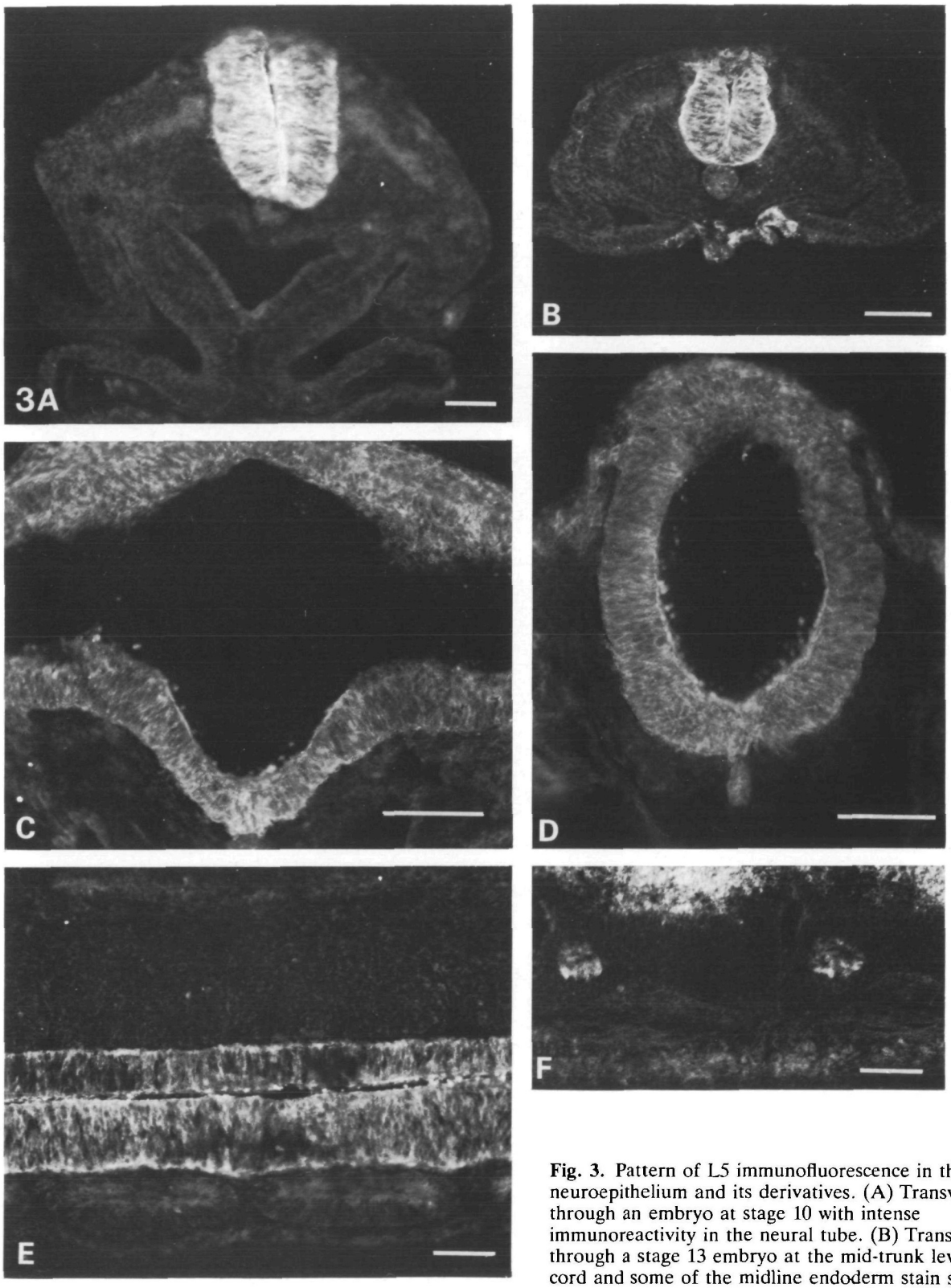


Fig. 3. Pattern of L5 immunofluorescence in the neuroepithelium and its derivatives. (A) Transverse section through an embryo at stage 10 with intense immunoreactivity in the neural tube. (B) Transverse section through a stage 13 embryo at the mid-trunk level. The spinal cord and some of the midline endoderm stain strongly with anti-L5 antibody. (C) Transverse section through the infundibular region of the prosencephalon at the level of the developing optic lobes. The neuroectoderm is immunoreactive for the L5 epitope, particularly ventrally, in the region where the pituitary gland develops. (D) Transverse section through the midbrain at stage 11. The neuroectoderm, notochord and neural crest are all immunoreactive. (E) Coronal section through the trunk (stage 12). The neural tube is strongly labelled. (F) Sagittal section through two somites at stage 15. The dorsal root ganglia are immunoreactive, as is a more dorsal, unsegmented mass of neural crest cells. Scale bars: A–D, 50 μm; E–F, 30 μm.

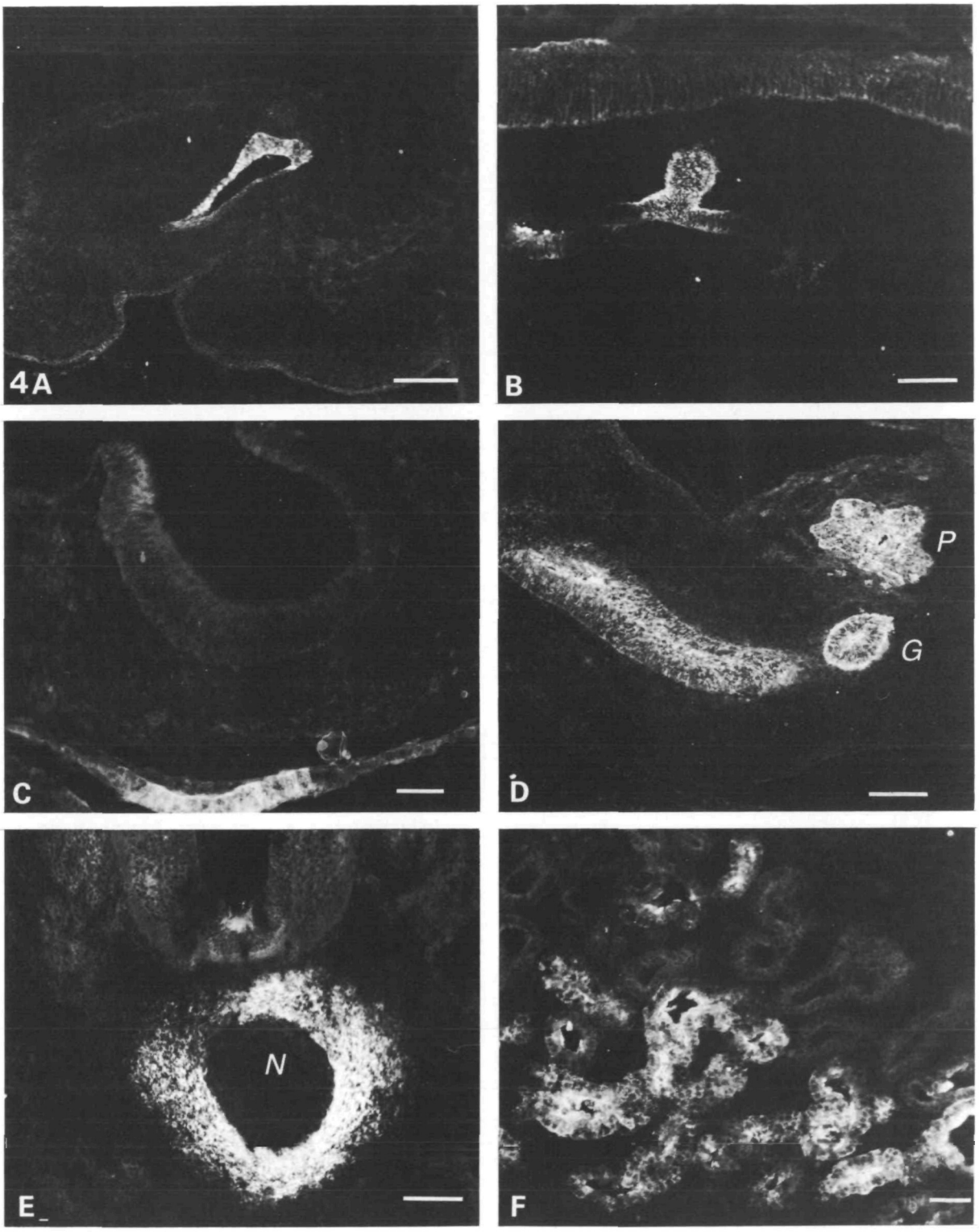


Fig. 4. Pattern of L5 immunoreactivity at later stages of development, in non-neural tissues. A–D show endodermal tissues, E–F mesodermal tissues. (A) Sagittal section through the pharyngeal arches at stage 25. The endoderm of the second pharyngeal pouch facing the third pharyngeal arch is strongly immunoreactive. (B) The primordium of the palatine tonsil and the hindbrain are labelled. (C) Section at the level of the otic vesicle. Immunoreactivity is seen at the level of the 5th rhombomere, in the endoderm facing the third pharyngeal arch. (D) Section through the trunk of a stage 25 embryo. The gut (G) and pancreatic primordium (P) show L5 immunoreactivity. (E) Transverse section through a stage 25 embryo. The sclerenchyme surrounding the notochord (N) is immunoreactive. (F) Section through a stage 25 embryo showing developing mesonephric tubules. Some of these tubules show strong immunoreactivity, whilst others do not. Scale bars: A, 150 μm; B–C, 50 μm; D, 100 μm; E, 25 μm; F, 50 μm.

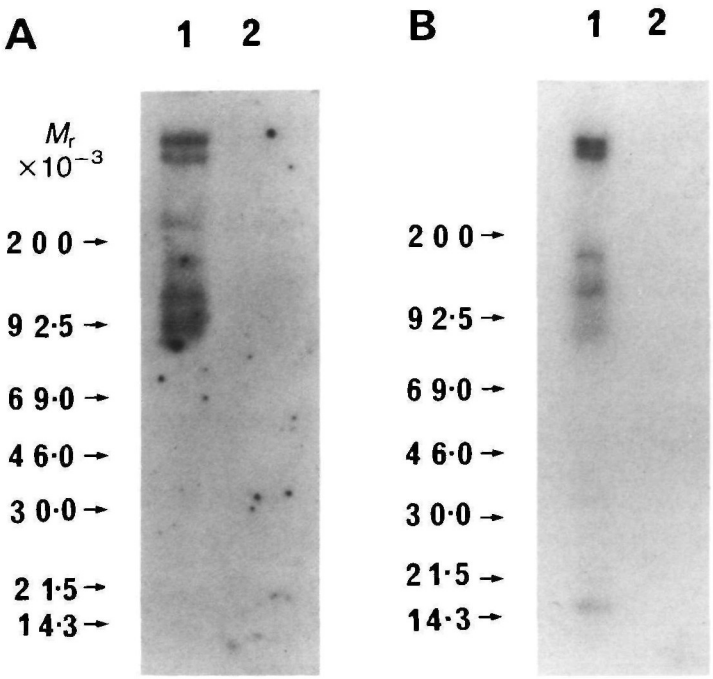


Fig. 5. Immunoblot analysis of chick embryo tissue, probed with anti-L5 antibody. (A) Neural tissue probed with anti-L5 (lane 1); several bands are visible, the most prominent of which has a M_r of about 500×10^3. No such bands are visible in non-neural tissue probed with anti-L5 (lane 2). (B) Immunoblots of neural tissue before (lane 1) and after (lane 2) digestion with *N*-glycosidase F. This enzymatic digestion removes all L5 immunoreactivity from chick neural tissue, confirming that the L5 epitope is a carbohydrate moiety. The molecular mass of reference markers is shown for each immunoblot.

The results were assessed in terms of the proportion of embryos in which secondary neural structures had been induced by the grafted Hensen's node. Table 1 and Figs 7–8 summarise the results of this experiment. In control embryos in which Hensen's node was grafted alone or with other, control (non-L5-secreting) hybridomas, supernumerary neural structures are seen in about 60 % of specimens within 24 h. Different types of control grafts (HNK-1, 4B5, non-secreting L5 or no hybridoma) gave similar results; the differences between them are not statistically significant (Table 1). Embryos grafted with a Hensen's node together with hybridoma cells secreting anti-L5 antibody, however, display a markedly decreased rate of supernumerary neural structure formation (29 %). The difference between control and experimental embryos is significant at the 2 % level (Table 1).

Table 1. *Statistical analysis of the effects of grafts of Hensen's node together with L5 hybridoma or control cells*

	n	Induced	%	Expected
(A) *Control grafts compared*				
4B5	20	13	65	11.8
HNK-1	18	9	50	10.62
L5-non sec.	16	11	69	9.44
Graft only	25	13	52	14.75
χ^2, 3 d.f.=0.833 (not significant)				
(B) *L5 grafts* versus *control grafts*				
L5	38	11	29	16.5
Controls	79	46	58	34
χ^2, 1 d.f.=6.07 $P<0.02$				

It is important to ascertain whether the supernumerary structures formed are the result of induction by the grafted node or of self-differentation of the graft. In a few embryos, the grafted node was labelled with DiI as described above, to determine whether the neural plates that developed were of graft or host origin; the sections obtained after photo-oxidation of the dye were stained with anti-L5 antibody. It was found that the epiblast overlying the graft did not contain L5 immunoreactivity, which was confined to host-derived, DiI-labelled tissues (Fig. 8). This result suggests that the rate of supernumerary neural plate formation, as assessed by observation of whole embryos, is an overestimate of the rate of induction. In most, if not all

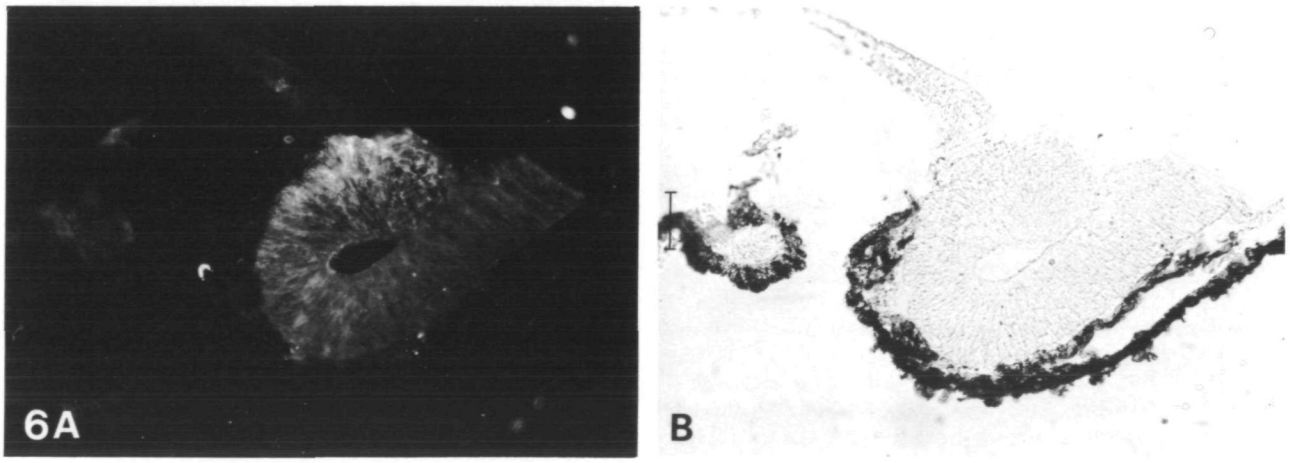


Fig. 6. L5 immunoreactivity in the *area opaca* (nomally non-expressing) after grafting a DiI-labelled Hensen's node into this region. (A) Immunofluorescence. L5 is expressed in the supernumerary neural plate. (B) Bright-field view of the same region, after photo-oxidation of DiI. The neural plate is of host (unlabelled) origin. Scale bar: 50 μm.

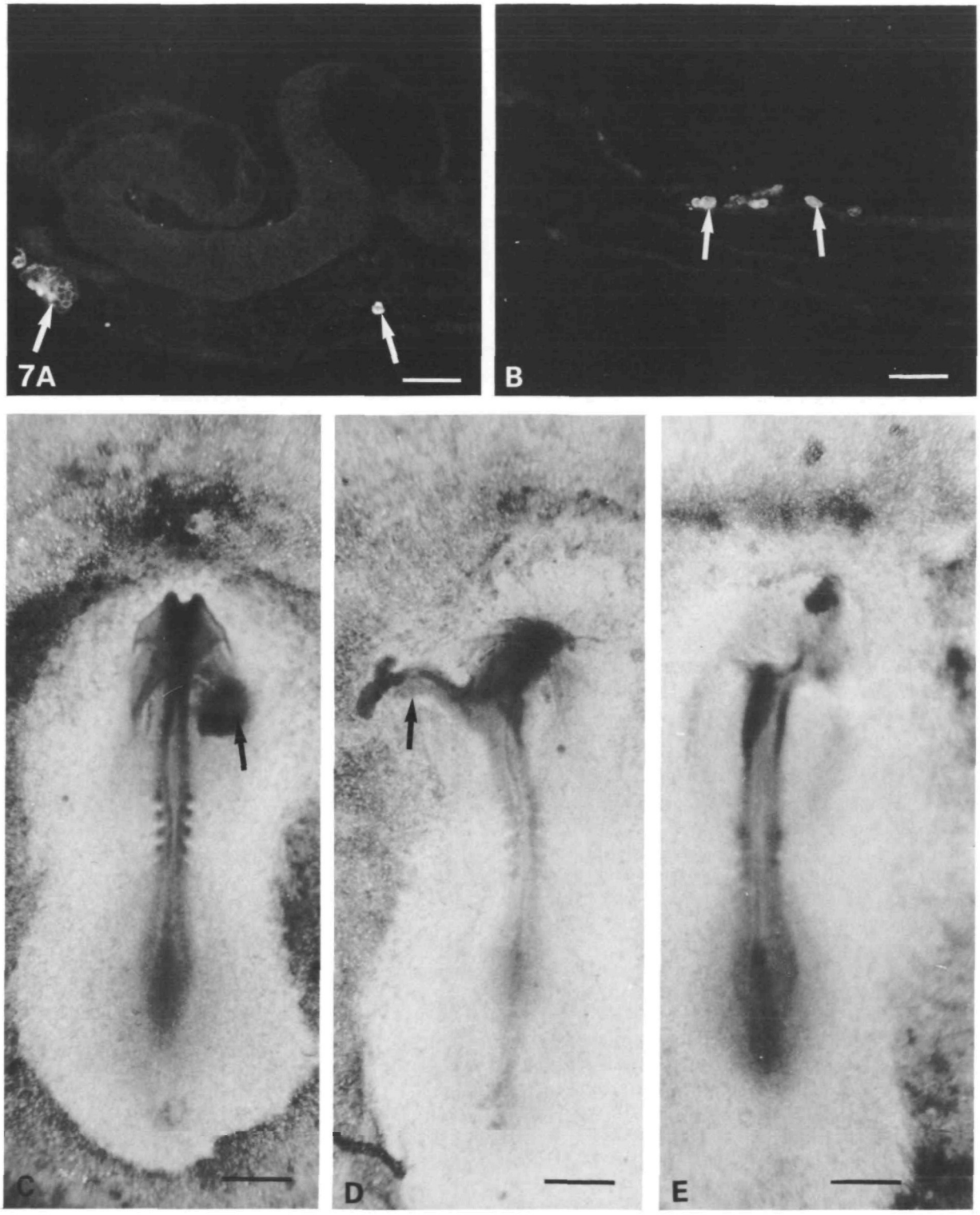


Fig. 7. Embryos in which Hensen's node and hybridoma cell lines had been grafted. (A,B) Transverse sections through embryos that had been grafted with L5 hybridoma cells and cultured to about stage 8. After fixation, they were sectioned and stained with FITC-conjugated anti-IgM antibody to reveal the position of the hybridoma cells (arrows). In A, the hybridoma cells are seen in the mesoderm and close to the neuroepithelium. (C) Whole mount of an embryo that had been grafted with a Hensen's node and anti-L5-secreting cells and cultured to stage 8. The grafted node has not induced supernumerary structures, although the graft is still visible (arrow). (D) Whole mount of a control embryo that had been grafted with Hensen's node without hybridoma cells. Induction of secondary embryonic structures can be seen (arrow). (E) Embryo grafted with Hensen's node and anti-L5 cells close to the host embryonic axis. No neural induction is seen and, in addition, head structures have failed to develop in the host embryo. Scale bars: 50 μm.

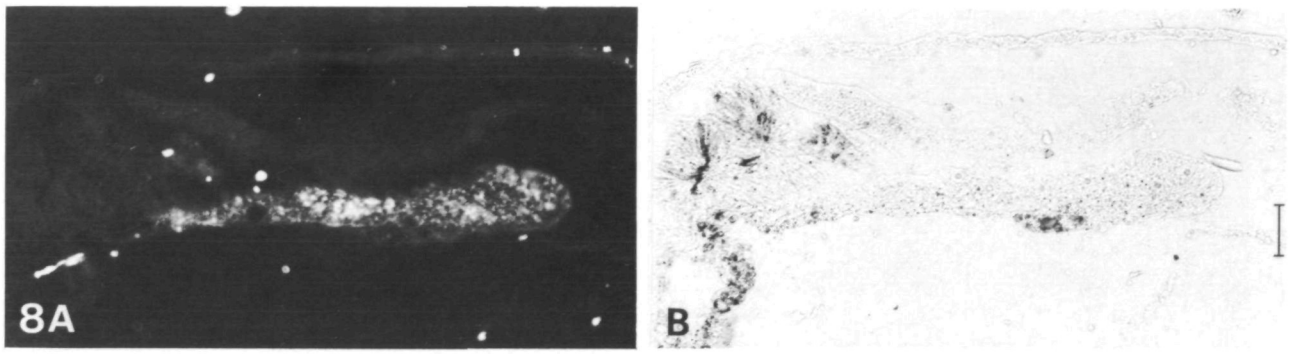


Fig. 8. Embryo that had been grafted with a DiI-labelled Hensen's node together with a pellet of anti-L5 hybridoma cells at the margin of the *area opaca*. (A) Immunofluorescence. The only tissue expressing L5 immunoreactivity is situated deep in the embryo. (B) Bright-field view of the same field, after photo-oxidation of the DiI. Somite tissue (left) and the tissue that is seen to be L5-positive in A are of donor origin. The host epiblast (top of photograph) has not been induced and does not express L5. Scale bar, 50 μm.

cases, the neural plates that develop may be of donor origin. This is in contrast with the results obtained after grafting Hensen's nodes in the absence of hybridoma cells, where most of the L5-expressing tissue is of host origin (see above and Fig. 6).

In some of the embryos that had been grafted with anti-L5 hybridoma cells, the position of the grafted cells in the cultured host embryo was visualized by staining cryostat sections with antibody against rat IgM. The grafted cells were often scattered over a wide area around the graft (Fig. 7A,B) but were not found in more remote regions.

It may be interesting that in one case, the graft of Hensen's node and hybridoma cells was placed close to the anterior tip of the primitive streak of the host embryo. The hybridoma cells were found among those of the host axis and head structures failed to develop (Fig. 7E). However, in 49 experiments (data not shown) in which hybridoma or control cells were grafted alone, without a Hensen's node, no consistent effects were seen on the development of the host axis.

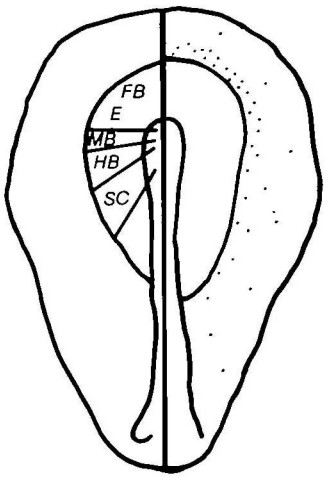


Fig. 9. Summary diagram of L5 expression at stage-4 (right), compared to a fate map of the same stage according to Rudnick (1935) (left). In the right half of the diagram, the region expressing strongest L5-immunoreactivity is delineated by a solid boundary. Outside this boundary, small groups of cells express, their number decreasing in anterior (top) to posterior direction. The outline of the primitive streak is also shown. FB, forebrain; E, eye; MB, midbrain; HB, hindbrain; SC, spinal cord.

Discussion

We have shown that a monoclonal antibody against the L5 epitope, which recognises several glycoproteins in neural tissue from early chick embryos, can be used as a neuroepithelium-specific marker during early stages of chick development. Grafts of Hensen's node induce expression of immunoreactivity in normally non-expressing regions (epiblast of the *area opaca*). Moreover, grafts of anti-L5-secreting hybridoma cells can inhibit induction of supernumerary neural structures by Hensen's node.

Developmental pattern of L5-immunoreactivity

Our results show that anti-L5 recognises all major cell types derived from the neuroepithelium of the chick embryo, including the neural crest and its derivatives, and all parts of the central nervous system at early stages of development. At later stages, during organogenesis (stage 25; 3.5 days' incubation), a few non-neural tissues are also immunoreactive (mesonephros, gut and primordia of the pharyngeal tonsil and pancreas). Streit *et al.* (1990) found that adult mouse astrocytes and neurons express the L5 epitope. We do not yet know whether the pattern of expression in adult fowl will be as restricted as that of the adult mouse or whether it will be as widespread as that of the embryonic chick, but Streit *et al.* (unpublished observations) also found that adult mouse kidney expresses L5-immunoreactivity.

The limits of expression of L5 in early embryos (stages 3+–4) corresponds closely to the edge of the presumptive neural plate at these stages, according to fate maps such as those of Rudnick (1935) (Fig. 9). The

finding that embryos as young as stage 3 (mid-gastrula stage) show immunoreactivity in the presumptive neural plate region therefore suggests that neural induction has begun by this early stage of development. This is consistent with current ideas on neural induction in amphibians (see Sharpe *et al.* 1987; Dixon and Kintner, 1989; Green and Smith, 1990; Sokol *et al.* 1990). It would be of interest to investigate whether the molecules carrying the epitope recognised by antibody against the L5 epitope are the same at these early stages of development as those found in more mature neuroepithelium.

Molecular nature of the L5 epitope and its carrier molecules

Streit *et al.* (1990) have carried out extensive studies on the molecules carrying the epitope recognised by the anti-L5 antibody in mouse brain extracts. They also find a major, high molecular mass component (the M_r of which they also estimate as 500×10^3) and several other components of lower M_r. The high M_r component was identified as a chondroitin sulphate containing proteoglycan, with three major core proteins of M_r 380, 360 and 260×10^3 because of results obtained after chondroitinase digestion. It is possible that the lower molecular mass components seen in our immunoblots correspond to these core proteins. However, attempts to probe such immunoblots with a monoclonal antibody against the glycosaminoglycan portion of chondroitin sulphate proteoglycan (CS56) in our laboratory have not been successful (data not shown). We were also unable to recognise the L5-positive, high molecular mass component from chick tissues using a rabbit antiserum raised against mouse L5-carrying proteoglycan (M_r 500×10^3).

Streit *et al.* (1990) also identified some other glycoproteins that contain the L5 epitope. In particular, they report that the neural recognition molecule L1 (see Rathjen and Schachner, 1984; Grumet *et al.* 1984; Keilhauer *et al.* 1985; Moos *et al.* 1988) and the immunoglobulin superfamily member Thy-1 (Perkins *et al.* 1988) carry the epitope. The low molecular mass of the latter raises the possibility that the lowest molecular mass component seen occasionally in our immunoblots corresponds to this glycoprotein, although it has not yet been reported that Thy-1 is expressed at such early stages of development.

Streit *et al.* (1990) found that L5 is a carbohydrate epitope, and our results with N-glycosidase-F digestion support this. However, the structure of the carbohydrate moiety is not yet known either in mouse brain or in chick neural tissues.

Functional involvement

We have found that grafts of Hensen's node into normally non-expressing regions of the embryo (*area opaca* epiblast) results in expression of L5-immunoreactivity by the host epiblast of this region. The host epiblast origin of the newly expressing tissue was confirmed using DiI-labelled nodes.

We also find that cells secreting anti-L5 interfere with neural induction by a grafted Hensen's node. This suggests that the epitope, or one or more of the molecules that carry it, might be involved directly in neural induction. We are not yet in a position to decide whether the involvement of these moieties in neural induction is restricted to the epitope, to all the carrying molecules or to one specific component.

It is interesting perhaps to note that grafts of hybridoma cells alone did not interfere consistently with formation of the neural plate of the host embryos. This may be due to the fact that neural induction in the host must have begun by the time of grafting. In addition, access of the secreted antibody to the host neural plate may be restricted. When the cells are grafted together with a donor Hensen's node, both problems are overcome: induction begins when the node is grafted (the hybridoma cells being present from this time), and the hybridoma cells are placed in intimate contact with the grafted node.

If L5-carrying molecules are involved in neural induction, what is the significance of the patterns of expression of L5 seen at later stages of development? L5 immunoreactivity was seen in the eye and ear placodes, mesonephric tubules and primordia of the pharyngeal tonsil and of the pancreas. In each of these cases, the organ systems are thought to be formed as a result of inductive interactions with neighbouring tissues (see Arey, 1965; Nieuwkoop *et al.* 1985). This raises the possibility that L5 or its carrying molecules may be involved in inductive interactions other than neural induction. In this context, it is interesting to compare the pattern of L5 immunoreactivity with that of HNK-1, whose carbohydrate epitope has also been suggested to be involved in inductive interactions (Canning and Stern, 1988). For example, HNK-1 stains the notochord of the early chick embryo (see Tucker *et al.* 1988; Stern *et al.* 1989) while anti-L5 recognises the neural tube and floor plate. The latter is thought to arise from an inductive interaction between the notochord and the overlying neuroepithelium (van Straaten and Drukker, 1985; van Straaten *et al.* 1987; Jessell *et al.* 1989; Smith and Schoenwolf, 1989; Schoenwolf and Smith, 1990).

It is now desirable to develop an *in vitro* assay for neural induction using amniote tissues, with which it should be possible to undertake a search for inducing substances and to study the response to neural induction in more detail.

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