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Sequential roles for Fgf4, En1 and Fgf8 in specification and regionalisation of the midbrain

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SUMMARY

Experiments involving tissue recombinations have implicated both early vertical and later planar signals in the specification and polarisation of the midbrain. Here we investigate the role of fibroblast growth factors in regulating these processes in the avian embryo. We show that *Fgf4* is expressed in the notochord anterior to Hensen's node before transcripts for the earliest molecular marker of midbrain tissue in the avian embryo, *En1*, are detected. The presence of notochord is required for the expression of *En1* in neural plate explants in vitro and FGF4 mimics this effect of notochord tissue. Subsequently, a second member of the fibroblast growth factor family, *Fgf8*, is expressed in the isthmus in a manner consistent with it providing a polarising signal for the developing midbrain. Using a retroviral vector to express En1 ectopically, we show that En1 can induce *Fgf8* expression in midbrain and posterior diencephalon. Results of the introduction of FGF8 protein into the anterior midbrain or posterior diencephalon are consistent with it being at least part of the isthmic activity which can repolarise the former tissue and respecify the latter to a midbrain fate. However, the ability of FGF8 to induce expression of genes which have earlier onsets of expression than *Fgf8* itself, namely *En1* and *Pax2*, strongly suggests that the normal function of FGF8 is in maintaining patterns of gene expression in posterior midbrain. Finally, we provide evidence that FGF8 also provides mitogenic stimulation during avian midbrain development.

Key words: Isthmus, FGF4, FGF8, En1, Midbrain, Ephrin, Polarity, Specification, Patterning, Vertical induction, Chick embryo

INTRODUCTION

Initial regionalisation of the neural plate along its anteroposterior axis occurs concurrent with, or soon after, neural induction and is believed to be the result of both vertical and planar signals. The former derive from underlying mesoderm while the latter originate from the organiser (the node in amniotes and the dorsal lip in *Xenopus*) and also, possibly, from the anterior margin of the neural plate (for reviews see Ruiz i Altaba, 1994, 1998). Subsequently, organising centres develop within the neuroepithelium and produce signals responsible for further refinement of anteroposterior pattern. The best-characterised of these is the isthmus (midbrain/hindbrain boundary) which has been implicated in the specification and patterning of the midbrain (reviewed by Bally-Cuif and Wassef, 1995; Wassef and Joyner, 1997).

Current evidence suggests that vertical, mesodermal influences are required to initiate the specification of that part of the neural plate which includes the midbrain primordium. Members of two families of transcription factors, *En* and *Pax*, are expressed in the posterior midbrain and the anterior part of hindbrain (often referred to as the mid/hindbrain region) in embryos of all vertebrate classes (see Joyner, 1996 for a review and references). These genes have been shown to be required for normal development of derivatives of that part of the neural tube (Joyner et al., 1991; Krauss et al., 1992; Millen et al., 1994; Urbanek et al., 1994; Wurst et al., 1994; Millen et al., 1995; Favor et al., 1996; Kuemerle et al., 1997; Schwarz et al., 1997; Urbanek et al., 1997). Using *En* expression as a marker for neuroectoderm specified to a mid/hindbrain fate, tissue recombination and grafting studies in mammalian, avian and amphibian embryos have demonstrated a requirement for underlying mesoderm in the early specification of that region (Hemmati-Brivanlou et al., 1990; Ang and Rossant, 1993; Darnell and Schoenwolf, 1997).

Subsequent polarisation of the midbrain involves planar signals originating from the isthmus which also has midbrain-inducing activity when grafted ectopically. The plasticity of posterior diencephalon and anterior midbrain in responding to

patterning signals from the posterior region of the midbrain vesicle was first recognised by Alvarado-Mallart, Nakamura and their colleagues and further developed in grafting studies by a number of groups. Respecification of these tissues was evinced by molecular, cytoarchitectural and retinal ganglion axonal projection criteria (reviewed by Alvarado-Mallart, 1993). 180° rotations of the midbrain, excluding the isthmic region, or grafts of anterior midbrain tissue posteriorly, adjacent to the isthmic region, result in the respecification of anterior midbrain to posterior character (Alvarado-Mallart et al., 1990; Ichijo et al., 1990; Martinez and Alvarado-Mallart, 1990). Similar rotations, but including the isthmic region, result in the midbrain having a mirror-image, double-posterior phenotype (Marin and Puelles, 1994). More dramatically, grafts of posterior forebrain (diencephalic prosomeres P1 and/or P2) into posterior midbrain or rotations of the entire midbrain vesicle, which place isthmic tissue adjacent to the posterior forebrain, cause respecification of the posterior forebrain to a polarised midbrain phenotype (Nakamura et al., 1986, 1988; Alvarado-Mallart et al., 1990; Nakamura 1990; Gardner and Barald, 1991; Itasaki et al., 1991; Martinez et al., 1991; Bally-Cuif et al., 1992; Bally-Cuif and Wassef, 1994; Marin and Puelles, 1994; Bloch-Gallego et al., 1996). Taken together, these studies suggest that a planar signal originating from the isthmic region is responsible for polarising the midbrain and is capable of specifying the posterior forebrain to a midbrain character. Grafting studies of Marin and Puelles (1994) examined the inductive capabilities of the isthmus and confirmed it as an organising centre for spatial patterning of the mid/hindbrain region. Furthermore, the molecular mechanisms involved in specification by the isthmus are conserved among vertebrates (Martinez et al., 1991; Bally-Cuif et al., 1992).

Candidates for vertical signals involved in specification of the midbrain have not been reported, however two secreted signalling molecules, Wnt1 and fibroblast growth factor 8 (Fgf8), are candidate isthmic signals. Expression of *Wnt1* in the midbrain region is initially dynamic but ultimately forms a narrow dorsoventral band anterior to the isthmic constriction and is also detected in the roofplate throughout the midbrain. This pattern of expression is conserved among vertebrates (Wilkinson et al., 1987; Parr et al., 1993; Eizema et al., 1994; Hollyday et al., 1995; Kelly and Moon, 1995; Rowitch and McMahon, 1995). Moreover, mutant mice that are homozygous null for *Wnt1* fail to form a normal mid/hindbrain border, progressively lose *En1* expression in the midbrain and anterior hindbrain and, ultimately, lack most of the midbrain, the isthmic nuclei and the anterior cerebellum (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Thomas et al., 1991; McMahon et al., 1992; Bally-Cuif et al., 1995a; Fritzsch et al., 1995). The presence of *Wnt1*-expressing cells in grafts of tissue from the mid/hindbrain region correlates well with the presence of isthmic patterning activities (Bally-Cuif et al., 1992). However, ectopic expression of Wnt1 has, so far, failed to mimic isthmic activities (cited in Wassef and Joyner, 1997).

Fgf8 is expressed at the isthmic constriction in all vertebrate classes (Heikinheimo et al., 1994; Ohuchi et al., 1994; Crossley and Martin 1995; Mahmood et al., 1995; Bueno et al., 1996; Crossley et al., 1996; Vogel et al., 1996; Christen and Slack, 1997; Riefers et al., 1998). Hypomorphic transgenic mice expressing reduced levels of Fgf8 protein show defects in midbrain and cerebellar development, although these have not been described in detail (Meyers et al., 1998) and the zebrafish *Fgf8* mutant (*ace*) exhibits similar defects (Riefers et al., 1998). Moreover, introduction of ectopic FGF8 into the embryonic chick diencephalon respecifies that region to a midbrain fate (Crossley et al., 1996). In addition, the use of a *Wnt1* enhancer to direct Fgf8 expression in transgenic mice induces *En2* expression and affects patterning events in the midbrain (Lee et al., 1997).

We have examined the effects of ectopic FGF8 protein on gene expression, cytoarchitecture and proliferation when introduced into the midbrain and contrast these data with those we obtained from introduction of FGF8 into the diencephalon and with the studies of Crossley et al. (1996) and Lee et al. (1997). A detailed temporal study of *Fgf8* expression in the avian mid/hindbrain, relative to several other genes implicated in mid/hindbrain development, showed it to be expressed after *En1* and *Pax2*, with *En1* being detected earliest. Ectopic expression of En1 is able to induce *Fgf8* expression in those regions of the neural tube competent to adopt a midbrain character in response to FGF8 protein. We find that *En1* expression in the developing neural plate is, like *En2*, dependent upon vertical signals from the notochord. We show that *Fgf4* is expressed transiently in the notochord underlying this region of the neural tube prior to the detection of *En1* transcripts. Finally, we report that FGF4, like FGF8, can induce *En1* when introduced ectopically into neural tube and, moreover, can substitute for notochord in regulation of *En1* in the neural plate in vitro.

MATERIALS AND METHODS

Incubation, in situ hybridisation and sectioning

Rhode Island Red chicken eggs were incubated as described by Mason (1999) and staged according to the criteria of Hamburger and Hamilton (Hamburger and Hamilton, 1951). In situ hybridisation and subsequent sectioning were performed as described by Shamim et al. (1999). Probes used for in situ hybridisation have been reported previously (Dressler et al., 1990; Cheng and Flanagan, 1994; Niswander et al., 1994; Drescher et al., 1995; Hollyday et al., 1995; Mahmood et al., 1995; Logan et al., 1996).

Implantation of FGF-coated beads

Heparin-coated acrylic beads (Sigma) were prepared by washing twice in L-15 (Leibovitz) medium (Life Technologies) and then incubated in 10 μl of 0.1 μg/μl FGF-4 (Sigma) or 0.1 μg/μl FGF8 (R & D Systems) for 1 hour. For control experiments, beads were incubated in L-15 medium. The beads were crushed with a pipette tip into smaller pieces before implantation into the neural tube; bead fragments were held in place in the neural tube more stably than intact spherical beads which tended to be extruded into the lumen during development. Chicken embryos were incubated to stages 10-12 (Hamburger and Hamilton, 1951), 'windowed' for manipulation as described by Mason (1999) and prepared for bead implantation by making an incision with a sharpened tungsten needle in the neural tube at the required level, ie. caudal diencephalon or anterior midbrain. A bead fragment was then inserted into the incision with a sharpened tungsten needle. Operated eggs were closed and incubated at 37°C in a humidified atmosphere for various periods prior to further analysis. Accuracy of the axial level of our bead implantation was confirmed by reference to the negative controls.

BrdU labelling

Embryos with bead implants were labelled with BrdU (Boehringer Mannheim) diluted according to the manufacturer's instructions and

injected into the lumen of the midbrain via a glass microelectrode. Embryos were incubated for a further hour before fixation overnight in 4% w/v paraformaldehyde in PBS. Embryos were embedded in wax (see above) and 10 μm sections were cut and mounted on gelatin-coated slides. Sections were dewaxed by washing twice in xylene (5 minutes), 100% ethanol (5 minutes), 95% ethanol (3 minutes) and 1× PBS (5 minutes). They were then incubated in 2 M HCl for 1 hour at 37°C, washed four times each for 5 minutes in 0.1 M Tris-HCl pH 8.3, three times in PBS before blocking non-specific antibody binding for 1 hour in 3% BSA in PBS. Sections were incubated in anti-BrdU antibody (Boehringer Mannheim) diluted in blocking solution according to the manufacturer's instructions overnight at 4°C. The following day, sections were washed five times each for 1 hour in PBS prior to incubation in alkaline phosphatase-coupled secondary antibody (Boehringer Mannheim) for 1 hour at room temperature. Sections were washed in PBS (3× 5 minutes), NTMT (3× 5 minutes) and BCIP/NBT substrate (Boehringer Mannheim) was applied. After sufficient reaction product had been produced, sections were washed in several changes of PBS prior to mounting in sterile 90% (v/v) glycerol, 1× PBS.

Staining sections with cresyl violet

Embryos were incubated to E9 and heads fixed in 4% w/v paraformaldehyde overnight. They were then prepared for wax sectioning with the following incubations prior to embedding: PBS (2× 10 minutes), saline (2× 10 minutes), 30% v/v ethanol, 50% v/v ethanol, 70% v/v ethanol (5 minutes each), 100% ethanol (3× 1 hour), Histoclear (National Diagnostics) (3× 1 hour) and wax (3× 1 hour and then overnight). 10 μm sections were cut and mounted onto TESPA (Sigma)-coated slides. Sections were dewaxed in Histoclear (2× 10 minutes), dehydrated in 100% ethanol, 90% v/v ethanol (1× 5 minutes), 70% v/v ethanol (1× 5 minutes) and distilled water (1× 5 minutes), then stained overnight in 0.5% w/v cresyl violet. Sections were dipped in distilled water, 1 M acetic acid, 70% v/v ethanol, 90% v/v ethanol and 100% ethanol. They were then cleared in Histoclear (2× 10 minutes) and mounted in Depex (BDH).

Introduction of En-1-expressing retrovirus into chick embryos

The construction, biological activity and experimental introduction of En1-expressing (RCAS) and control (RCAN) retroviral vectors has been described previously (Logan et al., 1996).

Collagen gel culture of neural plate explants

HH stage 4-6 chick embryos were dissected into Howard's Ringer as described by Mason (1999). With reference to fate maps (Muhr et al., 1997), a region including neural plate fated to form the midbrain primordium was excised together with underlying mesendoderm by transverse cuts with a tungsten needle. These were placed in Optimem medium (Life Technologies) supplemented with Glutamax and Pen/Strep (Life Technologies). Further dissection was performed with sharpened tungsten needles (removal of axial mesoderm) or after incubation with 1 mg/ml dispase, 5 μg/ml DNase-1 (Boehringer Mannheim) in Optimem for 5 minutes with subsequent mechanical dissection (separation of all mesoderm from overlying ectoderm). Tissue was then embedded in bovine dermal collagen supplemented with medium containing FGF4 (1-10 ng/ml final) and heparin (1 μg/ml final). Medium, FGF and heparin were added to the collagen as a 10× concentrated supplement. The collagen was set by adjusting its pH as described by Lumsden and Davies (1983) and medium containing appropriate factors added to the culture wells. Explants were cultured for 24 hours in a humidified atmosphere containing 5% (v/v) carbon dioxide at 37°C.

For inhibition experiments FGFR1 Fc chimera protein, containing the extracellular domain of the 3 Ig loop FGFR1 iiic isoform (Williams et al., 1994), was added to the collagen prior to setting and also to the culture medium at a final concentration of 20 μg/ml. Neutralising anti-FGF4 polyclonal antiserum (R and D systems) was added to collagen and medium at a final concentration of 50 μg/ml.

RESULTS

Temporal expression of *Fgf8* in the mid/hindbrain region

Fgf8 expression at the isthmus has been previously demonstrated in mouse and chick embryos and has been implicated as an inducing signal in the specification of midbrain character (see above). However, a detailed examination of its temporal expression relative to other genes also implicated in midbrain patterning has not been reported. We therefore examined the spatial and temporal expression of *Fgf8* transcripts and of *En1*, *En2*, *Pax2* and *Wnt1* in the presumptive midbrain region of the developing neural tube of chick embryos.

Weak expression of *Fgf8* transcripts was first detected in the 5-somite stage (5s; stage 8+ (HH 8+) of Hamburger and Hamilton, 1951) embryo in scattered cells across the neural plate (Fig. 1A) in the presumptive midbrain region (Muhr et al., 1997). Approximately 1 hour later, at HH9 (6s), expression was significantly stronger (Fig. 1B) and, when the isthmic constriction became apparent at HH 9 (7s), transcripts were

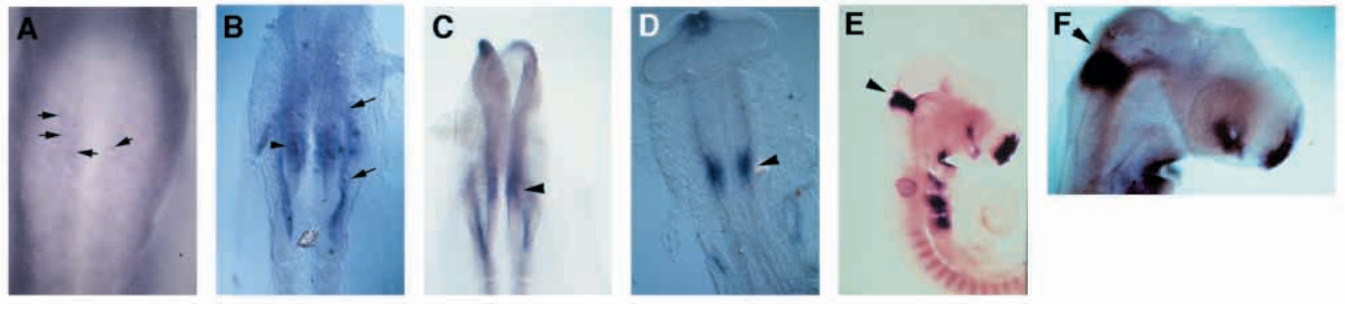

Fig. 1. Spatial and temporal expression of *Fgf-8* in the mid/hindbrain region of the developing chick brain. In situ hybridisation to *Fgf8* transcripts in whole chick embryos, the position of the isthmic constriction is indicated by an arrowhead in C-F. (A) Weak expression in the neural plate of a HH 8+ (5s) embryo is detected in scattered cells (arrows) in a region midway between the anterior tip of the neural plate and the first somite. (B) At HH 9– (6s) the apparent level of expression is much stronger in that region (arrowhead) and transcripts are also detected in the cardiogenic mesenchyme (arrows). (C,D) Transcripts are localised around the isthmic constriction; (C) HH 9 (7s), (D) HH 10+ (11s). (E) Expression is more extensive posterior to the isthmic constriction by HH 15 (24s). (F) All *Fgf8* RNA is detected posterior to the morphological constriction by HH 17 (30s).

detected at the caudal end of the midbrain vesicle spanning the isthmus (Fig. 1C). As previously reported for the mouse embryo (Mahmood et al., 1995), *Fgf8* RNA was absent from the floorplate (data not shown). During development to HH 10+ (11s), transcripts became increasingly localised to the isthmus (Fig. 1D). Subsequently, the domain of *Fgf8* expression increased in extent posterior to the isthmic constriction (anterior rhombomere 1) while becoming reduced anterior to it (Fig. 1E), until, by HH17 (30s), all *Fgf8* transcripts were detected posterior to the morphological constriction (Fig. 1F). Expression was also detected in the cardiogenic plate from HH 8+, the anterior forebrain from HH 8 (4s), the olfactory pits, branchial arches, primitive streak, tailbud and somites (Fig. 1B-F and HS unpublished observations).

We compared *Fgf8* expression with that of *En1*, *En2*, *Pax2* and *Wnt1*. Among these genes, *En1* transcripts were the first to be detected in the neural plate, being present from the 3-somite stage (HH 8–; Fig. 2A). Expression was seen in the presumptive midbrain region but *En1* RNA was absent from the floorplate. As development proceeded, transcripts were detected throughout the midbrain vesicle (Fig. 2B) and at HH 10+ (11s) extended from the diencephalic/mesencephalic boundary across the isthmus into the anterior of rhombomere 1 (r1; Fig. 2C). At later stages, however, expression of *En1* became localised closer to the isthmus in the midbrain while remaining in anterior r1 (Fig. 3C). *Pax2* transcripts were detected in a similar region of the neural plate to *En1* but were first detected at HH 8 (4s), approximately 60 minutes after *En1*. Greatest apparent expression was first detected adjacent to the floor plate, with weaker expression in lateral regions (Fig. 2D). As neuromeric boundaries became apparent, *Pax2* transcripts were detected in the caudal midbrain, across the isthmus and within anterior r1 (Fig. 2E,F), but occupied a less extensive region of the midbrain than *En1* at the same developmental stage. At later stages, *Pax2* transcripts persisted in the isthmus and extended into the caudal part of the midbrain and rostral part of r1 (Fig. 3D). *En2* RNA was first detected in the 6s embryo (HH 9–; Fig. 2G), by HH 10 (10s) expression was localised to the caudal midbrain, across the isthmus and in anterior r1 (Fig. 2H) and at stage 17 (30s) encompassed a broader region (Fig. 3B) than *En1*. By contrast, *Wnt1* transcripts were detected in the presumptive dorsal regions of the neural tube at HH 8+ (5s) but spanned a territory much greater than just the midbrain/r1 primordium (Fig 2I). By HH9– (6s) expression was detected more ventrally in the midbrain region while remaining dorsally restricted elsewhere (Fig. 2J) and at HH 10 (10s) transcripts were detected throughout the midbrain vesicle, across the isthmic constriction and into the anterior of r1 (Fig. 2L). At later stages, *Wnt1* RNA became restricted to the dorsal midbrain and a dorsoventral stripe anterior to the isthmus (Fig. 3A).

In summary, transcripts of all of the genes studied initially occupy a broader domain at the onset of their expression than they do at later stages. Both *En1* and *Pax2* are detected before *Fgf8* and *Fgf8* transcripts become localised to the isthmus before expression of other genes refines to the posterior midbrain. As development proceeds, *Fgf8* transcripts become localised posterior to the isthmic constriction.

Effects of ectopic FGF8 on midbrain and diencephalon (i) early regulation of gene expression

Spatial and temporal *Fgf8* expression are consistent with it being a candidate molecule for providing the isthmic polarising influence on the midbrain as identified in tissue grafting studies (see Introduction). To investigate this possibility, we introduced FGF8 protein ectopically within the anterior midbrain on heparin-coated acrylic beads and examined its ability to maintain and/or induce the expression of genes involved in specification of the posterior midbrain including *Pax2* and *En1*

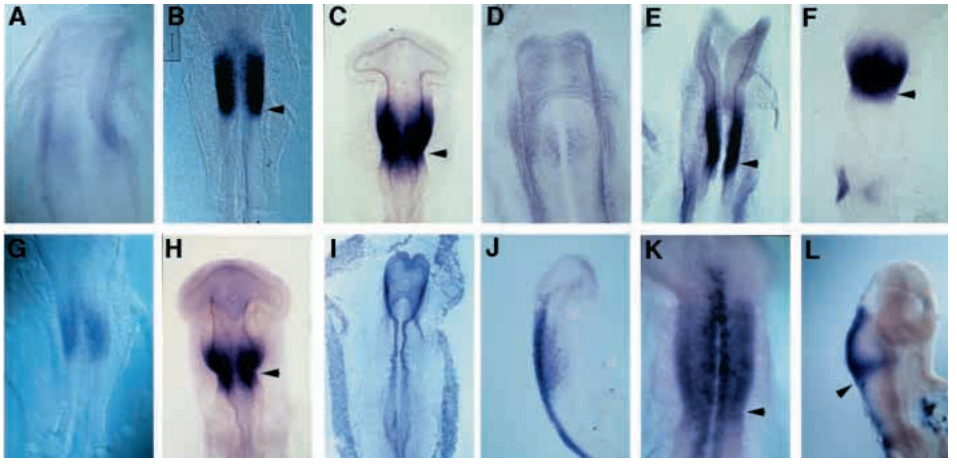

Fig. 2. Spatial and temporal expression of expression of *En1, Pax2, En2* and *Wnt1* genes required for normal development of the mid/hindbrain region. In situ hybridisation to *En1* (A-C), *Pax2* (D-F), *En2* (G,H) and *Wnt1* (I-L) transcripts in whole chick embryos. The arrowhead, present in certain panels, marks the position of the isthmic constriction. (A-I,K) Dorsal view, (J,L) lateral view. (A-C) *En1* expression in the neural tube of 3s (A; HH 8–), 6s (B; HH 9–) and 11s (C; HH 10+) embryos. At the onset of detectable expression (A), transcripts are present in the lateral and medial neural plate but are absent from the floorplate. Transcripts are initially detected throughout the midbrain vesicle, also extending into anterior r1 (B), but later become restricted to the posterior midbrain and anterior r1 (C). (D-F) *Pax2* RNA in the neural tube of 4s (D; HH 8), 7s (E; HH 9,) and 10s (F; HH 10) embryos. Earliest expression was detected in cells adjacent to the floorplate which, itself, was *Pax2*-negative (D). At later stages, transcripts were detected in the posterior midbrain and anterior of r1 (E), becoming progressively more localised to the isthmic constriction (F). (G,H) *En2* expression in the neural tube of 7s (G; HH 9) and 13s (H; HH 11) embryos. *En2* transcripts are first detected in the posterior midbrain and r1, although absent from the floorplate (H) and this pattern of expression is maintained subsequently. (I-L) *Wnt1* transcripts in 5s (I; HH 8+), 7s (J; HH9), 10s (K; HH 10) and 16s (L; HH12) embryos. RNA is first detected in the lateral margins of the neural plate extending from the posterior forebrain throughout the midbrain and hindbrain primordia (I). Expression begins to extend ventrally in the midbrain region at 7s (J) and, by 10s, transcripts are present throughout the midbrain and in anterior r1 (K). Later, expression is lost in r1 and *Wnt1* RNA becomes confined to the dorsal midline of the midbrain and a dorsoventral band of cells anterior to the isthmus (L).

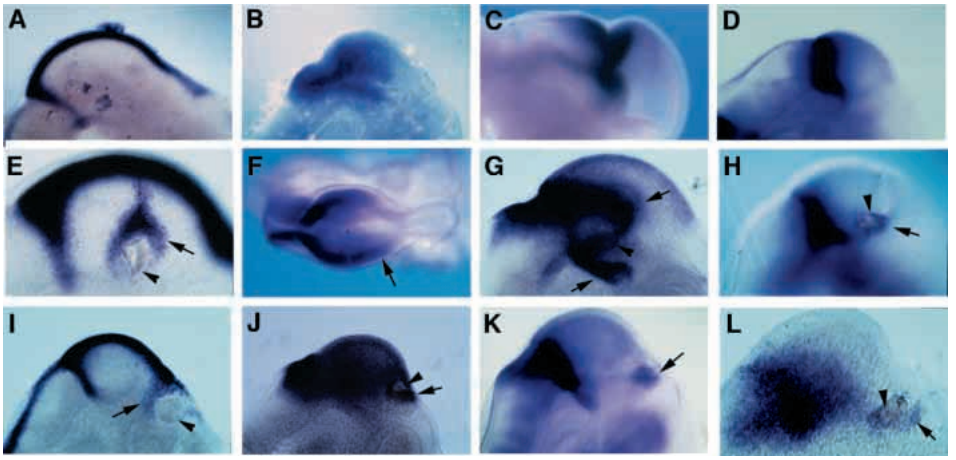

Fig. 3. Induction of gene expression by ectopic FGF8 in the midbrain and diencephalon. Expression of *Wnt1* (A,E,I), *En2* (B,F,J), *En1* (C,G,K) and *Pax2* (D,H,L) 24 hours after implanting beads. (A-D) Normal pattern of gene expression at HH 17-19; (E-H) gene expression after FGF8-coated bead grafted into the midbrain and (I-L) FGF8-coated bead grafted into the diencephalon. The position of the implanted bead is marked by an arrowhead and ectopic expression indicated by an arrow in E-L. All embryos are viewed laterally except F which is a dorsal view.

which are normally expressed before *Fgf8* (see above). Others have shown that this approach provides a focal source of functional FGF protein with the growth factor remaining bound to the bead or diffusing only a few cell diameters from it (Storey et al., 1998). We compared the effects of FGF8 on midbrain with its effects on posterior diencephalon.

Heparin-coated acrylic beads were incubated with the FGF8b protein isoform (nomenclature of MacArthur et al., 1995) and grafted into HH 10-12 (10-16s) host chick embryos, i.e. at a time after subdivision of the primary brain vesicles and formation of a morphological isthmic constriction and prior to the midbrain vesicle being irreversibly polarised along its AP axis (Itasaki et al., 1991). In these and in all subsequent experiments, control experiments using beads incubated in culture medium alone prior to implantation, failed to produce any of the responses observed when FGF protein was applied to the beads.

Following ectopic application of FGF8 in the anterior midbrain, ectopic expression of all of the genes examined was detected within 24 hours in the region of the implanted bead (compare Fig. 3A-D with Fig. 3E-H and see also Fig. 4A). Ectopic *Wnt1* (*n*=14) was generally induced in a strip of cells extending ventrally from the dorsal midline and in an arc around the bead (Fig. 3E). In a few embryos, ectopic induction was only observed around the bead and not extending from the dorsal midline and in some embryos there was a region of *Wnt1*-negative cells between the bead and the ectopic *Wnt1* domain (data not shown). Induction of *En* genes, *En2* (*n*=13) and *En1* (*n*=4) involved a rostral extension of their endogenous domain, although only in the case of *En2* did the ectopic domain extend as far rostrally as the midbrain/diencephalic border (Fig. 3F,G). By contrast, *Pax2* (*n*=4) was induced only in a small domain highly localised around the bead (Fig. 3H). In all cases, the induced gene expression by 24 hours was confined to the side of the neural tube into which the bead had been placed. This was also true for responses in the diencephalon (below) and identical results were obtained when embryos were examined 48 hours after implantation (data not shown). Alternative splicing can generate up to eight protein isoforms from the mouse *Fgf8* gene (designated Fgf8a-h; Crossley and Martin, 1995; MacArthur et al., 1995). Examination of the ability of several of these (Fgf8a,b,c and d) to induce *Wnt1* showed that all were capable of efficiently inducing ectopic expression (K. Mahony, H. S., I. M. and C. Dickson, unpublished data).

We examined the ability of FGF8 to induce ectopic expression of the same genes in the diencephalon. Induction of *En2* and *Wnt1* in the caudal diencephalon has been previously reported (Crossley et al., 1996), and we confirmed these results in our study of *Wnt1* (*n*=3; Fig. 3I) and *En2* (*n*=6; Fig. 3J). We also examined the ability of FGF8 to induce *En1* and *Pax2*, expressed before *Fgf8* in the developing midbrain region and not previously examined by others. Ectopic *En1* (*n*=7; Fig. 3K) and *Pax2* (*n*=3; Fig. 3L) were found to be induced in a restricted region adjacent to the bead. Only in the case of *En2*

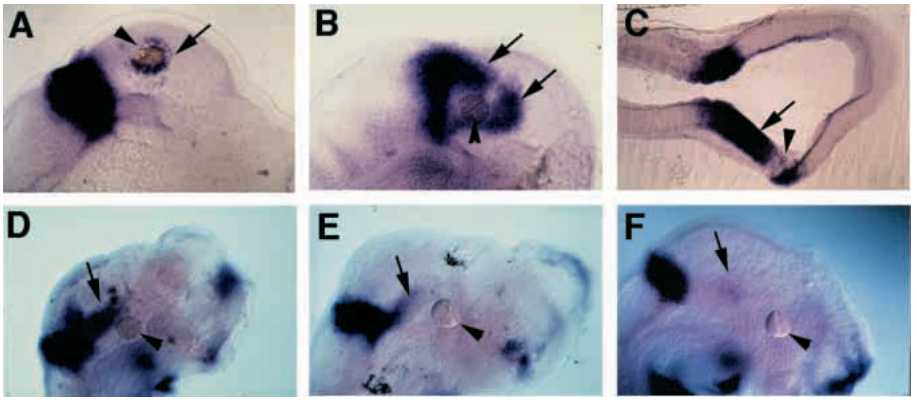

Fig. 4. Induction of *Fgf8* expression by FGF protein. FGF-coated beads (A: FGF8; B-F: FGF4) were implanted in the midbrain (A-C), midbrain/diencephalic boundary (D), prosomere 1 (E) or prosomere 2 (F). Embryos were incubated for 24 hours and examined for ectopic *Fgf8* expression. (A) FGF8 protein induces *Fgf8* RNA in the midbrain. (B) FGF4 induces more extensive *Fgf8* expression in the midbrain than FGF8. (C) Longitudinal section from embryo shown in B. (D) An FGF4-coated bead placed in the midbrain/diencephalic boundary induces *Fgf8* only in the midbrain. (E,F) FGF4-coated beads implanted in the posterior diencephalon fail to induce *Fgf8* in that region but weak ectopic expression is detected in the midbrain. The position of the implanted bead is marked by an arrowhead and ectopic expression indicated by an arrow.

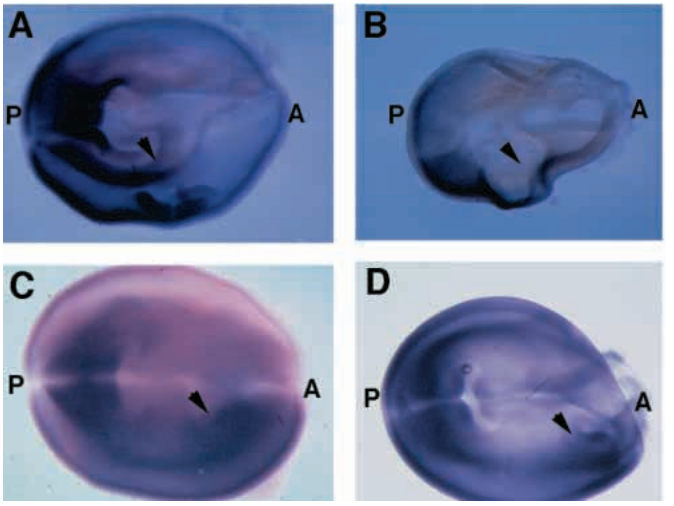

Fig. 5. Ectopic expression of *ephrin A2* and *A5* in the midbrain and diencephalon induced by FGF. FGF-coated beads were grafted into the midbrain (A,B) or posterior diencephalon (C,D) and embryos allowed to develop until HH 25 (E5). Embryos were examined for ectopic expression of *ephrin A2* (A,C) and *A5* (B,D) transcripts. Tecta have been dissected away from other brain structures and are viewed from the dorsal surface. Ectopic gene expression is indicated with an arrowhead and posterior (P) and anterior (A) poles are indicated.

were ectopic transcripts also detected in the midbrain following introduction of FGF8 protein into the diencephalon (Fig. 3J). Identical results were obtained when beads were implanted into either prospective prosomeres 1 or 2 of the caudal diencephalon and, in agreement with a previous report (Crossley et al., 1996), effects of ectopic FGF were confined to these posterior two prosomeres (P1 and P2) and were not seen in the adjacent P3.

By contrast, the ability of FGF8 to induce ectopic *Fgf8* expression differed between the midbrain and caudal diencephalon. FGF8 protein induced ectopic *Fgf8* transcripts in the midbrain vesicle when a bead was implanted into the anterior of that structure (n=4; Fig. 4A). Following a bead implant into the caudal diencephalon, however, no ectopic *Fgf8* RNA was detected in that tissue (n=10; data not shown). We also found that similar concentrations of FGF4 applied to beads could efficiently induce *Fgf8* in a more extensive region of the midbrain than FGF8 protein (n=22; Fig. 4B,C), suggesting greater activity. However, FGF4-coated beads also failed to induce ectopic *Fgf8* in the diencephalon when implanted into the midbrain/diencephalon border (Fig. 4D), P1 (Fig. 4E) or P2 (Fig. 4F). But, notably, beads implanted into the midbrain/diencephalon border did induce *Fgf8* in the midbrain (Fig. 4D) and implants into either P1 or P2 also induced weak ectopic *Fgf8* expression in the midbrain (Fig. 4E,F). FGF8 protein never induced *Fgf8* RNA in midbrain when a bead was introduced within the diencephalon. There are a number of possible explanations for this difference between FGF4 and FGF8, e.g. differing activity, stability or diffusion properties.

Effects of ectopic FGF8 on the midbrain and diencephalon (ii) later gene expression, morphology, cytoarchitecture and division

Polarisation of the midbrain is reflected by the differential projection of axons from nasal and temporal retinal ganglion cells to posterior and anterior optic tectum respectively. Two ligands of the ephrin family, ephrin A2 (ELF1) and A5 (RAGS), are expressed in a decreasing caudorostral gradient in the posterior tectum and have been implicated in the guidance of nasal axons and repulsion of temporal axons (Cheng et al., 1995; Drescher et al., 1995). Embryos were incubated until embryonic day 5 (E5, HH 26), when retinal axons would normally have reached the tectum, following grafting of FGF-coated beads into either the posterior diencephalon or midbrain at HH 10-12, and expression of *ephrin A2* and *ephrin A5* was examined. Following a graft into the anterior midbrain the expression of both genes extended further anteriorly in the tectal hemisphere that received the bead (Fig. 5A,B) but never extended to the most anterior region of the tectum. By contrast, following a graft into the diencephalon expression of both ligands extended throughout the midbrain and within the ectopic tectum induced from the diencephalon. In such embryos, the ectopic tectum exhibited reversed polarity and fused with the normal tectum (see below) and, consistent with this, expression of both genes decreased towards the centre of the fused structure (Fig. 5C,D). Identical results were obtained when either FGF4 or FGF8 was applied on the beads.

We allowed some embryos to develop until E9 (HH 35) following bead grafts at HH 10-12 in order to examine the effects of FGF, introduced ectopically into the midbrain or diencephalon, on the morphology and cytoarchitecture of those structures; again, identical results were obtained with FGF4 and FGF8 protein. Such embryos showed a range of morphologies, although these were similar regardless of whether the bead was implanted in the midbrain or diencephalon. In some instances, an additional tectal vesicle was apparent anteriorly on the side that received the bead (Fig. 6A,B). Loss of normal diencephalic derivatives was apparent in embryos that had previously had beads introduced into that structure and instead an expanded tectum was produced which was fused with the normal midbrain (Fig. 6C). Occasionally, ectopic tecta were produced bilaterally (data not shown) and in some instances were not clearly distinguishable from the normal midbrain by the presence of a cleft (compare Fig. 6A,B with C). Many embryos, however, showed a dramatic foliation of the normally smooth tectal surface and this was observed following application of a bead to either the midbrain or diencephalon (Fig. 6D,E).

The cytoarchitecture of the brains of these older embryos was examined by Nissl staining following sectioning. Effects of beads grafted into the midbrain were largely confined to the tectum. The tectal wall was both expanded and thinned (Fig. 6F), consistent with respecification to a phenotype associated with posterior midbrain. Embryos in which a smooth ectopic tectal structure had been produced following introduction of a bead into the diencephalon, were found to have had the normal derivatives of the posterior diencephalon, the synencephalon and parencephalon, replaced by ectopic midbrain structures, most noticeably an optic tectum (compare Fig. 6G and H). The cytoarchitecture of the ectopic tectum showed a reversed polarity with a reversed gradient of thickness and the presence of an ectopic torus semicircularis at the anterior of the structure (Fig. 6H). This was consistent with analyses of gene expression at earlier stages (see above). There was also an apparent duplication of ventral midbrain structures, again with reversed

polarity, and with an ectopic nucleus located anteriorly (Fig. 6H). The identity of the neurons constituting the latter could not be confirmed. We never found structures with a cerebellar morphology induced following grafts into either midbrain or diencephalon.

Sections of brains exhibiting foliation showed them to have greatly thickened tecta with the lumen of the midbrain being filled with folds of the tectal wall. These tecta had a normal number of layers but each layer was thickened (Fig. 6I,J). Expansion of the dorsolateral midbrain was also apparent in some embryos as early as 24 or 48 hours after grafting (see e.g. Fig. 4C). These data strongly suggested that ectopic application of FGF increased proliferation in the midbrain and diencephalon of some embryos. We examined this possibility by labelling dividing cells with a short pulse of BrdU after introducing the bead. Beads coated with FGF8 were grafted into either the midbrain or the diencephalon, embryos were incubated for 24 or 72 hours and BrdU was then introduced directly into the lumen of the neural tube via a microelectrode. Embryos were incubated for a further 60 minutes before

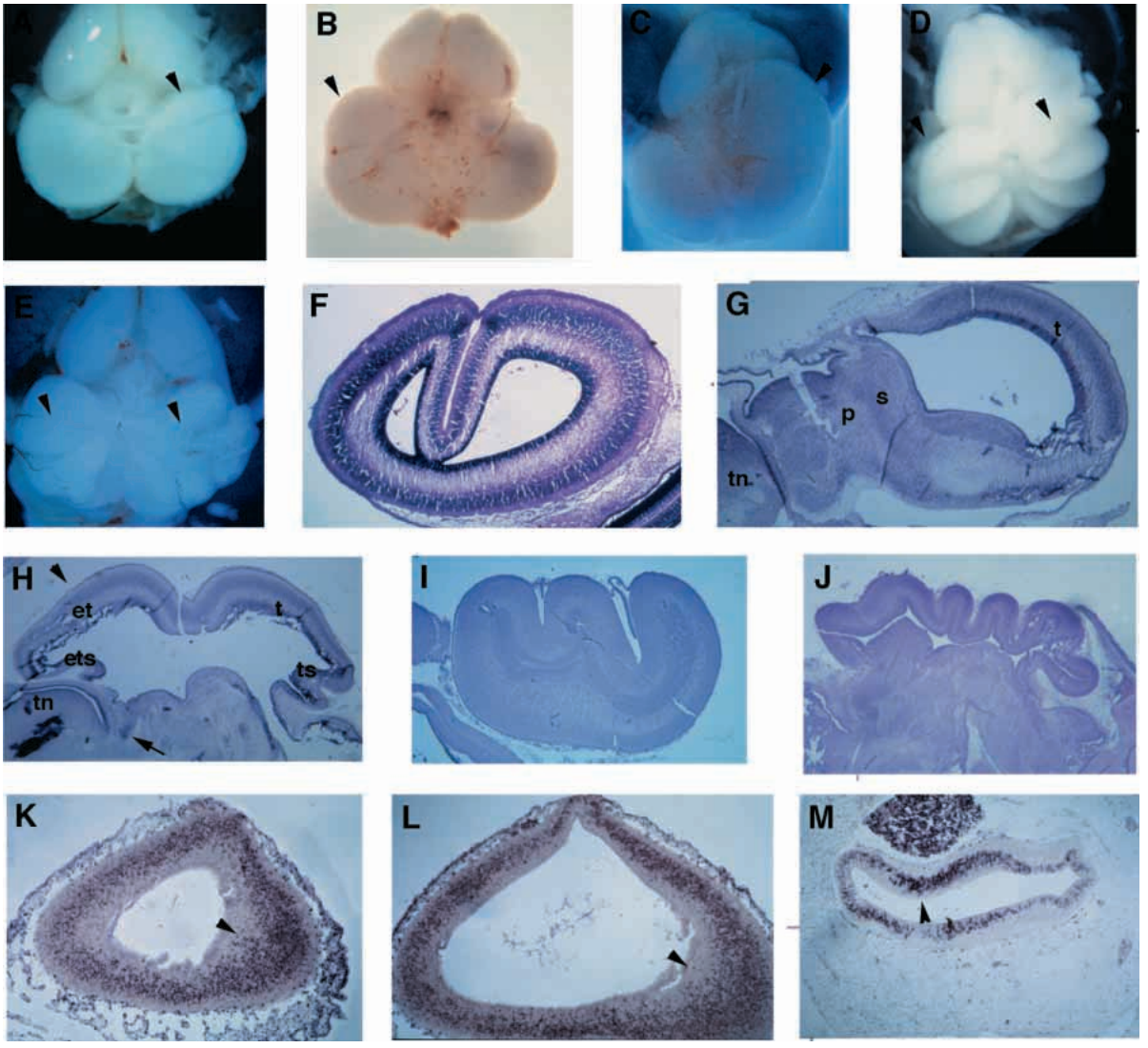

Fig. 6. Effects of ectopic FGF protein on morphology, cytoarchitecture and cell division in the midbrain and diencephalon. FGF-coated beads were grafted into the midbrain (A,D) or posterior diencephalon (B,C,E) and embryos allowed to develop until HH 35 (E9). Brains were dissected free of overlying tissue and photographed from the dorsal surface. (A,B) Ectopic vesicles produced when beads were grafted into the midbrain (A) or diencephalon (B) are indicated with an arrowhead. (C) A bead grafted into the diencephalon resulting in the unilateral expansion of the tectal structure (arrowhead) with apparent loss of posterior forebrain derivatives. (D,E) Bilateral foliation (arrowheads) of tecta following bead implantation into either the midbrain (D) or diencephalon (E). (F) Section from an embryo that received an FGF8 bead implant into the midbrain. (G,H) Sections taken from the control (G) and grafted (H) sides of the brain shown in C and stained for Nissl substance. The ectopic tectal structure is indicated by an arrowhead in H. An ectopic nucleus is indicated by an arrow. (I,J) Sections of foliated tecta taken from embryos having FGF-coated beads implanted into the midbrain (I) or diencephalon (J) and stained for Nissl substance. Anterior is to the left in F-J. (K,L,M) Increased BrdU incorporation (arrowhead) is associated with ectopic FGF8 protein expression in the midbrain (K,L) or diencephalon (M) and is observed 24 hours (K) and 72 hours (L,M) after implanting the FGF-soaked bead. et, ectopic tectum; ets, ectopic torus semicircularis; p, parencephalon; s, synencephalon; t, tectum; tn, telencephalon; ts, torus semicircularis.

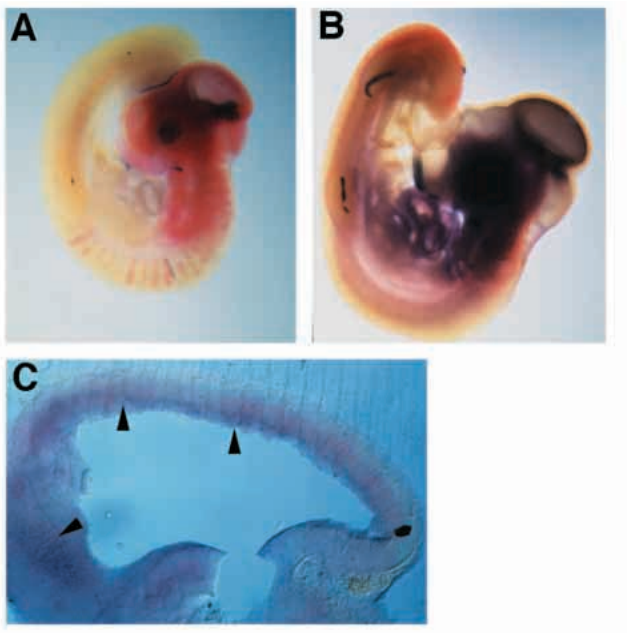

Fig. 7. En1 induces ectopic expression of *Fgf8* in midbrain and diencephalon. Embryos were infected with either a control (RCAN) retrovirus which produces transcripts containing mouse *En1* coding sequences but which cannot translate En1 protein due to the lack of a splice acceptor site or with a retrovirus (RCAS) which makes translatable *En1* mRNA. The extent of infection was determined by hybridisation with sequences specific for mouse *En1* (red colour) and *Fgf8* expression also analysed in the same embryos (blue/black colour). (A) Control embryo infected with the RCAN-En1 virus shows extensive infection of the head including the brain but no ectopic *Fgf8* transcripts are detected. (B) Embryo infected with the RCAS-En1 virus shows ectopic *Fgf8* expression in the head region including the midbrain and diencephalon. Note that the hindbrain is also infected but shows no ectopic *Fgf8* transcripts. (C) Section taken through the midbrain of an embryo infected with RCAS-En1 shows ectopic *Fgf8* RNA (arrowheads) in the ventricular zone.

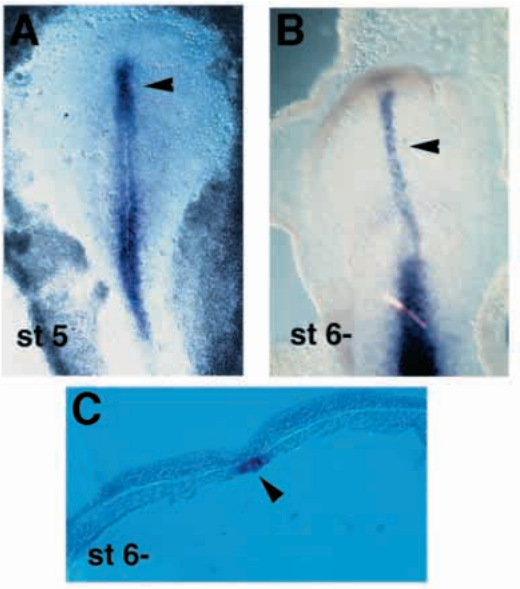

Fig. 8. Transient expression of *Fgf4* in the anterior notochord. (A,B) In situ hybridisation to *Fgf4* transcripts in HH 5+ (A) and HH 6– (B) chick embryos. (C) Transverse section of a HH 6– embryo taken anterior to Hensen's node shows *Fgf4* transcripts within the axial mesoderm (notochord). Arrowheads indicate expression in the notochord.

fixation, sectioning and immunohistochemical detection of incorporated BrdU. The number of dividing cells was greatly increased on the side of the neural tube that had received the grafted bead and this was associated with thickening of the neuroepithelium in the region of the implant. This increase in division was observed in embryos labelled either 24 or 72 hours after grafting the bead and was observed in both the midbrain (Fig. 6K,L) and the diencephalon (Fig. 6M and data not shown).

Ectopic En1 induces *Fgf8* expression in the midbrain and diencephalon

Examination of the temporal sequence of gene expression in the developing midbrain showed that both *En1* and *Pax2* were expressed before *Fgf8* could be detected, although both of these genes can be induced by ectopic FGF8 protein in older embryos. *En1* was the earliest of the midbrain-associated genes to be expressed during development of that tissue and we therefore examined the ability of ectopic En1 to induce *Fgf8*. Embryos were infected between HH 9 and 12 with an avian retrovirus which expressed high levels of transcripts encoding mouse En1 protein (Logan et al., 1996) and were examined for ectopic induction of *Fgf8* 36-48 hours later. Control embryos, infected with a retrovirus that produced RNA containing *En1* sequences which were not translated into En1 protein, showed no ectopic *Fgf8* expression (Fig. 7A). By contrast, embryos infected with virus producing translatable *En1* RNA had extensive ectopic expression of *Fgf8* within the neural tube. Notably, ectopic *Fgf8* transcripts were detected in the midbrain and diencephalon in these embryos (Fig. 7B); those regions in which ectopic FGF8 induces *En1*. Sections of these embryos showed that ectopic *Fgf8* RNA was induced in cells in the ventricular zone but was absent from the differentiated cells in the mantle layer including those expressing ectopic *En1* RNA (Fig. 7C).

Regulation of *En1* expression by FGF4 expressed in the notochord

The ability of FGF8 and En1 to induce transcripts for each other when expressed ectopically strongly suggests that each might be involved in the maintenance of the other during the development of the midbrain region. In addition, it raised the possibility that signalling by one or more FGF(s) might be involved in initiation of *En1* expression in the neural plate. Studies in amphibian, chick and mouse embryos have shown that expression of *En* genes is regulated by signals from underlying mesoderm, in particular, the notochord (Hemmati-Brivanlou and Harland, 1989; Hemmati-Brivanlou et al., 1990; Ang and Rossant, 1993; Darnell and Schoenwolf, 1997). It has previously been suggested (Crossley et al., 1996) that Fgf8 expressed in the cardiogenic plate might induce expression of *Fgf8* (and presumably, therefore, other Fgf8-responsive genes such as *En1*) in the overlying neural plate. However, we believe that this is unlikely since we find that *Fgf8* transcripts are first detected in the cardiogenic mesoderm at the same time as

expression is first detected in the neural plate and that the region of the neural plate expressing *Fgf8* does not overlie the cardiogenic plate at this time (Fig. 1B). A similar conclusion was reached by Lee et al. (1997) who reported that *Pax2* expression was induced in the murine neural plate prior to onset of *Fgf8* expression in cardiogenic mesoderm. We therefore examined other members of the FGF family for potential candidates for a vertical inducing activity for *En1*.

We found that *Fgf4* RNA had an appropriate spatial and temporal expression pattern to fulfil a vertical signalling role in midbrain development. Transcripts of this gene were detected transiently in the midline of the embryo anterior to Hensen's node between HH 5+ and HH7 (1s; Fig. 8A,B) but transcripts became undetectable between stages 7+ and 8– (2s-4s; H. S. and I. M., unpublished data). Transverse sections showed that expression of *Fgf4* was confined to the notochord (Fig. 8C). These data suggested that Fgf4 is a vertical signalling molecule from the notochord.

To investigate this possibility we first examined the ability of FGF4, applied to heparin-coated acrylic beads, to induce markers of the midbrain region when grafted into the midbrain and diencephalon as described for FGF8 above. We found that FGF4 was able to induce all of the genes studied in exactly the same manner as FGF8 (midbrain: *Wnt1* n=21, *En1* n=9, *Pax2* n=5, *En2* n=8; diencephalon: *Wnt1* n=6, *En1* n=3, *Pax2* n=3, *En2* n=11; data not shown) and could induce *Fgf8* in the midbrain (Fig. 4B-E). Indeed, FGF4 generally induced higher apparent levels of expression of all of the above genes than FGF8. The requirement for the notochord and the potential involvement of Fgf4 in the induction of *En1* expression in the neural plate was then examined using a collagen gel explant culture system.

The neural plate is induced at HH 4 (Roberts et al., 1991) and we isolated the region fated to give rise to the midbrain primordium (Schoenwolf and Sheard, 1990; Muhr et al., 1997) from HH 5+ and HH 6 embryos and cultured it together with notochord and paraxial mesendoderm for 24 hours. Explants were then examined for *En1* transcripts, which do not appear until HH 8– in ovo. *En1* expression was found to be induced in the explants (n=17/17, 5 experiments; Fig. 9A). However, explants comprising neural plate and paraxial mesoderm, but lacking the notochord, expressed little or no detectable *En1* transcripts (n=40/40, 5 experiments; Fig. 9B). When the collagen gel and medium was supplemented with physiological concentrations of FGF4 (1-10 ng/ml) significant expression of *En1* was detected in explants lacking notochords (n=25/37, 4 experiments; Fig. 9C). Control experiments to detect *Krox20* expression, characteristic of hindbrain levels, failed to detect transcripts in any of the above experimental conditions (n=9; data not shown). Moreover, when prospective hindbrain tissue from stage 7 embryos (the equivalent stage of neurulation to the prospective midbrain region examined above) was cultured with or without notochord and in the presence or absence of FGF4, no *En1* transcripts were detected (n=13; Fig. 9D,E and

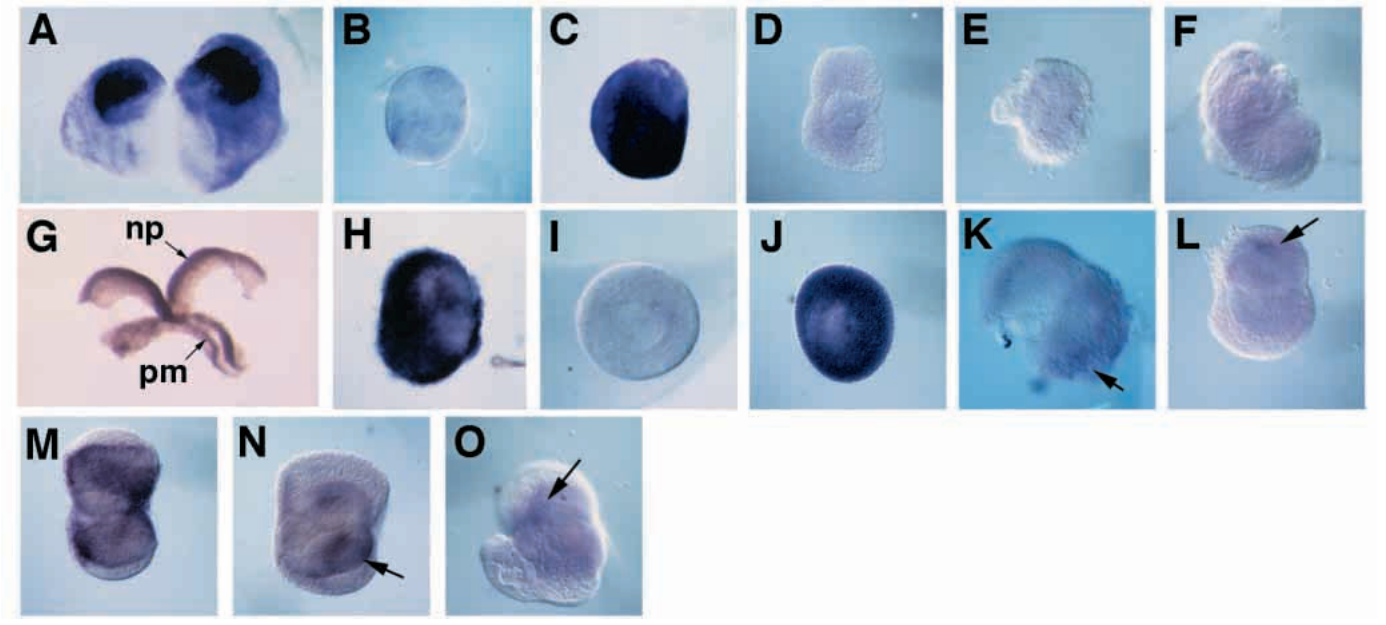

Fig. 9. FGF4 induces *En1* in explants of anterior neural plate. Explants were established from the region of neural plate fated to form midbrain from HH 5-6 embryos and cultured for 24 hours prior to in situ hybridisation for *En1* RNA. (A) Expression of *En1* in an explant comprising neural plate, paraxial mesendoderm and notochord. (B) Little or no expression of *En1* is detected when explants comprise neural plate and paraxial mesendoderm but lack notochord. (C) FGF4 added to explants of neural plate and paraxial mesoderm induces *En1* expression. Prospective hindbrain cultured intact with notochord (D) or without notochord but with FGF4 (E) fails to express *En1*. (F) Prospective anterior forebrain tissue cultured in the presence of associated mesoderm and FGF4 does not express *En1*. (G) Dispase treatment efficiently separates the neural plate (np) from underlying paraxial mesendoderm (pm). (H) FGF4 stimulates *En1* expression in isolated neural plate. (I) No *En1* transcripts are detected when FGF4 is applied to paraxial mesendoderm. (J-L) Prospective midbrain explants, including notochord, from the same experiment cultured with FGFR1-Fc chimera protein (K,L) show a considerable reduction in *En1* expression compared to control explants (J). (M-O) Culture of similar explants in the presence of anti-FGF4 neutralising antiserum (N,O) likewise show a reduction in *En1* expression when compared to sister control explants (M) although the degree of inhibition is less than with the FGFR1 Fc chimera. Arrows indicate residual *En1* expression in K,L,N and O.

data not shown). Likewise prospective anterior forebrain from stage 6 embryos cultured in the presence or absence of FGF4 also failed to express *En1* (*n*=6; Fig. 9F and data not shown).

To ascertain whether the effect of FGF4 was direct or whether it was acting indirectly via paraxial mesendoderm, we separated the neural plate from the underlying paraxial mesendoderm (Fig. 9G) and cultured each separately in the presence of FGF4. FGF4 was found to induce *En1* in isolated neural plate explants (*n*=10/10; Fig. 9H) but not in explants of paraxial mesoderm (*n*=0/5; Fig. 9I) suggesting that its action on the neural plate was direct.

To provide further evidence for a role for FGF4 in the regulation of *En1* expression, we sought to inhibit FGF4 action in explants containing notochord by using soluble inhibitors of FGF action. Intact explants were treated with an FGFR1-Fc chimeric protein, previously shown to inhibit FGF activity (Williams et al., 1994) in vitro and known to bind with high affinity to FGF4 (P. Doherty, unpublished data). Alternatively, explants were exposed to a neutralising antibody raised against human FGF4 protein. In both cases, a reduction in *En1* expression was observed relative to the untreated controls. The degree of inhibition was greatest using the FGFR1-Fc fusion protein (*n*=11/11; compare Fig. 9J with 9K and L) compared to the antiserum (*n*=11/12; compare Fig. 9M with 9N and O). In addition, the degree of inhibition using both reagents varied between explants from the same experiment (compare Fig. 9K with 9L and 9N with 9O), however a reduction in expression was observed in most explants.

DISCUSSION

FGF4, expressed in the notochord, can substitute for that tissue in regulating *En1* expression in vitro

Considerable evidence has accumulated suggesting that vertical signals from underlying mesoderm contribute to the regionalisation of the neural tube along its anteroposterior axis. In a number of these studies, expression of *En* genes has been used to identify neural tissue specified to a phenotype characteristic of posterior midbrain/anterior r1. These reports showed that either anterior mesendoderm or notochord, specifically, produced vertical signals required for the *En1* and/or *En2* expression (Hemmati-Brivanlou and Harland, 1989; Hemmati-Brivanlou et al., 1990; Ang and Rossant, 1993; Darnell and Schoenwolf, 1997). We find that FGF4 is able to mimic the ability of notochord tissue to regulate *En1* expression and that *Fgf4* is expressed in anterior notochord in a temporal manner consistent with it performing this function *in ovo*. However, our experiments do not distinguish between an instructive or a permissive role.

eFGF, the probable FGF4 homologue in *Xenopus*, is also expressed in the notochord (Isaacs et al., 1992) and the latter tissue regulates *En2* expression in that organism (Hemmati-Brivanlou et al., 1990). Expression of *Fgf4* has not been reported in the notochord of mouse embryos, although it is important to note that in the avian embryo expression is extremely transient in that tissue. Moreover, the increasing size of the FGF family, which currently comprises 18 members, and their frequently overlapping patterns of expression indicate redundancy in function; thus, it is possible that another FGF performs this function in mice. Notochord-derived Fgf4 may also perform other functions in the neural plate, and it is noteworthy in this context that a mesodermally derived FGF activity has been associated with the production of cranial neural crest cells (Muhr et al., 1997). In addition, a mesodermal FGF4-like activity has been implicated in the regulation of production of serotonergic neurons in the hindbrain (Ye et al., 1998).

Recently, fibroblast growth factors have been implicated in both neural induction and posteriorisation of neural tissue to a phenotype characteristic of spinal cord levels (see Doniach, 1995; Mason, 1996 for reviews and critique). How can this be reconciled with our observations of FGF4 function in specifying a more anterior phenotype to neural tissue? Fgf4 expressed in the notochord is unlikely to be involved in induction of the anterior neural plate as its transcripts are detected only after the neural plate has been induced (Roberts et al., 1991). Implantation of FGF4-coated beads into the anterior peripheral epiblast of chick embryos induces ectopic mesoderm formation and either directly or indirectly induces neural tissue expressing markers characteristic of spinal cord levels (Alvarez et al., 1998; Storey et al., 1998). However, this ability is lost after HH4 (Storey et al., 1998) indicating a change in competence of the anterior epiblast. This is reflected in the establishment of adjacent *Otx2*-positive (anterior; Bally-Cuif et al., 1995b) and *Gbx2*-positive (posterior; Shamim and Mason, 1998) domains in the epiblast including the neural plate. Thus, *Fgf4* expression is detected in the notochord anterior to Hensen's node only after the anterior epiblast has lost its ability to respond to FGF signalling by the induction of posterior neural tissue. It would seem likely, therefore, that the ability of Fgf4 to regulate expression of *En1*, a marker of prospective midbrain/r1 tissue, may also be dependent upon this change in competence. In support of this, targeted disruption of *Otx2* in transgenic mice indicates that it is required for the initiation of *En1* expression (Rhinn et al., 1998).

The region of the neural plate within which *En1* transcripts are induced is smaller than the extent of the neural tissue which overlies *Fgf4*-expressing notochord. The anterior limit of *En1* induction is probably defined by the interface between the *Fgf4*-negative prechordal plate and the notochord. How the posterior limit of the *En1*-expressing domain is defined is unclear but the neural plate is clearly regionally specified at the time of onset of *En1* expression (see above) and it has previously been reported that vertical signals, capable of inducing *En2*, are present in mesoderm that underlies *En2*-negative regions of neuroepithelium (Darnell and Schoenwolf, 1997). Thus, differential competence of the neural plate to respond to FGF4 may define the posterior limit of induced *En1* expression. *Otx2* expression and function suggest a role regulating such competence (Bally-Cuif et al., 1995b; Rhinn et al., 1998). Consistent with this, we failed to induce ectopic *En1* expression with FGF4-coated beads implanted into posterior hindbrain levels in HH7 (1s) embryos prior to the normal onset of *En1* expression in the mid/hindbrain region (data not shown) or in explants of presumptive hindbrain neural plate. Additionally, planar signals from the node may also influence the posterior limit of *En1* induction.

Confirmation of a role for Fgf4 in vertical signalling requires experiments in which its function is inhibited. To date, studies in which Fgf4 or FGF receptor (FGFR) function have been

compromised have failed to provide evidence for their function in the development of the mid/hindbrain region. Mouse embryos that are null for Fgf4 die at implantation (Feldman et al., 1995) and mice lacking functional FGFR1 and FGFR2 are not informative due to earlier developmental lesions (Deng et al., 1994; Yamaguchi et al., 1994; Arman et al., 1998). In this study, we show that both an FGFR1-Fc fusion protein and an anti-Hu FGF4 antiserum considerably reduce (but don't eliminate) *En1* expression in explants of presumptive midbrain which include notochord. The fusion protein is more effective than the blocking antiserum in this assay, this may be because the antiserum was raised against human FGF4 and does not cross-react as effectively with avian protein or, alternatively, that there are other FGFs expressed in the notochord as these stages which it does not bind. Those regions that continue to express *En1* RNA in the experiments with the Fc chimera, may be those in closest contact with the notochord in the explants. Thus our inhibition studies provide supporting evidence for a role for notochord-derived FGF4 in *En1* regulation but do not provide decisive proof.

Temporal expression of transcription factors and signalling molecules in the avian mid/hindbrain region

A detailed examination of the temporal expression of several genes (*Fgf8*, *En1*, *En2*, *Pax2* and *Wnt1*) that are required for normal development of derivatives of the midbrain and anterior hindbrain, reveals that both *En1* and *Pax2* are expressed before *Fgf8*. *En2* is detected subsequent to the onset of *Fgf8* expression, while *Wnt1* shows a complex pattern of expression, although dorsoventral expression throughout the midbrain and in anterior r1 occurs only after *Fgf8*. *Pax2* expression has not previously been reported in the chick embryo and the onset of *En1* expression has only been inferred from differential studies with an antiserum which detects all En proteins (Enhb-1) and a monoclonal antibody specific to En2 (4D9; Gardner et al., 1988; Davis et al., 1991; Gardner and Barald, 1992; Millet and Alvarado-Mallart, 1995).

Overall, complex spatial and temporal relative patterns of expression are observed for the genes studied. *En1*, *Pax2*, *Wnt1*, and probably also *Fgf8*, are initially expressed in a broader region of the neural tube than they come to occupy later and transcripts for all of the genes studied are also detected in r1 at least transiently (this study; Hollyday et al., 1995). These genes eventually come to occupy overlapping domains within the midbrain vesicle with *Fgf8* localised to the isthmic region, a dorsoventral stripe of *Wnt1* transcripts adjacent to the *Fgf8* domain and with *En1*, *Pax2* and *En2* spanning the isthmic constriction and occupying successively larger regions of the midbrain.

In contrast to our results in the chick embryo, *Pax2* expression precedes *En1, En2* and *Wnt1* in mouse and zebrafish embryos (Hatta et al., 1991; Puschel et al., 1992; Parr et al., 1993; Kelly and Moon, 1995; Rowitch and McMahon, 1995), whereas *Pax2* and *En1* expression are first detected at the same stage in *Xenopus* (Hemmati-Brivanlou and Harland, 1989; Davis et al., 1991; Heller and Brandli, 1997). This variation may be due to differences in the relative rates of transcript accumulation between species and/or in the sensitivity of the in situ hybridisation procedure for each transcript. However, in all vertebrate classes, *Pax2* and *En1* transcripts are detected either simultaneously or within a short time of each other. Taken together, these data suggest that *Pax2* and *En1* are unlikely to initiate each other's expression during development of the mid/hindbrain region (although they may serve to maintain one another's expression) and are possibly induced in response to the same signal(s). Rowitch and McMahon (1995) further show that the *Pax2* expression domain, at early stages, encompassed those of *En1* and *Wnt1* in the mouse. By contrast, the initial domains of *Wnt1* (in the lateral walls of the midbrain) and *En1* extend further anteriorly than that of *Pax2* in the chick brain. At later stages, the *Pax2* domain encompasses those of *Wnt1* and *En1* in both species.

Ectopic En1 induces *Fgf8*

En1 expression requires a vertical signal from underlying notochord, whereas others have shown that *Fgf8* is not induced by similar vertical signals (Darnell and Schoenwolf, 1997). We found that ectopic En1 can induce *Fgf8* expression in the neural tube and the regions that are competent to express *Fgf8* are those that are competent to respond to ectopic FGF8 protein by adopting a posterior midbrain fate. This is consistent with results of studies using the same strategy which show that ectopic En1 respecifies anterior midbrain to a posterior phenotype and consequently perturbs retinal ganglion axon guidance in the tectum (Friedman and O'Leary, 1996; Itasaki and Nakamura, 1996; Logan et al., 1996). Moreover, our data suggest that this respecification may be mediated, in part, by induction of ectopic *Fgf8* expression. These studies also suggest that *En1* is involved in the initiation of *Fgf8* expression in the neural plate. However, poor infection by the retroviral vector at stages prior to HH 7, combined with the approximately 18-hour delay after infection before the retroviral genome is activated (C. L. and J. Walshe, unpublished observations), prevent us from testing this hypothesis at the time of the normal onset of *En1* and *Fgf8* expression.

Although *En1* function has been disrupted in transgenic mice and results in loss of both midbrain and hindbrain derivatives (Wurst et al., 1994), studies of *Fgf8* expression have not been reported to date. Moreover, functional redundancy between *En1* and *En2* (Hanks et al., 1995) suggests that analysis of *Fgf8* expression in mice that are homozygous null for both genes might prove most informative. It is not clear whether or not *Fgf8* transcripts are induced throughout the *En1*-positive domain at the onset of expression of the former, but this seems unlikely and modulation of domains of gene expression by other, possibly planar, influences have been reported in this region of the neural plate (Darnell and Schoenwolf, 1997). However, it is noteworthy that, in the absence of either *Gbx2* function or in mice homozygous null for *Otx1* and hemizygous null for *Otx2*, *Fgf8* transcripts are induced in the neural tube but do not become refined to the isthmus, remaining expressed in a more extensive region of the mid/hindbrain territory (Acampora et al., 1997; Wassarman et al., 1997).

Role of FGF8 at the isthmus

Fgf8 transcripts are first detected after *En1* and *Pax2* and rapidly become associated with the isthmic constriction in the neural tube. Only subsequently do transcripts of *En1, En2*, *Pax2* and *Wnt1* become progressively restricted to the posterior

midbrain. At later stages *Fgf8* expression becomes localised to the region immediately posterior to the isthmic constriction. The latter finding is entirely consistent with the results of Millet et al. (1995) who showed, using lineage marking and in situ hybridisation for *Otx2* transcripts, that the isthmic constriction occupies a more anterior position by HH 20 than it does at HH 10. Taken together with fate-mapping studies of this region (Martinez and Alvarado-Mallart, 1989; Hallonet et al., 1990; Alvarez-Otero et al., 1993; Hallonet and Le Douarin, 1993), this indicates that the morphological isthmic constriction does not define regions specified to different fates and that the anterior limit of *Fgf8* expression marks the boundaries between the mid/hindbrain (mes/metencephalic; isthmocerebellar) and midbrain (mesencephalic; tectal) territories. This boundary of cell fate is also defined by the interface of *Otx2* and *Gbx2* expression domains (Millet et al., 1996; Shamim and Mason, 1998).

Our studies show that FGF8 mimics the activities of isthmus grafts in specifying posterior characteristics in the anterior midbrain and converts posterior diencephalon to the phenotype of a midbrain but with a reversed polarity. Other groups have reported similar properties for FGF8 (Crossley et al., 1996; Lee et al., 1997) but there are a number of differences between their findings and our own. Within the anterior midbrain we found that ectopic FGF8 protein efficiently induced the ectopic expression of genes associated with posterior midbrain: *En1*, *Pax2*, *En2*, *Wnt1* and *Fgf8* itself. By contrast, Lee et al. (1997) used a *Wnt1* promoter element to drive Fgf8 expression in the Wnt1-expressing cells of the midbrain (ie. dorsal midline cells and a ring of cells anterior to the isthmus) of transgenic mice. This group found that *En2* and possibly *Wnt1* but not *Pax2* or *En1* were induced. It is possible that this apparent difference in inductive capabilities reflects species-specific differences, however, it is more likely that the conflicting data arise from different experimental strategies in the chick and mouse e.g. the co-expression of Wnt1 and Fgf8 in the same cells in the latter animal. Lee et al. (1997) also used a different FGF8 isoform in their studies, FGF8a, from that used by ourselves and Crossley et al. (1996; FGF8b), however our preliminary data suggests that both behave identically when introduced into embryos on beads.

Crossley et al. (1996) reported the ability of ectopic FGF8 to induce *En2*, *Wnt1* and *Fgf8* in the diencephalon, associated with its respecification to a midbrain fate. We confirm these results for *En2* and *Wnt1* and extend the study to include *Pax2* and *En1* normally expressed before *Fgf8* during midbrain development and find that they are also induced in the diencephalon by FGF8. By contrast, we were unable to induce *Fgf8* transcripts in the diencephalon with FGF8 protein, suggesting a difference in competence between midbrain and diencephalon and that introduction of FGF8 protein on beads can respecify diencephalon in the absence of induction of ectopic *Fgf8* transcription. It is also possible that the ability of FGF8 and/or isthmic tissue to respecify the diencephalon may be mimicking an earlier vertical activity of FGF4.

Previous studies of ectopic gene expression following isthmic grafts are consistent with our findings. A number of studies have reported the ability of isthmic grafts to induce *En2* expression in host diencephalon or midbrain (Gardner and Barald, 1991; Bally-Cuif et al., 1992; Marin and Puelles, 1994; Bloch-Gallego et al., 1996). *Wnt1* is also induced in host diencephalic tissue by isthmic grafts and the ectopic expression occurs both around the graft and also extends dorsally to the roofplate (Bally-Cuif and Wassef, 1994). This pattern of expression is strikingly similar to that seen in grafts of FGF8-coated beads in our study. In addition, the ability of isthmic grafts to alter the topographic targeting of retinal ganglion axons in the tectum (Alvarado-Mallart and Sotelo, 1984; Ichijo et al., 1990; Itasaki et al., 1991) is mirrored by the induction by FGF8 of *ephrin A2* (this study; Lee et al., 1997) and *A5* (this study), which are associated with guidance of these axons, more anteriorly in the midbrain and in the diencephalon. We found that FGF-coated beads were able to induce ephrins throughout the enlarged and fused tectal structure when grafted into the diencephalon. By contrast, beads implanted in the midbrain only induced an anterior shift in their rostral limit of expression and ectopic transcripts never extended to the diencephalic/mesencephalic border. This was also true for *En1* and *Pax2* expression; only *En2* RNA was induced to the midbrain/diencephalic boundary. Taken together, these data suggest a possible influence of diencephalon on gene expression in the anterior midbrain which is lost if the former is respecified to a midbrain fate.

FGF8 can induce ectopic expression of genes which are normally expressed before it (*En1* and *Pax2*) and, indeed, which are capable of inducing *Fgf8* transcription themselves. This suggests that Fgf8 may normally function to maintain expression of genes associated with the posterior midbrain and anterior of r1, although it may also induce expression of *En2* which is first detected only after *Fgf8*. This would be consistent with our temporal studies which show that *Fgf8* expression becomes localised to the region of the isthmic constriction before transcripts of other genes become localised more posteriorly in the midbrain. Indeed, experiments using targeted gene disruption in transgenic mice to examine the functions of genes expressed in this region strongly suggest that they each might contribute to a regulatory loop maintaining each other's expression. Absence of Wnt1 function results in loss of *Fgf8* expression before other morphological changes are observed. It also results in loss of *En1* and *En2* transcripts, but the mutant phenotype is partially rescued by *En1* (Danielian and McMahon, 1996; Lee et al., 1997). In addition, ectopic Pax2 can induce *Wnt1* (Kelly and Moon, 1995) and mice homozygous null for *Pax5*, hemizygous null for *Pax2* lack expression of *Fgf8*, *En1*, *En2*, and *Wnt1* (Schwarz et al., 1997; Urbanek et al., 1997). However, the degree to which loss of tissue in these mutant mice contributes to the apparent loss of gene expression remains unclear.

Our cytological analyses show that effects of implanting FGF8 beads into either midbrain or diencephalon are frequently confined to the tectum and are consistent with FGF8 being the isthmic activity capable of repolarising the midbrain and respecifying posterior forebrain to a midbrain fate. In this respect our results are also consistent with those of others (Crossley et al., 1996; Lee et al., 1997). However, in contrast to experiments involving grafting of isthmic tissue (Marin and Puelles, 1994), ectopic FGF8 protein never produced ectopic structures with cerebellar morphology in either diencephalon or midbrain. In addition, nuclei associated with the isthmus and r1, e.g. the trochlear (IV) motor nucleus, were not duplicated anteriorly, although in one specimen an ectopic oculomotor nucleus, a midbrain derivative, may have been generated. The

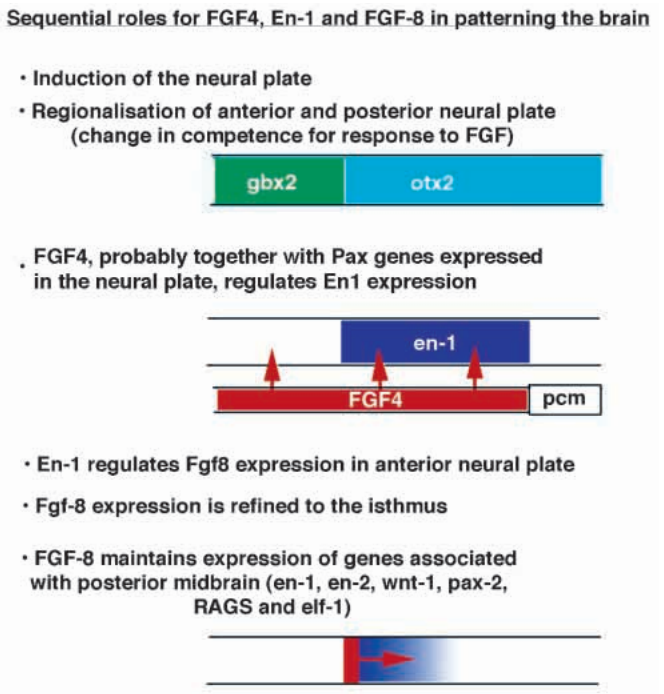

Fig. 10. A model for FGF function in patterning the midbrain. The temporal sequence of events progresses from top to bottom of the figure.

cerebellum and trochlear nucleus are derived from the extreme posterior midbrain and anterior hindbrain (i.e. *Gbx2*-positive region) and it may be that FGF8, alone, is insufficient to convert anterior midbrain or diencephalon to a fate characteristic of the isthmus and anterior hindbrain. However, Crossley et al. (1996) do report ectopic oculomotor and trochlear nuclei and it is noteworthy that these were present in very few of their experimental embryos.

Many embryos analysed at later stages after implanting FGF-coated beads showed dramatic thickening and foliation of tectal structures. In addition, enlargement of the walls of the diencephalon or midbrain were often apparent in embryos within 24 hours of introduction of ectopic FGF. These observations are strikingly similar to those of Abud et al. (1996) who showed that ectopic FGF4 protein expressed in chimeric mice produced foliation of the midbrain. Labelling dividing cells in grafted embryos indicates that this effect is due to a mitogenic activity of FGF8 which is apparent both 24 hours and 3 days after introduction of ectopic protein. Similar findings have been reported by others in mouse embryos (Lee et al., 1997). Since different embryos in the same experiment were found to have either the foliated phenotype or a tectal structure with reversed polarity, we suggest that variation in the amount of FGF protein bound to individual beads might determine which of the two morphologies was generated. Consistent with the latter interpretation, embryos generally exhibited the foliated phenotype bilaterally but most frequently exhibited a duplicated posterior phenotype unilaterally. These data are consistent with another property of the isthmus: that it is the region of highest mitotic activity in the developing midbrain and indicate a role for FGF8 in regulating both division and patterning in that region.

Conclusion: a model for FGF function in specification and patterning of the midbrain

Taken together with work of other groups, the results of this study suggest a model for the role of FGFs in specification and polarisation of the midbrain (Fig. 10). Neural induction occurs within epiblast which is already regionally specified along the anteroposterior axis as evinced by expression of transcripts for *Otx2* and *Gbx2*. The notochord extends anterior to Hensen's node beneath the cranial neural plate and transiently expresses Fgf4. The latter molecule regulates *En1* expression in the neural plate, possibly in combination with transcription factors, e.g. *Pax* genes expressed in the neural plate, with the anterior limit of En1 expression possibly defined by the boundary between the *Fgf4*-expressing notochord and the *Fgf4*-negative prechordal mesoderm. En1, possibly together with other factors, e.g. Pax2, regulates *Fgf8* expression within that region. Under the influence of interactions between adjacent Gbx2- and Otx2-expressing cell populations, *Fgf8* expression becomes localised to the isthmus. Fgf8 then functions to further refine and maintain expression of genes associated with the posterior midbrain.

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