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Relationship between *Wnt-1* and *En-2* expression domains during early development of normal and ectopic met-mesencephalon

LAURE BALLY-CUIF[1,*], ROSA-MAGDA ALVARADO-MALLART[1], DIANA K. DARNELL[2] and MARION WASSEF[1,*]

[1]*INSERM U106, Hôpital de la Salpêtrière, 75651 Paris cedex 13, France*
[2]*Department of Anatomy, School of Medicine, University of California, San Francisco, CA 94143-0452, USA*

*Corresponding authors

Summary

Grafting a met-mesencephalic portion of neural tube from a 9.5-day mouse embryo into the prosencephalon of a 2-day chick embryo results in the induction of chick *En-2* (*ChickEn*) expression in cells in contact with the graft (Martinez et al., 1991). In this paper we investigate the possibility of Wnt-1 being one of the factors involved in *En-2* induction. Since *Wnt-1* and *En-2* expression patterns have been described as diverging during development of the met-mesencephalic region, we first compared *Wnt-1* and *En-2* expression in this domain by in situ hybridization in mouse embryos after embryonic day 8.5. A ring of *Wnt-1*-expressing cells is detected encircling the neural tube in the met-mesencephalic region at least until day 12.5. This ring consistently overlapped with the *En-2* expression domain, and corresponds to the position of this latter gene's maximal expression. We subsequently studied *ChickEn* ectopic induction in chick embryos grafted with various portions of met-mesencephalon. When the graft originated from the level of the *Wnt-1*-positive ring, *ChickEn* induction was observed in 71% of embryos, and in these cases correlated with *Wnt-1* expression in the grafted tissue. In contrast, this percentage dropped significantly when the graft was taken from more rostral or caudal parts of the mesencephalic vesicle. Taken together, these results are compatible with a prolonged role of *Wnt-1* in the specification and/or development of the met-mesencephalic region, and show that *Wnt-1* could be directly or indirectly involved in the regulation of *En-2* expression around the *Wnt-1*-positive ring during this time. We also provide data on the position of the *Wnt-1*-positive ring relative to anatomical boundaries in the neural tube, which suggest a more general role for the Wnt-1 protein as a positional signal involved in organizing the met-mesencephalic domain.

Key words: *Wnt-1*, *En-2*, met-mesencephalic development, mouse/chick chimeras.

Introduction

Identifying the regulatory steps controlling central nervous system early organization has become the object of intense investigation. Recent molecular data concern the met-mesencephalic region of the neural tube in the vertebrate embryo. This region includes the cerebellar and tectal primordia, and is characterized by the specific expression of the *En* homeobox genes (homologous to *Drosophila engrailed* and *invected*), beginning with the earliest stages of neurogenesis in many vertebrate species, such as mouse (genes *En-1* and *En-2*, Davis and Joyner, 1988; Davis et al., 1988), chicken (gene *ChickEn* (chick *En-2*), Gardner et al., 1988), *Xenopus* (Hemmati-Brivanlou et al., 1991) and zebrafish (Fjöse et al., 1988). These genes encode homeodomain proteins, which are therefore believed to act as transcriptional regulators. On the basis of its specific expression pattern, and of transplantation experiments in the chick embryo (Martinez et al., 1991), the *En* genes may play a crucial role in the determination of the met-mesencephalic domain.

Experiments in which portions of the met-mesencephalic domain from a two-day quail embryo are grafted into a two-day chick host after a rostrocaudal inversion indicate that the fate of such portions of the neural tube, as well as their *En-2* gene expression, is regulated according to their new environment (Martinez and Alvarado-Mallart, 1990; Martinez et al., 1991). These results imply that the met-mesencephalic domain is not totally determined at that stage, that is, even after initiation of *En-2* expression. Rather, determination of this domain may involve at least two steps: the induction of the *En-2* gene, and the subsequent environment-dependent maintenance of *En-2* expression.

We are interested in determining what extracellular factor(s) might be responsible for the maintenance of *En-2* expression during the second step of determination of the met-mesencephalic domain. To attempt to answer this question, we used an embryological approach which made it

possible to induce the development of an ectopic met-mesencephalic region in the neural tube of a chick embryo. Ectopic transplantations of met-mesencephalic portions of quail neural tube in the diencephalon of a two-day chick embryo induced *ChickEn* expression in the chick host adjacent to the graft (Gardner and Barald, 1991; Martinez et al., 1991). The same induction could be obtained with a mouse or rat met-mesencephalic graft (Martinez et al., 1991). We therefore hypothesized that a secreted, phylogenetically conserved factor was present in the graft and responsible for *ChickEn* induction in the host tissue in contact with the graft. As *ChickEn* ectopic expression in quail/chick chimeras is followed by the development of ectopic cerebellum and optic tectum, this factor may be the same as the one involved in the maintenance of *En-2* expression during determination of the normal met-mesencephalic domain.

In the present paper, we study the possibility of the Wnt-1 protein being one of the factors involved in this process. Several arguments lead to this hypothesis: first, the *Wnt-1* gene has been shown to be expressed in the met-mesencephalic region (Wilkinson et al., 1987) (at least at early stages), and to be involved in the specification and/or early development of this region (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Thomas et al., 1991); second, the Wnt-1 protein is secreted, thus possibly acting as an extracellular communication signal (Bradley and Brown, 1990; Papkoff and Schryver, 1990); and third, the *Wnt-1 Drosophila* homolog, *wingless* (Rijsewijk et al., 1987), is involved at different steps in the regulation of *engrailed* expression during the specification of segmental compartments (Di Nardo et al., 1988; Heemskerk et al., 1991).

The initial data published by Wilkinson et al. (1987) showed *Wnt-1* to be expressed in the met-mesencephalic region until embryonic day 15 (E15). Other results, however, indicated that the *Wnt-1* and *En-2* expression domains diverged in the met-mesencephalic region before E12.5 (Davis and Joyner, 1988). To solve these discrepancies, we first compared the expression patterns of *Wnt-1* and *En-2* in the met-mesencephalic domain of the neural tube of a normal mouse embryo beginning with the early stages of neurogenesis. A spatiotemporal correlation is observed between *Wnt-1* and *En-2* expression in the met-mesencephalic domain until embryonic day 12.5, indicating that *Wnt-1* may be involved in *En-2* regulation until that stage. We subsequently tested whether the *Wnt-1*-positive region was involved in *ChickEn* induction in the mouse/chick ectopic grafts. The highest percentage of inductions was obtained when the graft originated from the level of the *Wnt-1*-positive ring, and in these cases *ChickEn* induction correlated with *Wnt-1* expression in the graft. The position of the *Wnt-1*-positive ring relative to anatomical structures of the mouse neural tube also suggests that *Wnt-1* could more generally act as a positional signal in the neural tube for the organization of the met-mesencephalic domain.

Materials and methods

In situ hybridization on tissue sections

Sample preparation

Embryos from outbred OF1 mice (IFFA Credo, Lyon, France) were removed from the uterus and fixed by immersion in 4% paraformaldehyde in phosphate buffer (0.12 M, pH 7.4-7.6) overnight at 4°C. They were then dehydrated and embedded in paraffin (Paraplast+). Serial sections (7.5 μm thick) were mounted on gelatin-coated slides. For hybridization of adjacent sections with different probes, a new slide was used every three serial sections, and one every second (Figs 1, 2) or third (Fig. 4) slide was hybridized with the same probe.

The embryos are staged E0.5 on the morning following breeding (the midpoint of the dark interval during which mating occurred is considered as day 0).

Preparation of probes

To generate suitable probes for the in situ analyses, we used the following cDNA clones: a 250 bp *Bgl*II-*Sst*I fragment of the mouse *En-2* cDNA subcloned into pGEM1 (Davis et al., 1988), a *Pst*I-*Eco*RI fragment of the *ChickEn* cDNA subcloned into pBluescript SK(+) (Gardner et al., 1988) and a 1879 bp *Hin*dIII-*Xba*I fragment of the *Wnt-1* cDNA cloned into pBluescript KS(+) (Fung et al., 1985). These *En-2*, *ChickEn* and *Wnt-1* cDNA clones have already been used in previous hybridization studies (Davis and Joyner, 1988; Gardner et al., 1988; Fung et al., 1985, respectively). The specificity of these cDNA fragments has already been checked by northern blot analysis (Davis et al., 1988; Gardner et al., 1988; Fung et al., 1985). The *En-2* subclone was linearized with *Hin*dIII and transcribed using T7 RNA polymerase, or with *Eco*RI and transcribed using SP6 RNA polymerase, to generate the antisense and sense probes, respectively. The *ChickEn* subclone was linearized with *Not*I and transcribed with T3 RNA polymerase, or linearized with *Xho*I and transcribed with T7 RNA polymerase, to generate the antisense and sense probes. Similarly, the *Wnt-1* subclone was either linearized with *Hin*dIII and transcribed with T7 RNA polymerase, or with *Not*I and transcribed using T3 RNA polymerase, for the antisense and sense probes. The RNA probes were labelled by incorporation of ^{35}S-UTP (Amersham, 1000 Ci/mmol.) during synthesis. The probes were then hydrolyzed to generate 150 nt fragments as described by Fontaine and Changeux (1989). The sizes of probe fragments were checked by gel electrophoresis.

In situ hybridization

Hybridizations were done as described by Fontaine and Changeux (1989), with minor modifications. Probes were used at a concentration of 5×10^4 cts/minute per μl, and hybridization was done overnight at 48°C under siliconized coverslips. The washed and dehydrated slides were dipped into Kodak NTB2 emulsion (undiluted) and exposed for 5 to 8 days. They were then counterstained with toluidine blue.

Whole-mount in situ hybridizations

Synthesis of probes was done as described above, except that the nucleotide mixture was 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.33 mM UTP, 0.17 mM digoxigenin-UTP (Boehringer Mannheim), and that the probe was not hydrolyzed after synthesis.

The protocol used for whole-mount ISH was provided by D.G. Wilkinson. Briefly, embryos were fixed in 4% paraformaldehyde in PBS for 4 hours and the neural tube was dissected for embryos older than E8.5. They were then gradually dehydrated in methanol/PBT (PBT: PBS; 0.1% Tween 20) up to 100% methanol, and stored at –20°C until use. They were rehydrated through a reverse methanol/PBT washing series, bleached with 2% hydrogen peroxide in PBT for 1 hour, and treated with 10 μg/ml proteinase K in PBT for 15 minutes, washed with 2 mg/ml glycine in PBT, then refixed in 0.2% glutaraldehyde/4% paraformaldehyde in PBT for 20 minutes, and washed with PBT prior to pre-

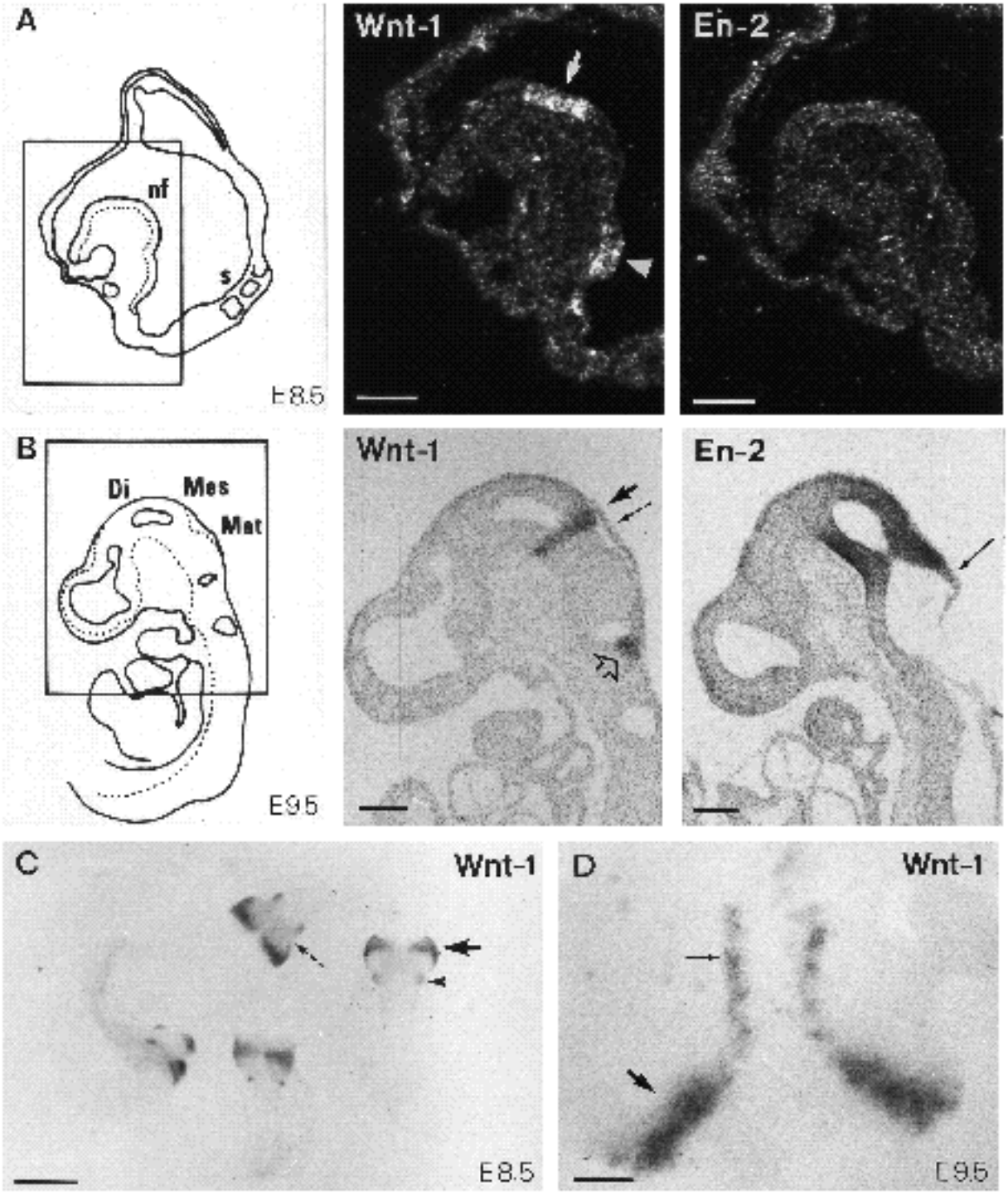

Fig. 1. Expression of the *Wnt-1* and *En-2* genes in the met-mesencephalic region of E8.5 (A,C) and E9.5 (B,D) mouse embryos. (A) and (B) are adjacent sagittal sections alternatively hybridized with a *Wnt-1* (middle panel) or *En-2* (right panel) antisense probe. Schematic representation of the sections are shown in the left panel. (C) and (D) illustrate whole-mounts of E8.5 (C) and E9.5 (D) embryos hybridized with a digoxigenin-labelled *Wnt-1* RNA probe. In D, a fragment of the ventral neural tube from the E9.5 embryo was flat-mounted (rostral is up and caudal down). At E8.5, *Wnt-1* is expressed on a broad domain in the met-mesencephalic region (arrows in A and C), as well as on two patches of cells in the hindbrain (arrowheads). Later, three domains of expression are visible: the dorsal midline (open arrow in B), the ventral midline (small arrow in D, note that two parallel rows of labelled cells are visible), and the met-mesencephalic ring (arrows in B and D; in D, lateroventral parts of the ring are shown). Note that the position of the *Wnt-1*-positive ring is rostral to the "met-mesencephalic" constriction (broken arrows in B and C). From E9.5 on, *En-2* expression is maximal at the level of the *Wnt-1*-positive ring, and decreases on the edge of the cerebellar plate (arrow in B, right panel). nf, neural folds; s, somites; Di, diencephalon; Mes, mesencephalon; Met, metencephalon. Bars, A, B, 150 μm; C, 500 μm; D, 80 μm.

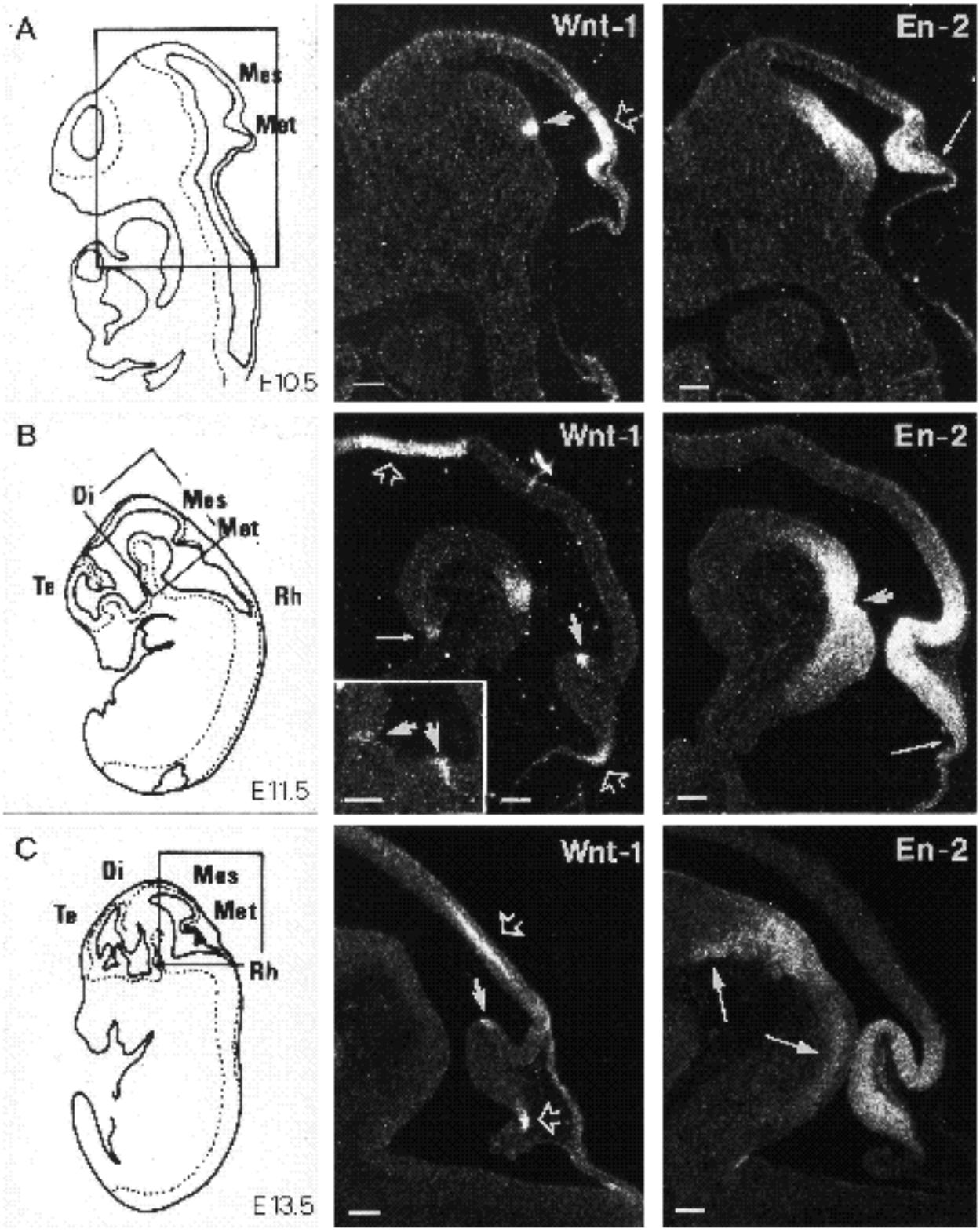

Fig. 2. Expression of the *Wnt-1* (middle panel) and *En-2* (right panel) genes in the met-mesencephalic region on adjacent sagittal sections of E10.5 (A), E11.5 (B) and E13.5 (C) mouse embryos. Symbols used are the same as those for Fig. 1. The groove appearing on the basal plate of the neural tube at the level of the *Wnt-1* ring is visible at E10.5 (A, middle panel), and on parasagittal sections at E11.5 (B, middle and right panels). At E13.5, *Wnt-1* transcripts are only detected in the dorsal region of the ring (large arrow in C), and *En-2* is expressed in post-mitotic nuclei (arrows in C). Te, telencephalon; Rh, rhombencephalon. Bars, A, 150 μm; B, C, 200 μm.

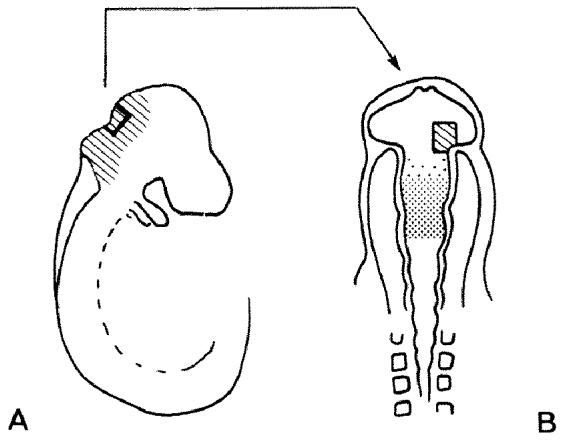

Fig. 3. Schematic representation of the grafting experiments. Various portions of the met-mesencephalic region of an E8.5 (not shown) or E9.5 (A) mouse neural tube were removed and transplanted into the prosencephalon of a HH10 chick host (B). The precise neuroepithelial areas taken as the graft are indicated in Table 1. Mouse *En-2* (hatched area in A) and *ChickEn* (dots in B) expressions are indicated.

hybridization. Prehybridization was done at 70°C for 1 hour in 50% formamide, 5×SSC, 50 µg/ml yeast RNA, 1% SDS, 50 µg/ml heparin. RNA probe was added to a final concentration of 1-2 µg/ml, and hybridization was done overnight at 70°C. Subsequent washes were as follows: twice 30 minutes at 70°C in solution 1 (50% formamide, 5×SSC, 1% SDS), 10 minutes at 70°C in 1:1 solution 1:solution 2 (0.5 M NaCl, 10 mM Tris-HCl pH7.5, 0.1% Tween 20), 5 minutes in solution 2, twice 30 minutes in 100 µg/ml RNAase A in solution 2, and finally twice 30 minutes at 65°C in solution 3 (50% formamide, 2×SSC). Embryos were then pre-blocked for 60-90 minutes in 10% decomplemented sheep serum in TBST (1.5 mM NaCl, 0.03 mM KCl, 0.025 M Tris-HCl pH 7.5, 0.1% Tween 20, 2 mM levamisole). Embryos were then incubated overnight at 4°C in anti-digoxigenin-alkaline phosphatase antibody (Boehringer Mannheim) diluted to 1/1250 in TBST. Alkaline phosphatase activity was revealed in 165 µg/ml 5-brom-4-chlor-3-indolyl-phosphate (BCIP, Boehringer Mannheim), 333 µg/ml 4-nitro blue tetrazolium chloride (NBT, Boehringer Mannheim), in NTMT (100 mM Tris-HCl pH 9.5, 50 mM $MgCl_2$, 0.1% Tween 20). Colour usually developed within 45 minutes.

Mouse/chick transplantations

We used 8.5- and 9.5-day OF1 mice embryos as donors, and HH10 (10-12 somites) White Leghorn chick embryos as hosts (staged according to Hamburger and Hamilton, 1951). After a sub-blastodermal injection of India ink to aid visualization of the chick embryo, a portion of the right alar plate of prosencephalon was ablated (see Fig. 3). The mouse grafts were prepared as follows: the mother was killed with chloroform vapors, and the embryos were quickly removed from the uterus by laparotomy. They were placed in a Tyrode saline solution, and a portion of the met-mesencephalic region was manually dissected and transferred to the host, with a glass pipette, to replace the ablated portion of the prosencephalon (see Fig. 3). The grafts were taken from various parts of the met-mesencephalic domain (see Table 1). The dorsoventral orientation of the graft was conserved, but its ros-

Table 1. *Analysis of* ChickEn *induction in grafted embryos*

Mouse donors	Origin of the graft	Grafted embryos	Integrated grafts	ChickEn induction			
				Analysis	Embryos tested	induction	
E8.5	A	18	9	mAb 4D9	9	0	0%
E9.5	B	21	7	ISH	7	1	14%
	C	22	7	ISH	7	5	71.4%
	D	20	11	mAb 4D9	11	1	9%
	E	17	7	mAb 4D9	3	0	28.6%
				ISH	4	2	

Schematic drawings of E8.5 and E9.5 mouse anterior neural tubes are shown to indicate the origin of the grafted tissue (black) compared to the "met-mesencephalic" constriction (arrow). Since no *ChickEn* induction was ever observed in the case of non-integrated grafts (Martinez et al., 1991), *ChickEn* induction was only analyzed in embryos where an integrated graft was visible (see text). The percentage of inductions (last column) is therefore calculated as a percentage of the number of embryos showing an integrated graft.

trocaudal orientation was generally random. After transplantation, the chick eggs were closed with a coverslip sealed with wax and kept at 38°C for 48 hours. The grafted embryos were then fixed in 4% paraformaldehyde in phosphate buffer, and either treated for whole-mount immunocytochemistry (as described in Martinez et al., 1991), or treated as described above for in situ hybridization on tissue sections. The embryos in which the graft had rolled up at the surface of the neural tube, or was not visible (see text), were not taken into account.

Results

We used in situ hybridization on tissue sections or on whole-mount embryos to study *Wnt-1* expression at one-day intervals between embryonic days 7.5 and 14.5. Most of our observations are in agreement with, and extend, the description made by Wilkinson et al. (1987). The *Wnt-1* gene is expressed in the neural tube from day 8.5 on. Its expression domain can be subdivided in three spatially (and probably functionally) different regions (see Figs 1 and 2): (1) the dorsal midline of the neural tube, (2) the met-mesencephalic region and (3) the ventral midline of mesencephalon and part of diencephalon. In the present study, we focused on *Wnt-1* expression in the met-mesencephalic domain.

1. Expression of the Wnt-1 *gene on the dorsal and ventral midlines*

Wnt-1 expression on the dorsal midline is detected from E9.5, from the diencephalon to the very caudal tip of the spinal cord, with a gap on the dorsal midline of the cerebellar anlage. In addition, we found that *Wnt-1* expression on the edge of the cerebellar anlage is not uniform but rather decreases rostrally (and medially), and the midline of the cerebellar edge remains *Wnt-1*-negative at least until E11.5 (not illustrated).

Wnt-1 expression is detected on the ventral midline of mes- and diencephalon at all stages examined from E9.5. In whole-mount embryos, this domain appears as two parallel rows of positive cells, separated by a narrow negative band (Fig. 1D). It has a sharp limit at its caudal end, corresponding to the *Wnt-1*-positive ring, and its rostral extension varies depending on the developmental stage: it reaches the tuberculum posterius (Kuhlenbeck, 1973) at E10.5, but then retreats and appears only in the mesencephalon at E14.5 (not illustrated).

On both the dorsal and ventral midlines, *Wnt-1* expression is restricted to the germinal zone.

2. Correlation between the expression patterns of the Wnt-1 *and* En-2 *genes during development of the met-mesencephalic region in the mouse embryo*

Expression of the Wnt-1 *gene in the met-mesencephalic domain*

The *Wnt-1* gene is expressed by 8.5 days in two large triangular-shaped lateral patches of cells in the neural folds on either side of the midline, in a region which probably corresponds to the presumptive met-mesencephalic domain (Fig. 1A and C). This *Wnt-1*$^+$ area corresponds to that described at this stage by Wilkinson et al., (1987) and Davis and Joyner (1988). In addition, we also detected at that stage a region of strong expression located more caudally, presumably in the hindbrain (Fig.1C).

At E9.5, *Wnt-1* is expressed in a ring of cells encircling the neural tube (see Fig. 1B and D). This ring, several cells wide, is located rostrally to the constriction that separates the metencephalic and mesencephalic vesicles ("met-mesencephalic" constriction). An entire ring of positive cells is still apparent at E10.5 (Fig. 2A) and E11.5 (Fig. 2B). Its position relative to the "met-mesencephalic" constriction remains unchanged in spite of the morphogenetic movements and asynchronous growth affecting this region of the neural tube at these stages. It is interesting to note that the number of positive cells on the mid-dorsal side of the ring approximately doubles between E9.5 and E11.5, increasing from 5-6 to 10-12 between these stages, whereas the absolute width of the ring in this location remains constant.

At E10.5 and E11.5, a groove becomes apparent on the ventricular surface of the neuroepithelium, and marks the caudal edge of the *Wnt-1*-positive ring (Fig. 2A and B). This groove, only visible in the basal plate, could correspond to a subdivision in the neural tube. It is not apparent at E9.5; hence, the *Wnt-1* gene marks the position of this subdivision before it is morphologically visible.

The *Wnt-1*-positive ring is still detected by E12.5 (not shown). It completely encircles the neural tube, in the same location as previously described. However, at that stage it becomes very narrow in its lateral and ventral parts. The coincidence between the ventral part of the ring and the groove mentioned above is still observed at E12.5.

Wnt-1 expression is turned off in the ventral and lateral regions of the ring between E12.5 and E13.5 (see Fig. 2C). However, *Wnt-1* expression persists in the dorsal part of the ring at least until E14.5 (not illustrated).

At E12.5, postmitotic neurons start to accumulate in the mantle zone of the neural tube. As was also observed for the *Wnt-1*-positive cells of the dorsal and ventral midlines, the *Wnt-1*-expressing cells are always restricted to the germinal zone.

We did not study later stages of development. We therefore cannot tell precisely when *Wnt-1* expression is totally turned off in this region.

Comparison with the expression of the En-2 *gene*

Adjacent sections of the same embryos were probed with the two *Wnt-1* and *En-2* antisense probes.

En-2 expression is never detected at E7.5. At E8.5, some of the embryos we examined show *En-2* expression, in a region apparently overlapping the *Wnt-1*-expressing domain (not shown), a result in agreement with the report of Davis and Joyner (1988). However, this expression is generally weak, and in most embryos *En-2* transcripts could not be detected at this stage (Fig. 1A). This fact can probably be explained by the presence in the same litter of embryos at slightly different stages of development. Since expression of the *Wnt-1* gene is detected without ambiguity in all E8.5 littermates, the *Wnt-1* gene appears to be turned on a few hours before the *En-2* gene (compare Fig. 1A and B).

At later stages (E9.5-E12.5), *En-2* is expressed in cells of the germinal zone in the met-mesencephalic region (Figs 1B, 2A,B), an area corresponding to the presumptive cerebellum, isthmus and colliculi, in agreement with the result of Davis et al. (1988). This domain encircles the neural tube and always overlaps the *Wnt-1*-expressing ring. The highest expression is found approximately at the level of the "met-mesencephalic" constriction as well as a little further rostrally. It then progressively decreases rostrally and caudally. This gradient is particularly apparent on the ventral side of the tube (it is more difficult to visualize on the dorsal side, especially in its caudal part, since *En-2* expression is rapidly interrupted on the choroid plexus). It is however clear on sagittal sections that *En-2* expression becomes fainter at the edge of the cerebellar plate (see Figs 1B, 2A,B). Thus, it appears that *En-2* expression is maximal around the *Wnt-1*-positive ring. This coincidence is found at least until E11.5. At E12.5, the maximum *En-2* expression is still observed in the *Wnt-1*-positive area. The latter, however, has become very narrow with respect to the peak of expression of *En-2* (not illustrated).

At E13.5 days, when the *Wnt-1* ring is turned off laterally and ventrally, *En-2* expression becomes more complex, and is in a more restricted domain of the germinal zone plus some post-mitotic nuclei, notably in the isthmus and cerebellum (Fig. 2C). We did not study these late stages of expression in detail.

In summary, a spatiotemporal coincidence is observed between *En-2* and *Wnt-1* expression in the germinal zone of the met-mesencephalic region between embryonic days 8.5 and 12.5, with highest *En-2* expression overlapping the *Wnt-1* expression domain.

3. Relationships between Wnt-1 *and* ChickEn *expression during development of an ectopic met-mesencephalic domain in the neural tube of a chick embryo*

The results presented above are compatible with the hypothesis that *Wnt-1* plays a role in the maintenance of *En-2* expression during determination and early development of the met-mesencephalic region in the mouse. We therefore subsequently studied whether *Wnt-1* could be one of the factors involved in the induction of *ChickEn* observed ectopically in the prosencephalon of a chick host embryo, when put in contact with a met-mesencephalic graft (Martinez et al., 1991).

Since we wanted to study *Wnt-1* expression in the grafted tissue, and since, with the stringency we used for in situ hybridization, the mouse *Wnt-1* probe did not cross-react with its chick or quail *Wnt-1* RNA homologs, we used mouse embryos as donors in our transplantation experiments. Various portions of the met-mesencephalic domain from E8.5 or E9.5 mouse embryos were ablated and grafted into the prosencephalon of a HH10 chick host (Fig. 3). The grafted embryos were analyzed after two days for *ChickEn* induction, either by whole-mount immunocytochemistry using the mAb 4D9 (Patel et al., 1989), or by in situ hybridization on tissue sections. The results are summarized in Tables 1 and 2. The analysis of such transplantations is however hampered by the lack of a specific marker for mouse met-mesencephalic neural tissue at that stage, therefore preventing an unambiguous identification of the grafted tissue. Our identification of the graft relied either on its thinner and clearer appearance compared to chick surrounding neural tissue in the case of whole embryos, or, as noted by Davidson et al. (1991), on its more intense staining on counterstained sections compared to chick tissue. It is however possible that a few cases of integrated grafts have been missed; the percentages of inductions given in Table 1 may therefore be overestimations.

The highest number of *ChickEn* inductions is obtained when the grafted tissue originates from the caudal portion of the mesencephalic vesicle (Table 1C, 71% of cases), that is a portion of the *Wnt-1*$^{+}$ neuroepithelial ring (compare with Fig. 1B). Two such cases (C1, C2 of Table 2) are illustrated in Fig. 4. The proportion of inductions considerably decreases a little rostrally (Table 1B) or caudally from this ring, even when the graft still includes the caudal-most region of the mesencephalic vesicle (Table 1A,D). In all instances, *ChickEn* induction gradually decreases away from the graft (see Fig. 4A,E), resembling the gradient of expression observed rostrally and caudally to the "met-mesencephalic" constriction in the normal embryo (Gardner et al., 1988; see also Fig. 4I). When these cases were analyzed with in situ hybridization (cases B and C), *ChickEn* induction was found to be correlated with *Wnt-1* expression in the graft (Table 2). In two instances (C1, C2, see Fig. 4B,C,F,G), *Wnt-1* transcripts seem concentrated in the periphery of the graft, that is in contact with the host tissue, suggesting that *Wnt-1* expression may be spatially modified during development of the ectopic met-mesencephalic region. In the other cases, however, *Wnt-1* is expressed throughout the grafted tissue, but the grafts are also very small (not shown), which may bias the analysis. *En-2* expression is only found in *Wnt-1*-expressing grafts, but, in contrast to the strong induction of *ChickEn*, its expression in the graft is generally weak (see embryo C2, Fig. 4D) or undetectable (other cases).

Surprisingly, a few other cases of *ChickEn* induction were obtained with grafts of purely caudal metencephalic origin (Table 1E, 28% of embryos), but, in contrast to cases B and C, no *Wnt-1* expression was ever observed in the grafts (Table 2), whether *ChickEn* was induced or not. Similarly, no *En-2* expression was detected in these grafts (not shown).

Table 2. *Expression of the* ChickEn *and* Wnt-1 *genes in grafted embryos*

Origin of the graft	Number of embryos	Cases	*ChickEn*	*Wnt-1*
B	1	B1	+	+
	6	B2-B7	−	−
C	5	C1-C5	+	+
	2	C6, C7	−	−
E	2	E1, E2	+	−
	2	E3, E4	−	−

The table concerns embryos analyzed with ISH (cases B, C, E, see Table 1). Again, the only embryos analyzed were those where an integrated graft was visible (column 4 of Table 1). + and − indicate expression or non expression, respectively, of the two genes, as determined by in situ hybridization.

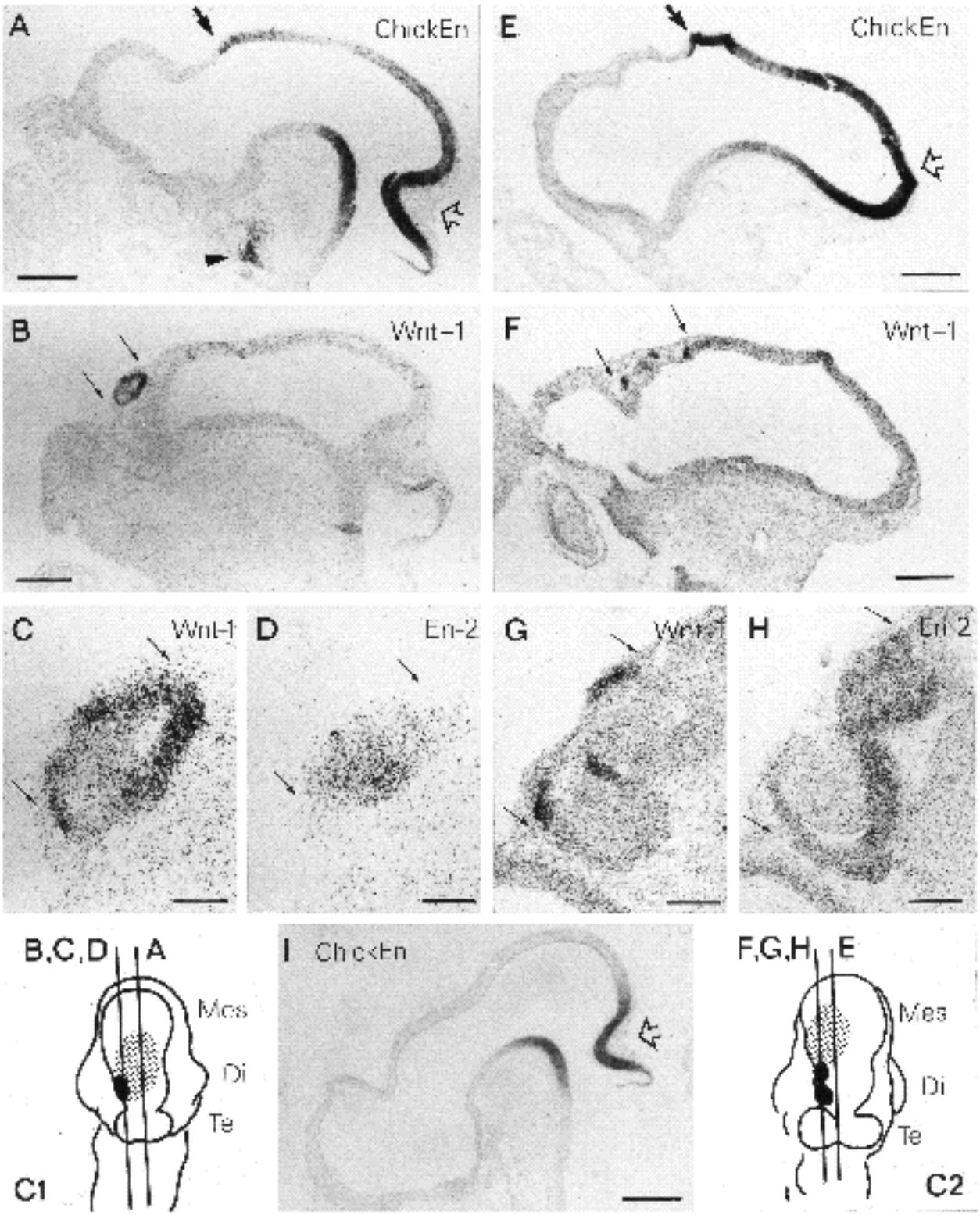

Fig. 4. Localization of *ChickEn* (A,E), *Wnt-1* (B,C,F,G) and mouse *En-2* (D,H) transcripts on nearby sagittal sections of the mouse/chick chimeras C1 (A,B,C,D) and C2 (E,F,G,H) (see Tables 1 and 2). The levels of the sections are indicated underneath for each case on a frontal external view of the grafted embryo (E4), where the position of the grafted mouse tissue (black) and *ChickEn* ectopic expression (dots) are also schematically represented. In B and F, the mouse graft is delimited by the small arrows, and is enlarged in C,D and G,H, respectively. Note the similar decreasing gradient in *ChickEn* expression in the ectopic (arrows in A and E) and normal met-mesencephalic (arrowheads in A and E) locations. I shows *ChickEn* expression on a sagittal section of an E4 unoperated chick embryo. Expression is only visible in the met-mesencephalic region (open arrow), and not in the diencephalon. Te, telencephalon; Di, diencephalon; Mes, mesencephalon. Bars, A,B,E,F,I, 300 μm; C,D,G,H 50 μm.

Discussion

The *Wnt-1* gene is expressed during development in three different domains: the dorsal midline of the neural tube, ventral midline cells and a ring of cells in the met-mesencephalic region. Surprisingly, however, the only affected region in *Wnt-1*$^{-}$ mutants is a broad met-mesencephalic domain overlapping the *Wnt-1* ring (McMahon and Bradley, 1990; Thomas and Capecchi, 1990), indicating, first, that *Wnt-1* most probably acts differently in its different expression domains, and second, that its role in met-mesencephalon is fundamental to the early development of this region and is, at early stages, non-redundant to that of any other gene. This role, therefore, appeared to us as the easiest to test experimentally, and we concentrated on this met-mesencephalic domain of *Wnt-1* expression. We tested the hypothesis of *Wnt-1* playing, in this portion of the met-mesencephalic domain, a role in *En-2* regulation. Additional data were also obtained suggesting a role of *Wnt-1* in the organization of this domain. These different points will be discussed below.

1. Localization studies and grafting experiments are compatible with the hypothesis of Wnt-1 *regulation of* En-2

In the met-mesencephalic region, *Wnt-1* is expressed from E8.5, and in a ring of cells encircling the neural tube from E9.5. Davis and Joyner (1988) reported that the *Wnt-1* ring had disappeared from this region by E12.5; it is however possible that their E12.5 embryos were slightly older than ours. In the mouse embryo, the *En-2* expression domain is consistently organized around the *Wnt-1*-positive ring (until E12.5), which, in addition, corresponds to the region of maximal *En-2* expression.

Moreover, quail/chick grafting experiments recently published by Gardner and Barald (1991) indicate that a factor responsible for the ectopic induction of *ChickEn* is produced in the caudal portion of the mesencephalic vesicle. Control experiments in which mesenchymal cells have been removed from the grafted tissue also indicate that the factor(s) responsible for *ChickEn* induction is attributable to neural cells in the graft (Martinez et al., 1991). The data presented in the present paper further demonstrate that the highest percentage of *ChickEn* inductions is obtained when the grafted neural tissue originates from a precise portion of the mesencephalic vesicle located at the level of the *Wnt-1*-positive ring. In these cases, there is a striking correlation between mouse *Wnt-1* expression in the graft and *ChickEn* induction in the surrounding host region. In addition, *ChickEn* expression progressively declines away from the graft in a similar manner to the gradient of *En-2* expression rostrally and caudally from the *Wnt-1*-positive ring in the unoperated neural tube.

We cannot tell, however, if *Wnt-1* expression is simply maintained in the graft, or reexpressed consequent to a respecification of the region as a whole. Also, since the mouse *Wnt-1* probe does not cross-react with the chicken *Wnt-1* RNA, we cannot tell whether *Wnt-1* expression can also be induced in the chick host in contact with the graft, which could favor the hypothesis of general regional respecification. We are presently investigating this point using a chick *Wnt-1* probe.

In any case, the fact that either both (Table 2, cases B1, C1-C5), or neither (Table 2, cases B2-B7, C6, C7) of the two genes are expressed strongly suggests that *Wnt-1* and *En-2* are part of a common regulatory pathway during development of this region.

On the basis of these results, it is tempting to speculate that one of the possible roles of *Wnt-1* during met-mesencephalic determination could be the regulation or maintenance of *En-2* gene expression around the *Wnt-1*-positive ring. Wnt-1 is a secreted protein (Bradley and Brown, 1990; Papkoff and Schryver, 1990), and can act via a paracrine mechanism in cell culture (Jue et al., 1992). It could therefore act as a cell-to-cell communication signal produced at the level of the ring and responsible for turning on a regulatory cascade resulting in the maintenance of *En-2* expression in the neighbouring cells.

Clearly, however, further experiments are needed to ascertain that the factor responsible for *En-2* maintenance in this region is the Wnt-1 protein itself. In the context of this hypothesis, it appears difficult to explain the very low level of *En-2* expression inside the graft itself. It is possible that a minimal number of cells are required for *En-2* maintenance, if a mutual stabilization phenomenon is necessary. In this context, the fact that *En-2* expression was detected only with large grafts (Table 2, cases C1, C2) may be significant. Or, it is possible that the mouse graft is at a slightly older developmental stage than the surrounding chick neural tube and is, therefore, no longer responsive to the factor(s) responsible for the strong induction of *ChickEn*. These hypotheses may also explain the absence of *En-2* expression in the grafted tissue of caudal grafts (Table 2, case E, see below).

2. Spatial variations in the mode of En-2/ChickEn *regulation in the met-mesencephalic domain*

The results discussed above are consistent with a role of *Wnt-1* in *En-2* stabilization in a portion of the met-mesencephalic region located around the *Wnt-1*-positive ring, until approximately E12 in the mouse embryo. In chick embryos at equivalent stages, this period of time corresponds to a still undetermined state for the met-mesencephalic domain (Martinez and Alvarado-Mallart, 1990). The Wingless protein however is known to diffuse only over a short distance (Van den Heuvel et al., 1989). If we assume a similar diffusion for the Wnt-1 protein, additional mechanisms are clearly required to account for *En-2* expression in the entire met-mesencephalic domain. As was already suggested, one possible mechanism might be a mutual stabilization of *En-2* expression in nearby cells. Such a mechanism has already been described during early stages of *engrailed* regulation in *Drosophila*, where it involves a positive autoregulatory loop (Heemskerk et al., 1991). A second possibility might be the *Wnt-1*-dependent production, at the level of the *Wnt-1* ring, of another, widely diffusible factor, activating (directly or not) *En-2* expression. The absence of such activators, or the presence of inhibitors, along the dorsal midline, might also explain why *En-2* is not normally expressed in diencephalon, whereas this domain expresses *Wnt-1* on the dorsal midline and is competent for *En-2* expression.

A few cases of *ChickEn* inductions were also obtained

with purely caudal metencephalic grafts (Table 1E), and in such cases no *Wnt-1* expression was found in the grafts (Table 2). Other factor(s), produced in metencephalon and distinct from Wnt-1, may therefore be capable of inducing *En-2* expression. The fact that a caudal portion of cerebellum can develop in some cases in *Wnt-1*$^{-}$ (Thomas and Capecchi, 1990) or *swaying* (Thomas et al., 1991) mutants also suggests that the formation of caudal cerebellar structures can be independent of *Wnt-1*.

3. Wnt-1 *may act as a positional marker in the neural tube for the organization of the met-mesencephalic domain*

Two lines of reasoning lead to this hypothesis.

First, it is striking that the caudal border of the *Wnt-1*-positive ring corresponds to a groove that appears only at E10.5 on the basal plate of the neural tube. This groove corresponds to the sulcus intraencephalicus posterior, described in early anatomical studies (Palmgren, 1921; Vaage, 1969, 1973; Kuhlenbeck, 1973) as being located rostrally to rhombomere 1, and forming a remnant of the undeveloped ventricular cavity of the second mesencephalic neuromere. We also observed that this groove corresponded at E10.5 to a zone of interruption in the expression of the Neural Cell Adhesion Molecule (*NCAM*) gene, the expression of which is apparently confined to postmitotic neurons in the neural tube at this age (M-J. Santoni and L. Bally-Cuif, unpublished observations). This groove most probably indicates a transition to a region of different mitotic activity, and may constitute an important boundary inside the neural tube.

Second, the position of the *Wnt-1* ring in the alar plate of the chick embryonic neural tube (unpublished results) is rostral to the "met-mesencephalic" constriction, in the caudal third of the mesencephalic vesicle, a location that is reminiscent of the limit between the cerebellar and tectal presumptive territories defined using quail/chick chimeras (Martinez and Alvarado-Mallart, 1989; Hallonet et al., 1990). The *Wnt-1* ring may therefore represent the boundary separating these two territories of the neural tube.

Together, the data may indicate that the *Wnt-1* ring corresponds to an important position in the met-mesencephalic region.

In the *Drosophila* embryo, the Wingless protein is secreted and internalized by adjacent cells (Van den Heuvel et al., 1989), probably allowing their maintenance of *engrailed* expression. The vertebrate Wnt-1 protein has also been shown to be secreted in in vitro assays (Bradley and Brown, 1990; Papkoff and Schryver, 1990), and is able to induce various cell physiological responses, such as transformation of C57 mammary epithelial cells via a paracrine mechanism in culture (Jue et al., 1992), or modulation of gap-junctional communication when ectopically expressed in a *Xenopus* embryo (Olson et al., 1991). Even if one must remain careful in interpreting these results, because the *Wnt-1* gene is not normally expressed in such conditions and may simply mimic the effects of another member of the Wnt family of related proteins, these results nevertheless clearly indicate that the *Wnt-1*-expressing cells are able to signal their presence to neighbouring cells. Similarly, cells of the *Wnt-1*-positive ring may signal their position during neural tube development, and act as a positional marker in the neural tube.

Taken together, the results presented in this paper are consistent with a role for *Wnt-1* as a positional signal for the early organization of the met-mesencephalic domain, and strongly suggest that the Wnt-1 protein may be one of the factors involved in the regulation or maintenance of *En-2* expression during the progression of this domain towards determination.

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