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The cellular environment controls the expression of *engrailed*-like protein in the cranial neuroepithelium of quail–chick chimeric embryos

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Summary

We have previously shown that one of two chicken *engrailed*-like genes, *chick En-2*, is expressed in a restricted region of the early chick embryo brain: the mes/metencephalon (Gardner *et al.* 1988). In this study, we examine the role of the cellular environment in regulation of *engrailed*-like (En) protein expression in quail–chick chimeric embryos. Two types of transplant surgery were performed at the 9–15 somite stage to produce chimeric embryos. In the first, the mid-mesencephalic vesicle or caudal mesencephalic vesicle alar plate (which is *En* protein-positive) was transplanted from a quail embryo into an *En* protein-negative region of chick neuroepithelium, the prosencephalon (mMP and cMP grafts, respectively). In the second reciprocal surgery, prosencephalic alar plate which is *En* protein-negative, was transplanted into the *En* protein-positive mesencephalic vesicle (PM grafts). A polyclonal antiserum, *αEnhb-1*, which recognizes *chick En* proteins (Davis *et al.* 1991) was used to identify *En*-positive cells 48 h after surgery. In mMP embryos, 71 % of integrated grafts had lost *En* expression ($n=17$). In contrast, in cMP grafts, 93 % of integrated grafts continued to stain with the antiserum ($n=14$). In addition, in 86 % of these embryos, the graft induced adjacent chick host diencephalic cells to become *En* protein-positive as well. All PM grafts contained *αEnhb-1*-positive cells; such cells never expressed this protein in their normal environment. These early changes in *En* protein expression correlate well with the morphological changes observed in similar graft surgeries assayed later in development. Thus, our results are consistent with the hypothesis that *En* genes play a role in the regionalization of the early cranial neuroepithelium.

Key words: chick embryo, development, *engrailed*, neural tube transplantation, quail, optic tectum, cerebellum.

Introduction

Recent experiments have shown that the cranial neuroepithelium of stage 8–15 chick embryos may have a limited ability to regulate its developmental fate, at least when examined for gross morphological development (Nakamura *et al.* 1986; Nakamura *et al.* 1988; Martinez and Alvarado-Mallart, 1989*a*; Nakamura, 1990; Alvarado-Mallart *et al.* 1990; Ichijo *et al.* 1990; Matsuno *et al.* 1991). When sections of neural tube and overlying cells were excised and transplanted into heterotopic regions of the chick host neuroepithelium, in some cases, grafts developed histologically according to their new surroundings. For example, when prosencephalon-derived grafts were placed into a mesencephalic environment, they developed optic tectum-like laminae appropriate for their new location and even became innervated by optic nerve fibers from the host retina (Nakamura *et al.* 1988).

Investigations into the molecular basis of these morphological phenomena, as well as the mechanisms that underly regional differentiation in the normal development of the brain have led several investigators to propose a role for the *engrailed* gene family in these events (Gardner *et al.* 1988; Patel *et al.* 1989; Kavka *et al.* 1989; Martinez and Alvarado-Mallart, 1990; Davis *et al.* 1991). The *En* genes are a subfamily of homeobox-containing genes that have been highly conserved in vertebrate evolution (Joyner *et al.* 1985; Darnell *et al.* 1986; Joyner and Martin, 1987; Fjose *et al.* 1988; Holland and Williams, 1990; Davis *et al.* 1991; Hemmati-Brivanlou *et al.* 1991). *En* genes were first described in *Drosophila*, in which the prototype *engrailed* gene is known to play a role in the early segmentation of the embryo along its antero-posterior axis (Kornberg, 1981). There are two *En* genes in the chick, *chick En-1* and *chick En-2* (Darnell *et al.* 1986; Davis *et al.* 1991). Antibodies raised against *En* proteins localize to the presumptive midbrain–hindbrain region of the chick neural tube beginning at stage 8–9 (Gardner *et al.* 1988; Patel *et al.* 1989; Davis *et al.* 1991). Because similar patterns of *En* localization have been found in all vertebrates examined (Davis *et al.* 1988; Gardner *et al.* 1988; Davidson *et al.* 1988; Davis and Joyner, 1988; Njolstad and Fjose, 1988; Patel *et al.* 1989; Hemmati-Brivanlou and Harland, 1989; Davis *et al.* 1991; Hemmati-Brivanlou *et al.* 1991; Hatta *et al.* 1991), and because *En* expression delineates, among other

things, presumptive optic tectum and cerebellum, a role for the gene in foreshadowing the emergence of these structures is envisioned.

In the chicken, the monoclonal antibody, mAb *4D9* (Patel *et al.* 1989), recognizes only the *chick En-2* gene product (Davis *et al.* 1991). Our laboratory (Kavka *et al.* 1989), as well as that of Martinez and Alvarado-Mallart (1990), has used mAb *4D9* to investigate the role of the cellular environment in *chick En-2* expression. In those experiments, the right-side alar plate of the mesencephalic vesicle of a 2 day old chick embryo was removed and replaced with a left-side mesencephalic vesicle graft from a quail embryo of the same age. The graft had been rotated 180 degrees so that the originally caudal *4D9 En*-positive cells were now placed in a rostral *4D9 En*-negative environment. Regulation seemed to occur within 20 h after surgery; the new *4D9* staining pattern in the graft was indistinguishable from antibody localization on the unoperated side or in control embryos of the same age. Phenotypic regulation, including normal topographic retinotectal projections to the graft (Ichijo *et al.* 1990), has been reported to occur when similar surgeries are performed and embryos are allowed to continue to develop to near hatching (Alvarado-Mallart *et al.* 1990; Ichijo *et al.* 1990; Matsuno *et al.* 1991).

In all of the previous studies, however, there was no way to exclude the possibility that grafts had either physically reoriented after implantation or that cells within a graft may have simply migrated through the neuroepithelium back to their original rostro-caudal positions. To eliminate that possibility in the present study we grafted patches of neuroepithelium; rather than rotate a single large graft we have transplanted smaller regions which can then be easily distinguished from host tissue later in development. To examine the regulation of *En* protein in early brain primordia, we have transferred grafts of quail mid-mesencephalic vesicle or caudal mesencephalic vesicle alar plate, which express *En* protein, into the presumptive diencephalon of chick embryos, where *En* protein-positive cells are not observed in the course of normal development. In separate reciprocal experiments, *En* protein-negative grafts from quail embryos were transplanted into the mesencephalic vesicle of chick hosts. Localization of *En* protein was determined through antibody staining using the polyclonal antiserum, *αEnhb-1* (Davis *et al.* 1991). Graft tissue was distinguished from host due to the presence of condensed heterochromatin within quail but not in chick cell nuclei (Le Douarin, 1969; Le Douarin, 1973).

Materials and methods

Fertile eggs from White Leghorn chickens (*Gallus gallus domesticus*) and quails (*Coturnix coturnix japonica*) were obtained from Dave's Eggs and Poultry, Ann Arbor, MI. All eggs were maintained in a force-draft incubator at 38–39 °C for the first day of incubation. Several hours before surgery, chick eggs were removed to a Forma Scientific incubator (38 °C, 100 % humidity, model #3325); chick eggs were maintained at 100 % humidity from that time on (except during surgery: 30–40 min in a humidified room). All operations were performed at stage 10–12 (Hamburger and Hamilton, 1951). In all experiments the donor tissue came from quail, the host was chick.

Surgeries

To prepare host embryos, 2.5 ml albumen were removed from each egg with a syringe. A window was cut approximately 1.5 cm^2 on the upper side of the egg and Hank's Balanced Salt Solution (GIBCO #310–4020) containing penicillin-streptomycin (diluted 1:100, SIGMA #P 0781) was added (2–3 drops initially and every few minutes during surgery to prevent desiccation). The window was then covered with surgical tape (Blenderm, 3M #1525–1), and eggs were returned to the 100 % humidity incubator until needed.

Donor and host were stage-matched (stage 10–12) by observation of cephalic development. A fine tungsten needle was used to extirpate a square or slightly rectangular patch of donor neuroepithelium. The graft consisted of cells from the dorsal neural tube (alar plate), along with some adherent dorsal mesenchyme and ectoderm (Fig. 1B, top). An effort was made during dissection to minimize the non-neuroepithelial contribution to the graft. The graft was transferred to a chick egg in a bead of fluid on a small loop of tungsten wire. A matching-size patch of host neuroepithelium was removed and discarded. The quail graft was then gently moved to the space prepared for it and pressed flush with the host neuroepithelium. Care was taken to maintain the rostro-caudal orientation of grafts. In 5 control surgeries (discussed with results) grafts were intentionally taken from the left side of the donor, rotated 180 degrees and joined with the host neuroepithelium in this new orientation.

After surgery, eggs were covered again with surgical tape, allowed to heal for 20 min at room temperature, and then returned to the 100 % humidity incubator. Embryos were removed 24–48 h after surgery and fixed overnight in methanol:DMSO (4:1).

Wholemount immunohistochemistry

For *αEnhb-1* localization in whole mount, embryos were fixed and stained according to a modification of the method used by Davis *et al.* (1991). Embryos were fixed overnight in methanol:DMSO (4:1) at 4 °C, followed by bleaching in methanol:DMSO:30 % H_2O_2 (4:1:1) for 4–5 h at room temperature. This destroyed endogenous peroxidase activity in the tissue. Embryos were stored at −15 °C until needed. For immunolocalization embryos were hydrated and washed in PBS containing 0.1 % Triton X-100 and 2 mg ml^{-1} BSA (PBT). A part of the left (unoperated) side of the brain was cut open for better penetration of Ab and reaction solutions. Embryos were incubated in *αEnhb-1* diluted either 1:50 or 1:75 in PBT on a rotator for 2–3 days at 4 °C. The embryos were then washed at least five times for 1 h each in PBT and incubated in peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch), diluted 1:200 in PBT, for up to 2 days on a rotator at 4 °C. Final washes were performed as above. After washing, embryos were incubated for 40–45 min in an incubation mixture containing diaminobenzidine (0.3 mg ml^{-1}), $CoCl_2$ (0.025 % aq.) and $Ni(NH_4)_2(SO_4)_2$ (0.02 % aq.). This incubation solution was then replaced with a thoroughly mixed fresh solution of the same composition but containing 0.003 % H_2O_2 (reaction solution). The reaction was quenched after 2 min by passing the embryos through several washes of PBT.

Embryos were then embedded in TissuePrep 2 (Fisher Scientific #T555) and sectioned. Alternate sections were

mounted on gelatin subbed slides, dewaxed, and either coverslipped immediately to observe the diaminobenzidine-peroxidase reaction product or passed through bleach solutions and stained for DNA (Feulgen and Rossenbeck, 1924) to distinguish quail from chick cells (Le Douarin, 1969; Le Douarin, 1973).

Bleaching the diaminobenzidine-peroxidase reaction product

In order to visualize the condensed heterochromatin in quail cell nuclei we used a simple method adapted from Lillie (1965) to bleach the tissue before performing the Feulgen-Rossenbeck reaction. Sections were dipped for 30 s in $KMnO_4$ (1 % aq.), followed by a 30 s wash in H_2O, followed by a 30 s immersion in oxalic acid (1 % aq.), followed by at least two H_2O washes. This cleared the tissue but left a faint color difference between *En* protein-positive and *En* protein-negative cells allowing, in some cases, direct comparison between the extent of antibody localization and the graft border on a single section. Adjacent sections were not bleached; they were examined for conclusive definition of the extent of the immunoperoxidase reaction.

The Feulgen-Rossenbeck reaction on methanol-fixed and bleached tissue

The following procedure was optimum for tissue sections previously treated as described above. Tissue was incubated for 12 min in 5 N HCl at room temperature. Sections were then washed twice in H_2O, 1–2 min ea. and immersed in Schiff's reagent (SIGMA S-5133) for 40 min in the dark. Sections were then passed through three freshly made solutions (1–2 min ea.) containing: 1 part 1 N HCl: 20 parts H_2O: 1 part 10 % aq. $Na_2S_2O_5$ (Humason, 1979). Following this second mild bleach, slides were dehydrated to 95 % ethanol, immersed briefly in Fast Green (1 mg ml^{-1} 95 % ethanol), and then further dehydrated to 100 % ethanol, passed through xylenes and coverslipped under Permount (Fisher Scientific #SP15–100).

Results

At the time we performed surgery (stage 10–12), the neural tube had closed and the brain was divided into three vesicles: the prosencephalon, mesencephalon and rhombencephalon (Fig. 1A). By the time of analysis, 24 to 48 h after surgery, the prosencephalon had divided into telencephalon (rostral) and diencephalon (caudal), while the rhombencephalon had divided into metencephalon (rostral) and myelencephalon (caudal). The mesencephalon remains undivided.

Fate-mapping of the avian brain, from the division of the 3 primary brain vesicles into 5, has been somewhat complicated by the finding that rostral cerebellum (considered originally to come from the metencephalon) actually arises, in part, from cells of the caudal mesencephalic vesicle (Martinez and Alvarado-Mallart, 1989*b*; Hallonet *et al.* 1990; Fig. 1A). In our experiments, grafts that were taken from the caudal border of the mesencephalic vesicle, though they might in the course of normal development have contributed to metencephalic structures, will be referred to as 'caudal mesencephalon.'

In surgeries of either the mMP (mid-mesencephalon into prosencephalon) or cMP (caudal mesencephalon into prosencephalon) type, a graft was introduced into the roof of the host prosencephalon between the optic vesicles. After 24–48 h, these grafts were invariably

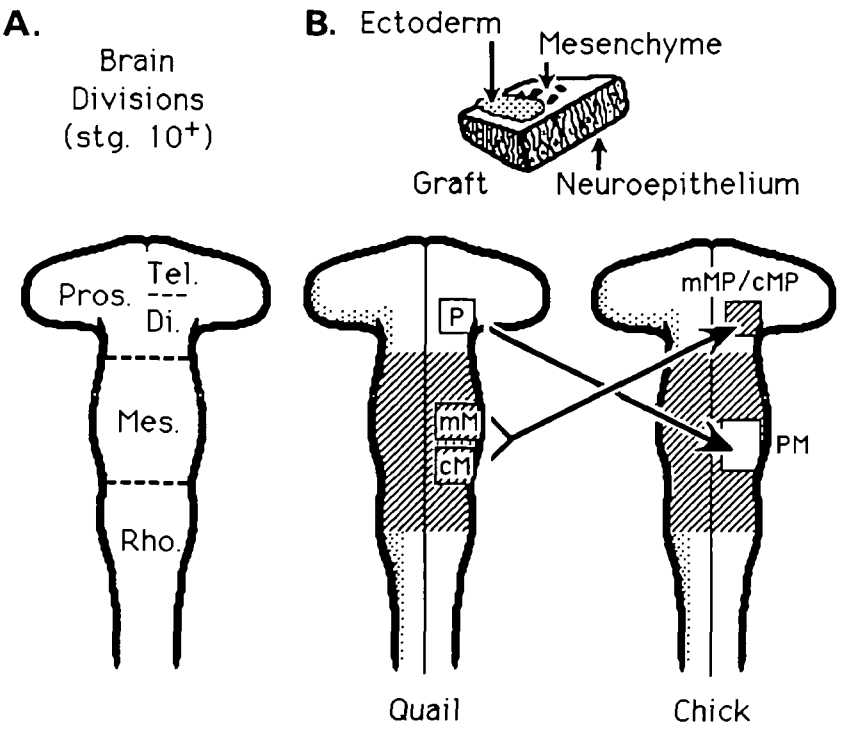

Fig. 1. Explanation of surgeries. (A) At the time surgeries were performed (stage 10–12) the brain was divided into prosencephalon (Pros.), mesencephalon (Mes.) and rhombencephalon (rho.). The borders of these divisions are shown with thick dotted lines. The presumed border between presumptive diencephalon (Di.) and presumptive telencephalon (Tel.) is shown with a thin dotted line. (B, bottom) Production of chimeric embryos. Hatched lines show approximate extent of *En* protein expression at the time of surgery. mMP grafts were excised from a quail mid-mesencephalon roof and transplanted into the roof of the diencephalon in a chick host embryo. cMP grafts were identical except that the graft neuroepithelium originated from the caudal-most part of the mesencephalic vesicle. PM grafts were removed from the roof of the prosencephalon (quail) and transplanted into the roof of the mesencephalic vesicle (chick). (B, top) Grafts were composed primarily of neuroepithelium, though some adherent mesenchymal and ectodermal cells were included.

found in or adjacent to the host diencephalon. We conclude that the region of the prosencephalon that we have investigated in the present study includes and does not extend beyond the presumptive diencephalon.

In reciprocal experiments, grafts were taken from the prosencephalon (presumptive diencephalon) and transplanted into the chick mesencephalic vesicle (Fig. 1B). These surgeries will be referred to as 'prosencephalon into mesencephalon' grafts (PM grafts; Fig. 1B).

All operations were performed on two day old embryos, stage 10–12. Variations within this range of embryo ages had no effect on the outcome of experiments. This is consistent with the findings of Alvarado-Mallart *et al.* (1990), who demonstrated, in similar embryo surgeries, that regulation or restriction of gross morphological development was independent of graft or donor age within a defined range of stages (stage 8–15).

En protein-positive grafts transferred to an En *protein-negative cell environment (Fig. 1B; Table 1)*

When the alar plate of the mid-mesencephalon (*En* protein-positive at time of surgery) was grafted into the host prosencephalon (mMP surgeries, $n=22$) and examined 48 h after surgery, 17 of the grafts were found to be integrated into the host neuroepithelium. We define 'integrated' as 'cells directly apposed.' This contact ranged from perfect continuity between graft and host to, in the most extreme few cases, a continuous stalk of cells between graft and host neuroepithelia. Among integrated grafts the extent of integration, unless otherwise noted, had no effect on the outcome of the experiments. In the 5 mMP embryos in which the graft did *not* integrate, the grafts were found in the diencephalic mesenchyme between host neuroepithelium and overlying ectoderm (Fig. 2A and B). All 5 unintegrated mMP grafts still contained some *αEnhb-1*-positive cells 48 h after surgery, though overall immunoreactivity was much diminished. Of the 17 integrated mMP grafts, 12 no longer expressed *En* protein (Fig. 3C–E).

Four of the 5 integrated mMP grafts that remained *αEnhb-1*-positive had integrated near the di–telencephalic border (Fig. 3A and B) whereas only 1 embryo contained a well integrated *En* protein-positive graft in mid-diencephalon. This last was scored as antibody-positive even though graft cells appeared to stain less intensely than did cells of the host mesencephalon, suggesting that in this graft some loss of *En*-protein may have occurred in the 48 h following surgery.

In contrast, when grafts were taken from the caudal mesencephalon and transplanted into the prosencephalon (cMP surgeries, $n=17$), graft cells remained *αEnhb-1*-positive 48 h after surgery in all but 1 case (Table 1). In addition, 12 out of 14 embryos with integrated grafts not only contained *αEnhb-1*-positive graft cells, but also contained *αEnhb-1*-reactive chick cells in the host diencephalon (Fig. 4A–D). Similarly, 2 of the 5 *αEnhb-1*-positive mMP embryos discussed above contained a very small number of adjacent antibody-stained host cells (Fig. 3B). The 1 cMP embryo scored as graft-*αEnhb-1*-negative (Table 1), did stain very faintly with the antibody, continuous with faint staining in the host neuroepithelium. *En* protein-positive cells were never seen in the diencephalon of 3–5 day old unoperated control embryos (Gardner and Barald, unpublished data).

In 3 cases where cMP grafts did not integrate into the host diencephalon and in 3 control cMP embryos in which the graft was intentionally placed adjacent to but not in the host neural tube, no *En* protein-positive cells were found in the host diencephalon when embryos were examined 48 h after surgery (total cMP-like surgeries=20; Table 1).

In all cases, when host diencephalic cells became *αEnhb-1*-positive, the immunoreactivity was strongest at the graft–host border and appeared to diminish in a gradient with distance from the graft. The extent of immunoreactivity in the host diencephalon was usually greater lateral and caudal to the graft than in the rostral direction (compare Figs 4A and 4C).

All grafts contained a very small amount of adherent mesoderm and ectoderm. The contribution of these cells was so small that non-neuroepithelial quail cells could rarely be found in host embryos 48 h after surgery. Nevertheless, control experiments were performed ($n=5$) in which a large graft from the caudal mes-metencephalon region containing mesoderm and ectoderm, but without associated neuroepithelium, was transplanted rostrally, adjacent to the presumptive diencephalon (Fig. 5). These embryos were examined after 48 h. Many quail-derived mesodermal cells were found adjacent to the host diencephalon but *αEnhb-1*-positive cells were never observed in the host diencephalic neuroepithelium (Fig. 4E and F).

In summary, these results indicate that transplantation of the alar plate from the mid-mesencephalon into the diencephalic region most often leads to a loss of *En* expression in the grafted cells. In contrast, cells from the caudal mesencephalon remain *En* protein-positive 48 h after surgery and, in addition, may induce adjacent host diencephalic cells to express *En* protein as well. This last phenomenon seems to require neuroepi-

Table 1. *Summary of surgery results:* En *protein (+) graft into an* En *protein (−) region*

Surgery	n=X	Graft *En* 48 h		Host diencephalon *En*-positive
mMP		(+)*	(−)	
integrated:	17	5	12	2
not integrated:	5	5‡	0	0
total:	22			
cMP		(+)†	(−)	
integrated:	14	13	1	12
not integrated:	6	6	0	0
total:	20			

* Some cells in graft remained *En* protein-positive.
† All cells in graft remained *En* protein-positive.
‡ Immunoreactivity diminished.

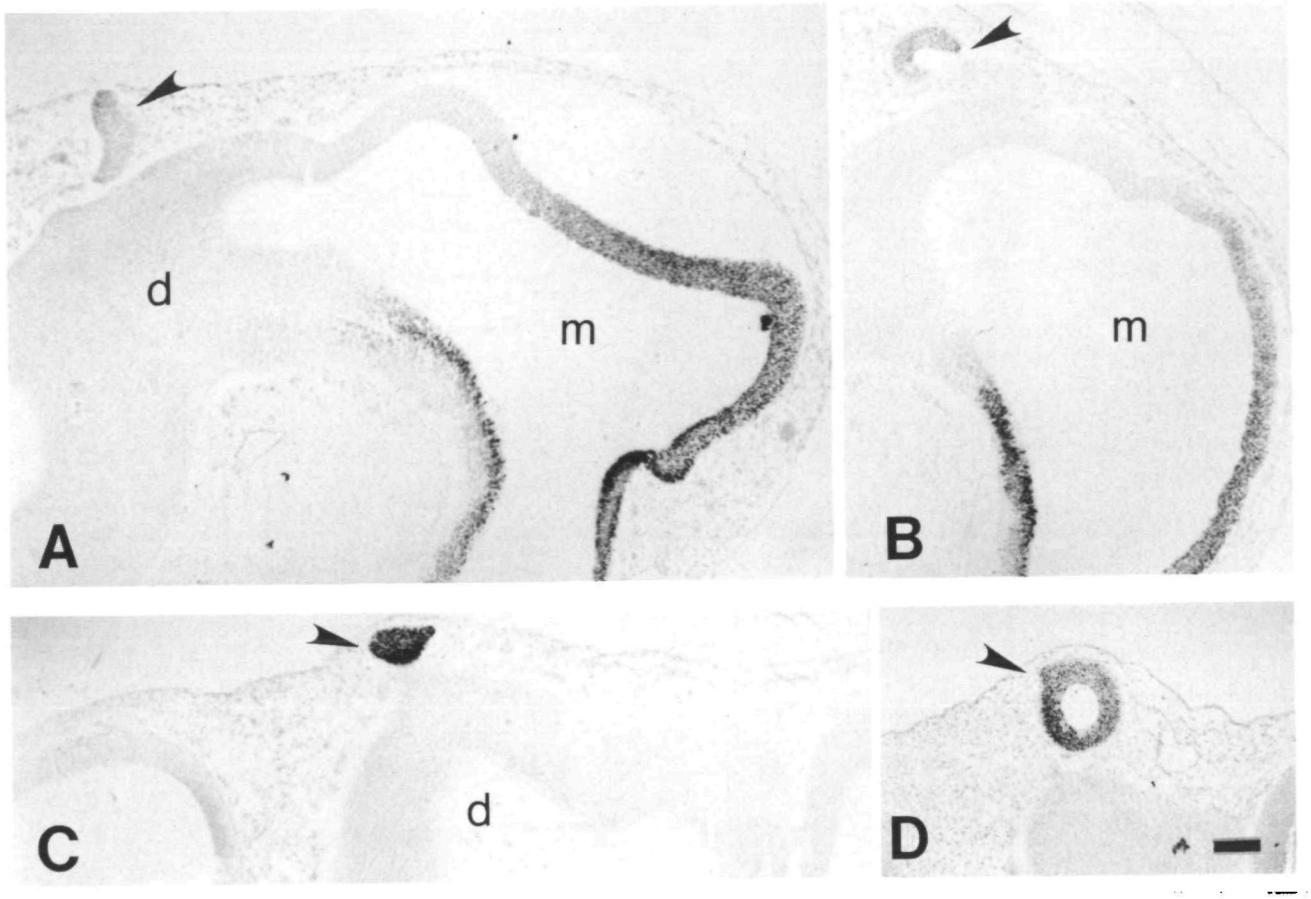

Fig. 2. Unintegrated mMP and cMP grafts, 7 μm parasagittal sections, 48 h after surgery (grafts indicated by arrowheads). In all photos, rostral is to the left, caudal to the right. Grafts were originally *En* protein-positive. Results differed depending on whether the graft had originated from the mid-mesencephalon (mMP; see A and B) or the caudal mesencephalon (cMP; see C and D). Antiserum reactivity diminished in the former but remained strong in the latter (*En* protein localization is shown in the host mesencephalic neuroepithelium of A and B for comparison). Abbreviations: d, diencephalon; m, mesencephalon. Scale bar=100 μm.

thelial cell–cell contact. Finally, caudal mesencephalon-region mesoderm alone does not have the ability to up-regulate *En* protein expression in the diencephalon.

En *protein-negative grafts transferred to an* En *protein-positive cell environment (Fig. 1A; Table 2)*

When a patch of prosencephalic alar plate was transplanted into the mesencephalic vesicle (total $n=36$), all but one of the integrated grafts after 24 h and all PM grafts after 48 h were found to contain *En* protein-positive cells (Fig. 6A–D). Roughly half of PM grafts were *αEnhb-1*-positive throughout the graft (24 h, 50 %; 48 h, 62 %). In some grafts (39 % overall), *αEnhb-1*-positive cells could be seen only in the caudal region of the graft, adjacent to the host–graft border with immunoreactivity diminishing in a caudo-rostral gradient (not shown). *En* protein upregulation in PM grafts, therefore, appeared to be variable from one embryo to the next. Roughly equal percentages of largely antibody-positive and partially antibody-positive grafts were seen at 24 and 48 h after surgery. Variability in the numbers of antibody-positive cells seen in the grafts may have been due, in part, to the quality of graft integration; imperfectly integrated grafts were less likely to be antibody-positive throughout. In 5 embryos the prosencephalic graft was intentionally rotated 180 degrees before insertion into

Table 2. *Summary of surgery results:* En *protein (−) graft into* En *protein (+) region. PM grafts 24 and 48 h after surgery*

Hours after surgery	n=X	Graft *En* after surgery		
24 h		(+)*	(+/−)†	(−)‡
integrated:	19	10	8	1
not integrated:	1	0	0	1
total:	20			
48 h				
integrated:	16	10	6	0
not integrated:	0	0	0	0
total:	16			

* All cells in graft *En* protein-positive.
† Some cells in graft *En* protein-positive.
‡ No cells in graft *En* protein-positive.

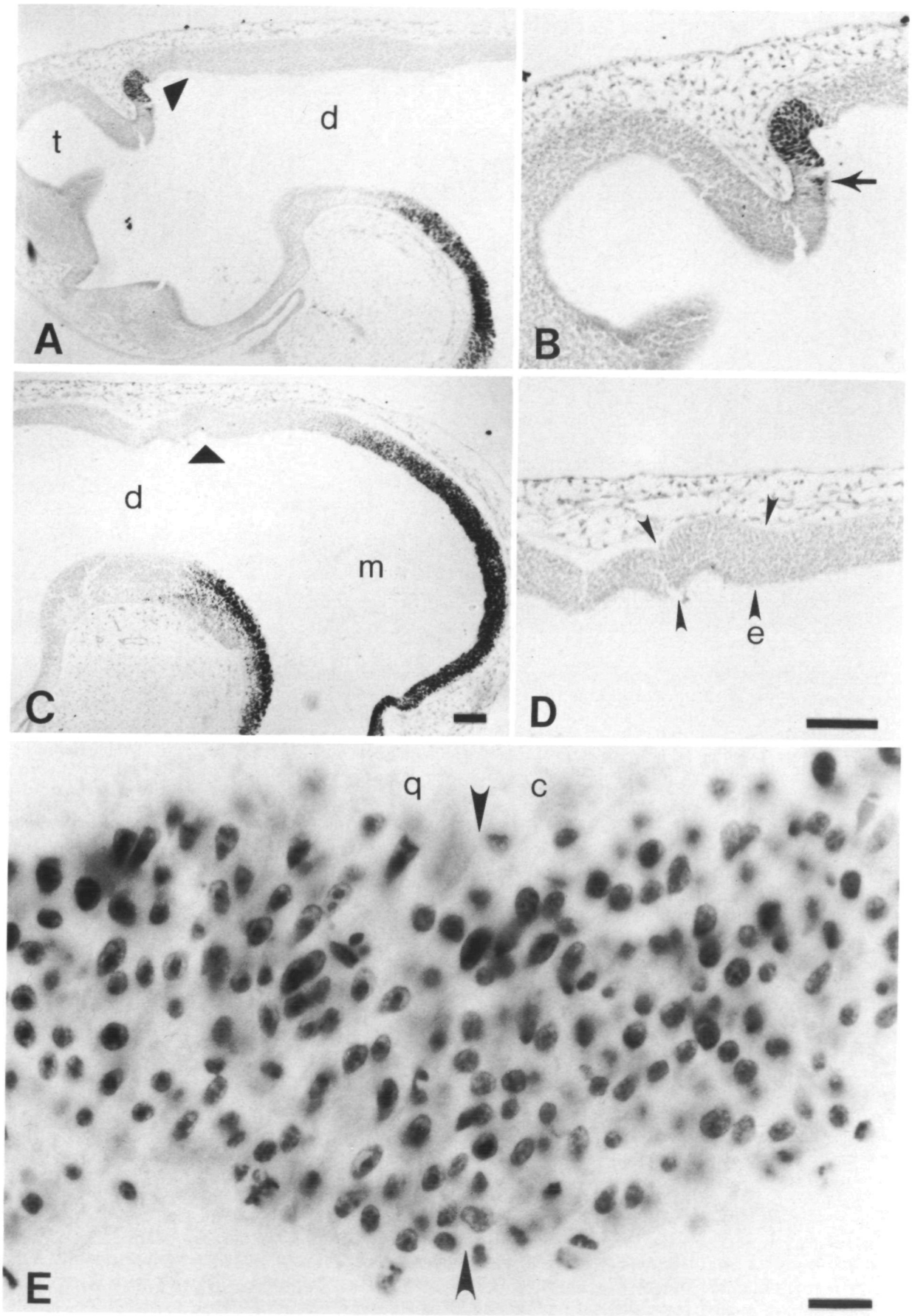
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Fig. 3. Chimeric embryos with integrated mMP grafts, 7 μm parasagittal sections, 48 h after surgery. (A–D), *αEnhb-1* localization; (E), Feulgen-Rossenbeck reaction. In all photos, rostral is to the left, caudal to the right. Some integrated mMP grafts (29%) remained *En* protein-positive 48 h after transplantation to the diencephalon. One of these embryos is shown in A; the graft is indicated by a triangle and shown at higher power in B. The arrow in B indicates antiserum-positive cells in the chick host neuroepithelium. Most integrated mMP grafts (71%) were no longer *En* protein-positive 48 h after surgery (C and D). (D) Higher power of region indicated with a triangle in (C); graft borders shown with small arrowheads. (E) Caudal border (large arrowheads) of the graft shown in C and D, adjacent section, stained for DNA to indicate quail *vs* chick cells. Cracks in the tissue were probably due to shrinkage during methanol fixation. Abbreviations: t, telencephalon; d, diencephalon; m, mesencephalon; e, graft–host border shown at high power in E; q, quail cells; c, chick cells. Scale bar for A and C shown in C=100 μm. Scale bar for B and D shown in D=100 μm. Scale bar for E=10 μm.

the host mesencephalic neuroepithelium. This had no effect on the extent of *αEnhb-1* immunoreactivity in the graft.

Some *En* protein-positive cells may extend rostral to the mesencephalic vesicle at the time surgeries were performed (stage 10–12; Davis *et al.* 1991; Gardner and Barald, unpublished data). However, these cells are found only in the dorsal midline, which was excluded from PM grafts, and grafts were taken from a still more rostral region of the prosencephalon to eliminate the possibility of their containing *En* protein-positive cells at the time of surgery. The most likely explanation for the graft *En*-reactivity seen in Fig. 6 is that *En* protein expression was upregulated in graft cells following surgery.

In summary, both cMP and PM surgeries resulted in the juxtaposition of *En* protein-negative (prosencephalic) and *En* protein-positive (caudal mesencephalic) cells. In both cases the result was identical: upregulation of *En* protein occurred in the prosencephalic cells.

Discussion

Within the homeobox region the *chick En-1* and *-2* genes share extensive sequence similarity with the mouse *En-1* and *En-2* genes, respectively (Darnell *et al.* 1986; Davis *et al.* 1991). Because the patterns of *En* expression in mouse and chick development have been largely conserved, Davis *et al.* (1991) have suggested that the function of *En* genes in mouse and chick development may have been conserved in evolution as well. In murine development it is now believed that the two *En* genes may serve complementary or overlapping roles in development; targeted deletion of the *En-2* gene in transgenic mice produces only subtle alterations in late cerebellar development (Joyner *et al.* 1991). For this reason, in the present study, we have used the polyclonal antiserum, *αEnhb-1*, which recognizes epitopes on both of the *chick En* gene products as a marker for overall *En* expression.

Our experiments have shown that the expression of *En* protein in the dorsal neuroepithelium of stage 10–12 chick embryos can be influenced by alterations in the cellular environment within 24 h after surgery. Most mid-mesencephalon grafts, isolated from their *in situ* environment and either integrated into the diencephalon or left unintegrated, adjacent to the diencephalon, were not able to maintain *En* protein expression (mMP grafts). In contrast, when caudal mesencephalon grafts were removed from their *in situ* environment, they were able to maintain *En* protein expression independent of degree of integration (cMP grafts). When integrated, cMP grafts were further able to induce host diencephalic cells to become *En* protein-positive. In reciprocal surgeries, when prosencephalon grafts were transferred to the mesencephalic vesicle, graft cells became *En* protein-positive.

The loss of En *protein in mMP grafts*

mMP grafts were *En* protein-positive at the time of surgery; if left undisturbed in the course of normal development these mid-mesencephalic cells would have continued to be *En* protein-positive at least until embryonic day 6 (Gardner and Barald, unpublished data), and possibly until hatching (Gardner *et al.* 1989; Gardner and Barald, unpublished observation). After surgery, *En* expression decreased in most mMP grafts whether they were integrated or not, suggesting that a positive signal may be required for the maintenance of *En* expression in the mid-mesencephalic vesicle and that such a signal is not present in the graft's new environment. Alternatively, an inhibitory signal, absent in the mesencephalon, may be present in the diencephalic region. However, maintenance of *En* expression in cMP grafts argues against this second explanation.

In a few cases, mMP graft cells continued to express *En* protein 48 h after surgery. In 4 out of 5 of these embryos, the graft had integrated close to the host telencephalon. Aside from stochastic events, there are 2 possible explanations for continued *En* expression in these grafts: either the grafts themselves were different from those that lost *En* protein in that they contained some cells from the caudal mesencephalon (similar to cMP grafts) or, alternatively, the rostral diencephalon may have unique properties not present in the mid- to caudal diencephalon. In the rostral-most diencephalon there may exist a signal capable of maintaining *En* protein expression. That signal presumably would not function in the course of normal development because *En* protein is never detected in the diencephalon of 3–5 day old control embryos (Gardner and Barald, unpublished data).

Evidence that a positive inductive signal arises from the caudal mesencephalic neuroepithelium

cMP grafts appeared to induce surrounding host diencephalic cells to become *αEnhb-1*-positive in a

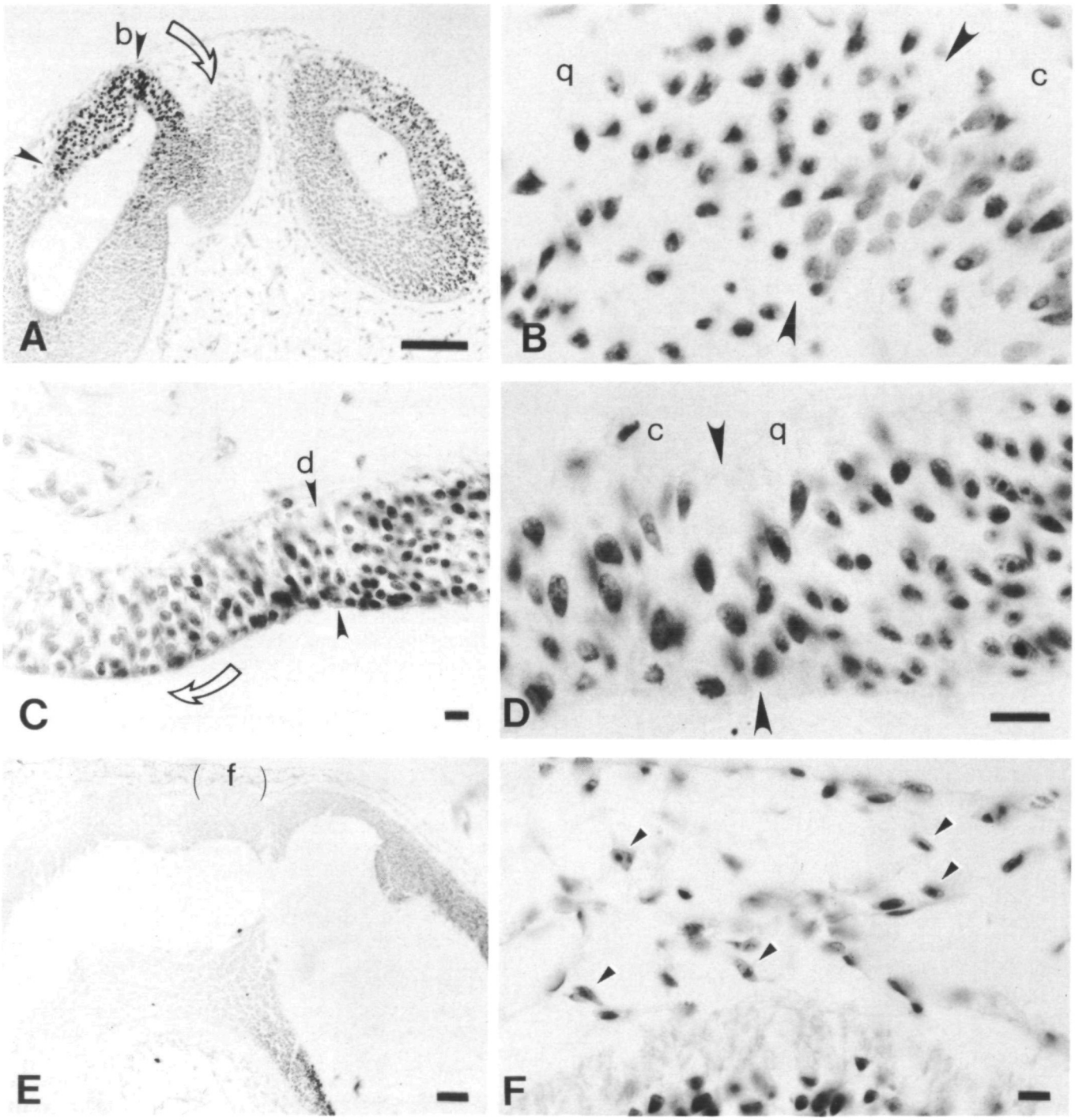

Fig. 4. Chimeric embryos with integrated cMP grafts, 7 μm (A–D) or 15 μm (E and F) parasagittal sections, 48 h after surgery. (A, C, E), *αEnhb-1* localization; (B, D, F), Feulgen-Rossenbeck reaction. In all photos, rostral is to the left, caudal to the right. Most cMP grafts remained immunoreactive 48 h after surgery. A cMP graft is indicated by arrowheads in A. The rostral border of a second cMP graft is indicated by arrowheads in C. Most integrated cMP grafts also appeared to induce adjacent host diencephalic cells to become *En* protein-positive, as shown by open arrows in A and C. (B) Caudal border (large arrowhead) of graft shown in A, adjacent section. (D) Rostral border (large arrowhead) of graft shown in C, adjacent section. In control experiments, mesenchyme and ectoderm alone, without neuroepithelium, was transplanted from the mes-metencephalic region into the region of the presumptive diencephalon (E and F; see Fig. 5). In these embryos, En protein-positive cells were never seen in the host diencephalon (E). (F) Adjacent section to that shown in E; quail graft mesenchymal cells indicated by small arrowheads. Abbreviations: b, graft–host border shown at high power in B; q, quail cells; c, chick cells; d, graft–host border shown at high power in D; f, graft–host border shown at high power in F. Scale bar for A=100 μm. Scale bar for B and D shown in D=10 μm. Scale bar for C=10 μm. Scale bar for E=100 μm. Scale bar for F=10 μm.

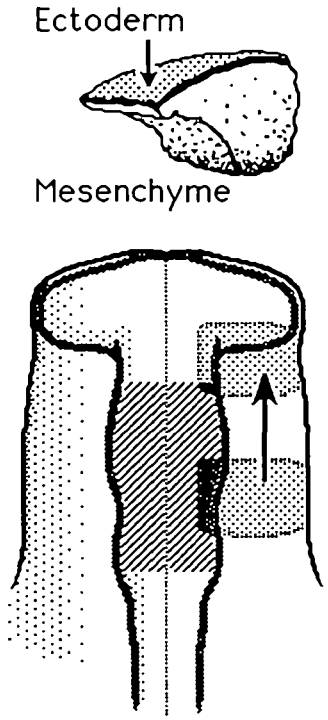

Fig. 5. Control surgeries, composite illustration. In 5 embryos a large region composed of mesenchyme and overlying ectoderm was transplanted from the quail mes-metencephalic region into a chick host, adjacent to and over the presumptive diencephalon. Graft is shown above.

gradient, increasing with proximity to the graft. PM grafts that did not become completely *αEnhb-1*-positive at 24 or 48 h after surgery usually exhibited a gradient of immunoreactivity with the most darkly stained cells at the caudal border of the graft (this was also true for grafts that had been rotated 180 degrees). While the loss of *En* protein seen in mMP grafts was not integration-dependent, the upregulation of *En* expression seen in PM and cMP embryos did require good integration. In particular, it involved contact with cells of or from the caudal mesencephalon. A positive inductive signal arising from the caudal mesencephalon could explain these phenomena and might, as well, be responsible for the maintenance of *En* protein expression in the mid-mesencephalic vesicle during normal development. This possibility is strengthened by the observation that isolated mid-mesencephalon grafts cannot maintain strong *En* protein expression, whereas isolated caudal mesencephalic grafts can do so.

Several lines of evidence suggest that the proposed inductive signal passes through the neuroepithelium. First, the level of *En* protein upregulation in PM grafts seemed to depend on the quality of graft integration. Second, in cMP embryos with unintegrated grafts, even though the graft continued to be antibody-positive and remained adjacent to the host diencephalon, host cells never became *En* protein-positive. Third, in control experiments in which a large block of mesoderm and ectoderm was transplanted from the *En* protein-positive (mes-metencephalic) to the *En* protein-negative (prosencephalic) region, *En* protein-positive cells were never seen in the adjacent host diencephalon.

From experiments in both frog and chick it is known that neural induction signals can pass through neuroepithelial cell–cell contact as well as through mesoderm–neuroepithelial contact (for review see Neiuwkoop *et al.* 1985). In the frog it is clear that a regionalizing signal can pass through the neuroepithelium alone, without mesoderm–neuroepithelial influences (Nieuwkoop *et al.* 1952; Dixon and Kintner, 1989). Evidence does exist for mesodermal induction of initial *En* protein expression in *Xenopus* neuroectoderm (Hemmati-Brivanlou and Harland, 1989). Hemmati-Brivanlou *et al.* (1990) have suggested that the rostral notochord may be primarily responsible for this. However, our surgeries were performed on chick embryos after neural induction, after neural tube closure and after *En* protein expression had commenced. While some mesenchymal cells were transferred along with the grafts, their dorsal origin excludes the possibility that notochordal tissue could be involved. As mentioned above, we have shown that mesenchymal cells from the mes-metencephalic region alone are not capable of inducing *En* protein expression in the host diencephalon. Further, in our laboratory, preliminary results from experiments performed after neural tube closure (stage 10–12) have shown that neither removal of the cephalic notochord nor implantation of an additional cephalic notochord affect the already established pattern of *En* expression in the neural tube. These results are in agreement with the findings of Darnell and Ordahl (personal communication) that notochordless chick embryos have a normal overall expression pattern of *chick En-2* protein. From all of the preceding considerations, therefore, we believe that the upregulation of *En* protein expression in host or graft diencephalic cells (in cMP or PM embryos, respectively) may be the result of a regulatory signal arising from the caudal mesencephalic neuroepithelium itself. We propose that, in normal development, *En* gene expression in the mesencephalon is maintained by a regulatory signal originating from the caudal part of the mesencephalic vesicle.

Martinez and Alvarado-Mallart (1990) have suggested that a regulatory signal affecting *chick En-2* gene expression may emanate from the metencephalon. Since these authors consider the metencephalon to include a part of the caudal mesencephalic vesicle, their suggestion is in agreement with our own findings.

En *protein expression may be involved in regional differentiation of the neuroepithelium*

The present report, our previous work (Kavka *et al.* 1989), and that of Martinez and Alvarado-Mallart (1990) all correlate well with previously reported morphological studies in which heterotopic neuroepithelial transplantations were performed (Alvarado-Mallart and Sotelo, 1984; Nakamura *et al.* 1986; Nakamura *et al.* 1988; Martinez and Alvarado-Mallart, 1989*a*; Nakamura, 1990; Alvarado-Mallart *et al.* 1990; Ichijo *et al.* 1990; Matsuno *et al.* 1991). We have found that when mid-mesencephalon was transplanted to the

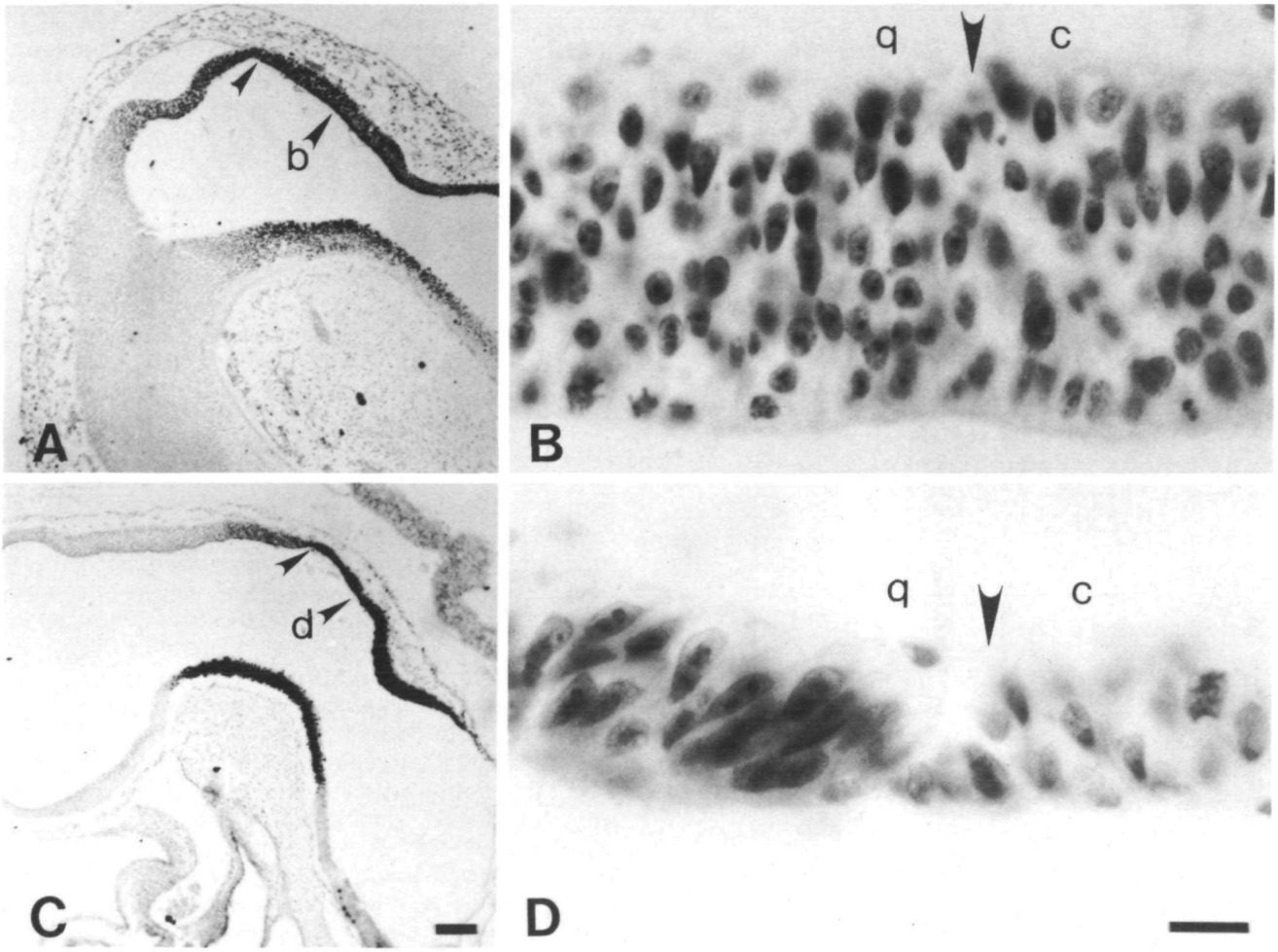

Fig. 6. Chimeric embryos with PM grafts, 7 μm parasagittal sections, 48 h (A and B) and 24 h (C and D) after surgery. (A, C), *αEnhb-1* localization; (B, D), Feulgen-Rossenbeck reaction. Grafts were taken from an *En* protein-negative region of the quail prosencephalon and transplanted into the roof of the chick mesencephalic vesicle. If well integrated, grafts, whether examined after 24 or 48 h, contained antiserum-positive cells, in most cases throughout the graft. Graft borders are indicated by small arrowheads in A and C; the caudal graft–host border of each embryo is shown in B and D, indicated by a large arrowhead. Abbreviations: b, graft–host border shown at high power in B; d, graft–host border shown at high power in D; q, quail cells; c, chick cells. Scale bar for A and C shown in C=100 μm. Scale bar for B and D shown in D=10 μm.

prosencephalon (mMP surgeries), 71 % of integrated grafts lost all *En* protein expression. In similar surgeries (Martinez and Alvarado-Mallart, 1989*a*; Alvarado-Mallart *et al.* 1990), 53 % of mMP-type grafts failed to develop the optic tectal laminae appropriate for their rostro-caudal level of origin, suggesting that mid-mesencephalic cells may be able to change their developmental fate.

Other laboratories have shown that cMP-type grafts in all cases develop tectal or cerebellar morphologies (Alvarado-Mallart and Sotelo, 1984; Alvarado-Mallart *et al.* 1990; Nakamura, 1990). This suggests that the caudal mesencephalon is determined at an early stage of development. Similarly, we found that in cMP surgeries, 16 out of 17 grafts continued to express *En* protein 48 h after surgery. Alvarado-Mallart *et al.* (1990) observed, further, that in two mMP-type embryos (among those that maintained their tectal phenotype), the grafts seemed to induce host diencephalic cells to make optic tectum-like laminae. That finding is reminiscent of our own observation that the caudal mesencephalon can induce host diencephalic cells to become *En* protein-positive (cMP grafts). Similarly, in two mMP embryos in which the grafts remained *En* protein-positive, we observed some *En* protein-positive host cells surrounding the graft as well.

Nakamura has shown that the prosencephalic alar plate, when transplanted into the mesencephalic vesicle (similar to our PM surgeries), has the capacity to differentiate into optic tectum (Nakamura *et al.* 1986; Nakamura *et al.* 1988; Nakamura, 1990). These grafts develop characteristic tectal phenotypes and are capable of receiving optic nerve fibers from the host retina (Nakamura *et al.* 1988). In the course of normal development, presumptive optic tectal cells in the chick embryo express *En* protein (Gardner *et al.* 1988; Patel *et*

al. 1989; Gardner *et al.* 1989; Kavka *et al.* 1989; Martinez and Alvarado-Mallart, 1990; Davis *et al.* 1991). We would expect and did in fact observe that PM grafts begin to express *En* protein after surgery.

Since *En* gene expression in neuroepithelial grafts correlates well with later morphological development, our findings are consistent with previous suggestions (Gardner *et al.* 1988; Patel *et al.* 1989; Martinez and Alvarado-Mallart, 1990; Davis *et al.* 1991) that *En* genes play a role in neurogenesis and the regionalization of early chick cranial neuroepithelium.

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Note added in proof

In a recent report, Martinez *et al.* (*Neuron* **6**, 971–981, 1991) have observed induction of *chick En-2* protein in host cells when a graft from the mes-metencephalon was transplanted into the prosencephalon. In older embryos these grafts formed an ectopic cerebellum, while adjacent diencephalic cells of the host appeared to form an ectopic optic tectum. These observations substantially support our own findings and conclusions with regard to cMP grafts.

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