A spinal cord fate map in the avian embryo: while regressing, Hensen's node lays down the notochord and floor plate thus joining the spinal cord lateral walls

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SUMMARY

The spinal cord of thoracic, lumbar and caudal levels is derived from a region designated as the sinus rhomboidalis in the 6-somite-stage embryo. Using quail/chick grafts performed in ovo, we show the following. (1) The floor plate and notochord derive from a common population of cells, located in Hensen's node, which is equivalent to the chordoneural hinge (CNH) as it was defined at the tail bud stage. (2) The lateral walls and the roof of the neural tube originate caudally and laterally to Hensen's node, during the regression of which the basal plate anlage is bisected by floor plate tissue. (3) Primary and secondary neurulations involve similar morphogenetic movements but, in contrast to primary neurulation, extensive bilateral cell mixing is observed on the dorsal side of the region of secondary neurulation. (4) The posterior midline of the sinus rhomboidalis gives rise to somitic mesoderm and not to spinal cord. Moreover, mesodermal progenitors are spatially arranged along the rest of the primitive streak, more caudal cells giving rise to more lateral embryonic structures. Together with the results reported in our study of tail bud development (Catala, M., Teillet, M.-A. and Le Douarin, N.M. (1995). *Mech. Dev.* 51, 51-65), these results show that the mechanisms that preside at axial elongation from the 6-somite stage onwards are fundamentally similar during the complete process of neurulation.

Key words: axial elongation, sinus rhomboidalis, gastrulation, neurulation, quail-chick chimaera, Hensen's node regression, chick

INTRODUCTION

In vertebrate development, one of the decisive events in laying down the anteroposterior axis of the body is the formation of the prechordal mesoderm and notochord during gastrulation. According to the classical view, the presumptive chordal material invaginates from caudal to rostral at the level of the dorsal lip of the blastopore in Amphibians, and of its equivalent, Hensen's node, in Amniotes (Vogt, 1929; Wolff, 1935a,b; Pasteels, 1937; Rudnick, 1944; Tam and Beddington, 1987; Lawson et al., 1991; Smith et al., 1994; Sulik et al., 1994; Wilson and Beddington, 1996). The morphogenetic processes through which the dorsomedial mesoderm becomes elongated into a notochord as it 'invaginates' underneath the superficial ectoderm have raised much interest among embryologists, most investigations being carried out on the amphibian and avian embryos.

In amphibians, various mechanisms have been proposed to account for the elongation of the notochordal material. According to Keller et al. (1989), this process can be divided into 3 phases. (1) During the late gastrula stage, notochordal cells become distinct from those of the paraxial mesoderm. (2) From late gastrula to early neurula stages, the notochord narrows, thickens and lengthens by cell intercalation. (3) From mid to late neurula stages, cells from the circumblastoporal ring of the mesoderm could be added to the caudal end of the notochord by a mechanism called accretion (Keller et al., 1989). However, Gont et al. (1993) have shown that, in fact, from early neurula stage (stage 13 of Nieuwkoop and Faber (1967)), involution stops and notochord formation is due to elongation from rostral to caudal of a distinct population of cells located in the late blastoporal lip designated as the 'chordoneural hinge' (CNH).

In the avian embryo, the presumptive notochordal material has been localized at the primitive streak stage in the area of Hensen's node (Pasteels, 1937; Spratt, 1955; Nicolet, 1965, 1970 and 1971; Rosenquist, 1966 and 1983; Selleck and Stern, 1991; Schoenwolf et al., 1992; Garcia-Martinez et al., 1993). Very few studies, however, have been devoted to the mechanisms controlling its elongation. By using carbon particles as a marker of embryonic territories, Spratt (1957) suggested that cells located in the primitive streak caudally to Hensen's node

contribute to notochord elongation. Similarly, Sausedo and Schoenwolf (1993, 1994), who worked on gastrulating chick and mouse embryos in culture, concluded that extension of the notochord occurs principally through accretion of cells to its caudal end, together with active cell rearrangement and proliferation within the already formed notochord.

The notochord plays a crucial role in neural induction and later on in patterning the early neural primordium, the neural plate (Yamada et al., 1991; and see Placzek, 1995 for a review). In the neural plate anterior to Hensen's node, a midline region can be distinguished, closely apposed to the notochord, which will give rise to the floor plate and two lateral zones that will form the walls of the neural tube, the roof plate and the neural crest. As the neural tube forms, it rapidly acquires a ventrodorsal polarity characterized by distinct morphological and molecular features. Experiments based either on the graft of an extra notochord laterally to the neural tube (van Straaten et al., 1988; Smith and Schoenwolf, 1989; Yamada et al., 1991) or on early extirpation of the notochord in the chick embryo at the level where the paraxial mesoderm is still unsegmented (van Straaten and Hekking, 1991; Artinger and Bronner-Fraser, 1993; Monsoro-Burq et al., 1994) have led to the current view that the appearance of a floor plate results from an induction arising from the notochord. According to this view, the neural plate is originally a uniform structure induced to form neural epithelium by signals vertically transmitted from the underlying mediodorsal mesoderm together with a homeogenetic or planar induction travelling within the plane of the neuroepithelium itself (Nieuwkoop, 1952a-c; Ruiz i Altaba, 1993; Doniach, 1993).

Our previous investigations on the 25-somite chick and quail embryos (Catala et al., 1995) have introduced a new factor to current notions on the formation of the notochord and floor plate in the caudal part of the body. We found that, in the process called secondary neurulation, which takes place from the lumbar region down to the extremity of the tail, the notochord and floor plate share the same embryological origin from a small population of cells located rostroventrally in the tail bud. These cells form the CNH (Pasteels, 1937) or the prospective notochordal region (Schoenwolf, 1977) and correspond to the remains of Hensen's node where chordal and neural material merge (Catala et al., 1995). Thus, from 25-somite stage onwards, the notochord elongates from cranial to caudal as CNH moves caudalward without involving any other addition of cells at its caudal part as proposed by the accretion hypothesis (e.g. Keller et al., 1989). The mechanism thus revealed in birds is similar to the one described by Gont et al. (1993) in the tail bud of *Xenopus laevis*. The most striking result in our findings was that, when a quail CNH was substituted isotopically for its chick counterpart in the prospective caudal region, floor plate of the neural tube, as well as the notochord, was derived from the graft.

The grafts including the CNH carried out in the tail bud of the 25-somite embryo contribute to spinal cord corresponding to the level of sacral nerves 3 to 6 and all the floor plate posterior to it (Catala et al., 1995). This part of the spinal cord forms by secondary neurulation, a process by which the medullary cord cavitates through multiple lumens that eventually coalesce and form a single central canal (Holmdahl, 1925; Criley, 1969). More anteriorly, the spinal cord forms by primary neurulation, in which the neural plate folds upwards and fuses in the midline. Primary neurulation ends when the posterior neuropore closes at the 22-somite stage. Because our previous findings applied only to secondary neurulation in the tail bud, it was essential to investigate the mechanism of notochord and floor plate formation during primary neurulation and in the region of transition between the two modes of spinal cord formation.

In the present study, we carried out chick-quail grafts (Le Douarin, 1969) at the 6-somite stage. We present a fate map of the sinus rhomboidalis, which contains Hensen's node at its center (Fig. 1A), at a stage when primary neurulation is taking place. In order to obtain long-term survival, which is necessary to determine whether a continuity exists between the primary and secondary neurulation mechanisms, we performed all operations in ovo which, although technically challenging, permit long-term incubation. By performing a detailed fate map of the entire sinus rhomboidalis and of the anterior primitive streak, we were able to map the origin of the spinal cord and of the floor plate-notochord unit laid down by the regression movement of the CNH.

The results show that Hensen's node at 6-somite stage contains all the material destined to form the notochord and floor plate from the upper thoracic level down to the extremity of the tail. The neural tube is in fact composed of two lateral territories produced by bisection of the neural plate and joined together by the insertion of the floor plate material arising from a more rostral region of the embryonic axis.

MATERIALS AND METHODS

Chick (*Gallus gallus domesticus*) and quail (*Coturnix coturnix japonica*) eggs from commercial sources were incubated in a humidified atmosphere at 38°C. The experiments were carried out on 6-somite-stage embryos. Chick embryos were operated in ovo and quail embryos were used as donors in all our experiments. Operated embryos were staged after killing according to either the number of somites formed or the criteria of Hamburger and Hamilton (HH) (1951).

The avian embryo at 6-somite stage (Fig. 1A)

At 6-somite stage (embryonic day 1.5 = E1.5), the neural tube is generally closed down to the level of the last formed pair of somites. The caudal part of the embryo comprising the open neural plate forms the so-called sinus rhomboidalis (Fig. 1A). In the anterior sinus rhomboidalis, the notochord can be easily distinguished underneath the medial neural plate as a thin cord (Fig. 1B). At its caudal end, the notochord is enlarged down to a median depression corresponding to Hensen's node. At the level of Hensen's pit, the superficial neural plate region of the sinus rhomboidalis is interrupted by a mass of tissue of different morphology that lies in continuity to the already formed notochord and to the midline cells of the neural plate (i.e. the prospective floor plate). This disposition is similar to that of the CNH as it appears later in the tail bud (Pasteels, 1937; Catala et al., 1995) (Fig. 1C). Caudally to this median pit, the superficial layer of the sinus rhomboidalis is separated from most lateral ectodermal regions by extensions of the neural folds (Fig. 1D). Remnants of the primitive streak are visible medially from the caudal-most part of the sinus rhomboidalis to the limit of the area pellucida (Fig. 1E).

Microsurgery experiments

6-somite-staged chick embryos are prepared for operation in ovo as previously described for 25-somite-stage embryos (Catala et al., 1995). Fragments of tissue to be excised are precisely delimited and
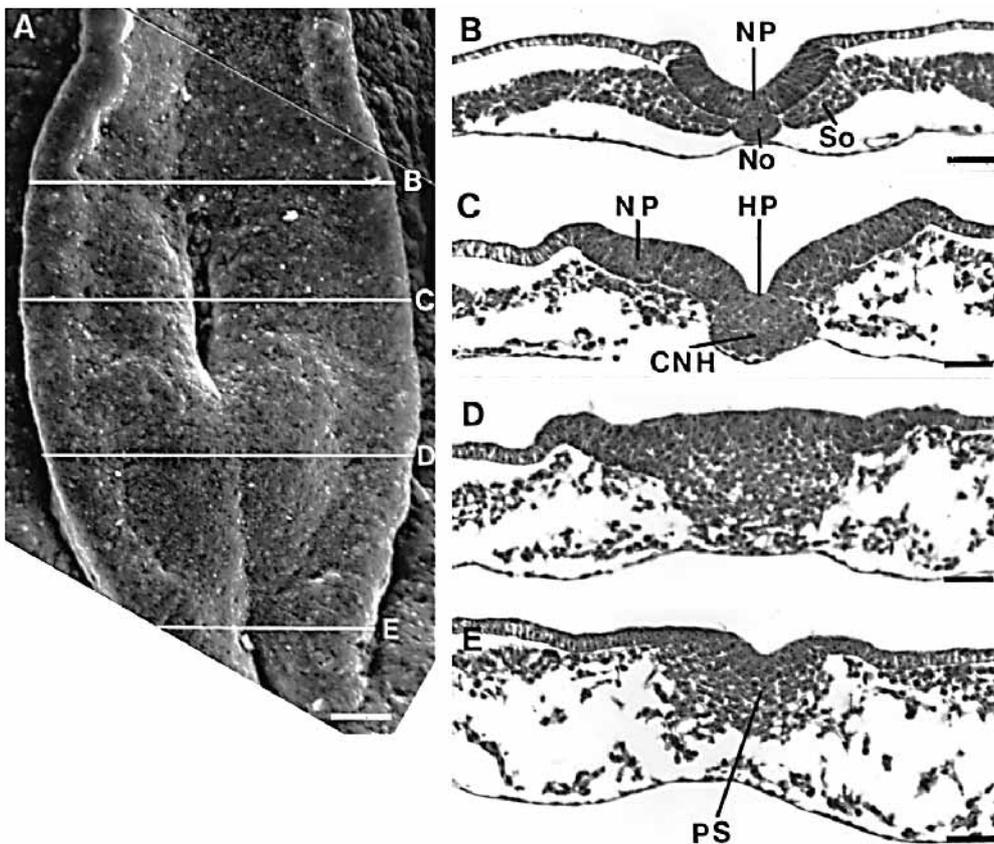
Fig. 1. Scanning electron micrograph of the sinus rhomboidalis in a chick embryo at the 6-somite stage shown in dorsal view (A) and successive transverse sections stained by Cresyl Violet (B-E). Hensen's node appears as a depression located on the midline. At rostral levels (B), both the neural plate (NP) and notochord (No) are individualized but still in close apposition. Somite precursors (So) are arranged on both sides of the notochord. (C) Section at the exact level of the Hensen's pit (HP); the neural plate (NP) is splitted into two halves by Hensen's node material, which forms the chordoneural hinge (CNH). (D) Just caudally to HP, the superficial layer forms a continuous sheet of cells over the midline. (E) At the most caudal levels, one can see the remnants of the primitive streak forming a groove (PS). Bars = 25 μm (A) and 50 μm (B-E).

removed from the chick embryo using steel microscalpels and glass micropipets. In graft experiments, the quail equivalent areas are dissected from pinned out donor embryos. Quail grafts are transplanted isotopically into the host chick embryos and the chimaeras are reincubated. Fixations occurred at various times, ranging from E1.5 to E8.5.

Experimental series

In a first series, the area including the median pit and its rostral region corresponding to the caudal-most part of the notochord, together with the underlying endoderm and overlying ectoderm, was removed without replacement. The rostral and caudal limits of the excision varied somewhat according to the embryos. The posterior limit of the excision extended from the mid-level to the extremity of the median pit. In all other experimental series, tissues were transplanted isotopically and isochronically from quail to chick (Fig. 2).

In a second series (experiment 2 in Fig. 2), the excised chick material was replaced by its quail counterpart; either (1) experiment 2A, excision and graft included the endoderm, or (2) experiment 2B, the chick endoderm remained in situ.

Experiment 3: the graft involved the superficial layer and a few cells of the underlying mesoderm in the midline region lying immediately caudal to the median pit.

Experiment 4: the graft comprised the neural fold and the lateral moiety of the neural plate on one side of the median pit. Some deep mesodermal cells may have been transplanted with the ectoderm since the graft was not subjected to enzymatic dissociation before implantation.

Experiment 5: the graft involved the superficial layer (and possibly some underlying cells) located at the caudolateral border of the sinus rhomboidalis.

Experiments 6-8: isotopic transplantations in the posterior part of sinus rhomboidalis and at various anteroposterior levels of the

primitive streak as indicated on Fig. 2. Underlying endoderm was not included in the graft.

Histological analysis

Most of the operated embryos were fixed in Carnoy's solution, embedded in paraffin and serially sectioned at 5 μm. For identification of the quail and chick nuclei in chimaeras, the sections were stained either according to the Feulgen-Rossenbeck's procedure (Feulgen and Rossenbeck, 1924) or by using the monoclonal antibody (mAb) QCPN (Developmental Studies hybridoma Bank), which recognizes a quail-specific antigen on all cell types and does not stain chick cells. The dewaxed and rehydrated sections were incubated with the primary antibody overnight at 4°C. They were washed in phosphate-buffered saline (PBS) and incubated with anti-mouse second antibody linked with peroxidase for 1 hour at room temperature. The enzymatic activity was revealed by diaminobenzidine tetrahydrochloride and the sections were counterstained by glychemalun.

RESULTS

39 operated embryos were analyzed between 1 hour and 7 days after surgery (E1.5 to E8.5).

Experiment 1. Excision of Hensen's node region

The median pit, the posterior end of the already formed notochord and the overlying neural plate together with the underlying endoderm were ablated in 6-somite chick embryos. The posterior limit of the ablation within the median pit varied, including or excluding the caudal tip of Hensen's node. Twelve operated embryos were killed at various stages between 22-
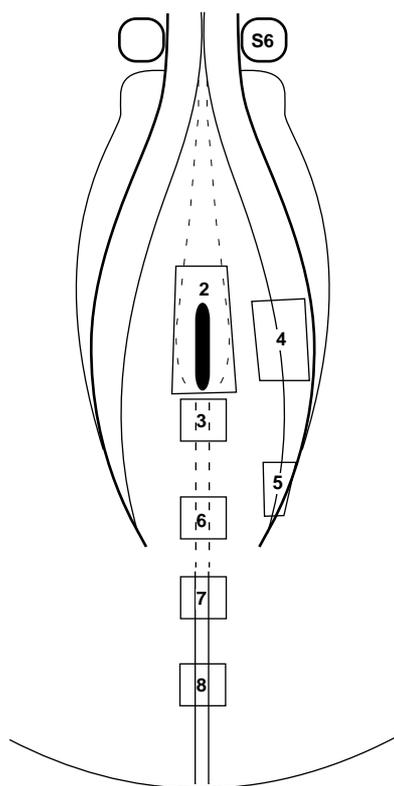
Fig. 2. Schematic representation of the quail to chick transplantations performed. Regions 2 (300×150 μm), 3, 6, 7 and 8 (100×100 μm each) are located on the midline in a rostrocaudal sequence. Region 2 corresponds to the caudal part of the already formed notochord plus the Hensen's node. Region 4 (200×100 μm) involves the lateral neural plate at the transverse level of Hensen's node. Region 5 (150×100 μm) is located at the caudal and lateral border of the sinus rhomboidalis.

somite and stage 35 HH (E2 to E8.5). They all showed an interruption of the notochord from the thoracic region to posterior levels that depended on the caudal limit of the excision in the median pit. The part of the notochord situated immediately ahead the interruption was considerably enlarged.

Two embryos were fixed one day after surgery, at 22- and 27-somite stage (stages 14 and 16 HH) and were analyzed on histological serial transverse sections. In both cases, the posterior 1/3 of the median pit was left in situ. Rostrally to the excision (somites 18-19), the enlarged segment of notochord coexisted with a normally polarized neural tube (Fig. 3A). The notochord was lacking from somite 20 to the segmental plate region (level of somite 30). Caudally to the enlarged notochord (somites 20 to level of somite 28), the neural tube was rounded in shape (Fig. 3B). Somites 21 to 25 were fused pairwise in the midline, forming a single mass located ventrally to the rounded neural tube (Fig. 3B). Immediately rostral to the tailbud, a segment of notochord was present equal to the length of 3 somites and was overlain by a normally polarized neural tube (Fig. 3D). Both caudal neural tube and notochord merged in the CNH at the anterior part of the tailbud. Interestingly, rostral to the caudal fragment of notochord the neural tube presented a floor plate-like morphology over the length of 2 somites (Fig. 3C).

Although no notochord was present at this level, paraxial mesoderm was segregated on either side of the neural tube.

In embryos killed 2 and 4 days after surgery (E3.5 and E5.5 i.e. stages 20 to 28 HH, n=6, not shown), the enlarged segment of notochord was seen at the posterior limit of the forelimbs. The neural tube, at this level, was deprived of a floor plate but dorsal root ganglia and somitic derivatives were normally differentiated and localized. More caudally, the neural tube was not polarized and dorsal root ganglia were fused ventrally on the midline. The only somitic derivatives were muscle masses located transversally underneath the neural tube. Absence of notochord and floor plate was found down to the tail in two cases where the median pit had been totally removed. In contrast, when a caudal fragment of the median pit had been left in situ, a fragment of notochord was present in the terminal region of the embryo. As in the cases described above, a floor plate morphology was evident not only in the region where the notochord was present but also on a certain length rostrally to its anterior limit.

Similar observations have been made at E7.5 and E8.5 (stages 32 to 35 HH, n=4, see Fig. 3E-G). All of these embryos had a fragment of caudal notochord. As seen before, the neural tube was polarized at its caudal end and a few segments rostrally to the notochord anterior limit where somitic derivatives differentiated normally (Fig. 3G).

The fact that the rostral segment of notochord ends at thoracic level as an enlarged structure, suggests that surgery prevents its normal caudal growth. This interpretation is also supported by the enlarged notochord that is found, at later stages, under a portion of neural tube lacking a floor plate (Fig. 3E). Our provisional interpretation is that the normal caudal growth of the preexisting notochord is stopped by the absence of endoderm and neural plate at the site of excision. The presence of a fragment of notochord in the caudal part of the embryo, when the median pit has not been completely removed, shows that the notochordal material that is left in place in these experiments, is capable of moving caudally in spite of the absence of the rostral portion of the median pit.

Experiment 2A. Grafting of quail Hensen's node with underlying endoderm

In a second experimental series, we replaced the excised region (the median pit and the caudal portion of the already formed notochord, together with the underlying and overlying tissues) by its quail counterpart. One grafted embryo fixed at E3.5 (40-somite, i.e. stage 20 HH) was analyzed in serial transverse sections stained either with the Feulgen-Rossenbeck's technique or with the QCPN mAb. In this embryo, as in the above described cases, the rostral notochord ended in a swelling at the upper-thoracic level (posterior limit of the forelimbs), which corresponds to the rostral limit of the operation. At the same transverse level, the neural tube was perfectly normal in size and shape. Caudally, the notochord was absent on a length corresponding to several segments. From this level down to the tail, the floor plate of the chick neural tube was of quail type. A quail notochord appeared from the level of the genital ridges (mid-thoracic level) downwards. Interestingly, quail endodermal cells were found dorsally in the intestine of the same region (Fig. 4A), suggesting that the region of Hensen's node contains not only floor plate and notochord precursors, but also progenitors of the dorsal-most endoderm of the gut.
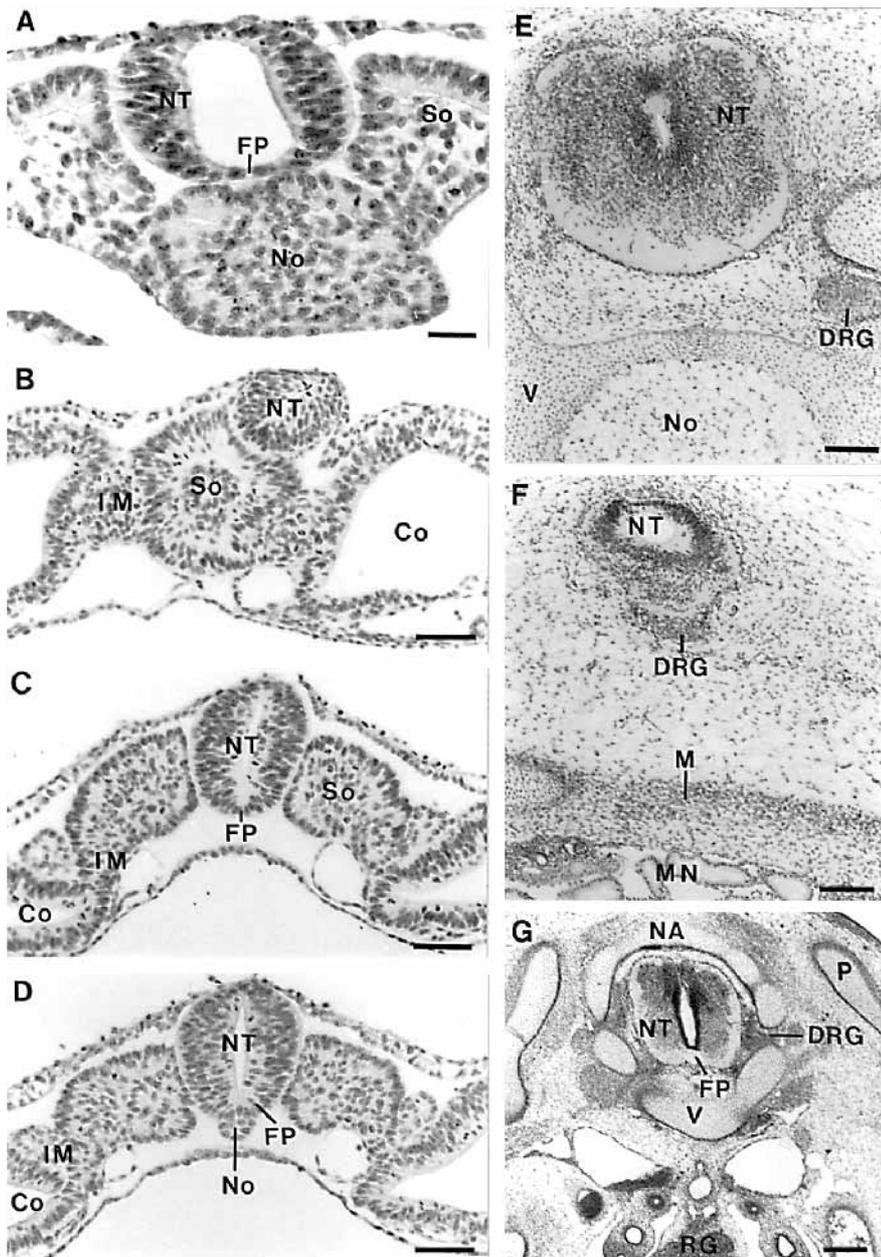
Fig. 3. Partial excision of Hensen's node performed in 6-somite-stage chick embryos (the caudal part of Hensen's node was not included in the operation, see text). (A-D) Transverse sections 1 day after surgery. (A) 22-somite-stage embryo. Section just ahead the excision region where the notochord (No) ends in an enlarged structure. At this stage, the enlarged notochord is found under a normally polarized neural tube (NT). (B-D) 27-somite-stage embryo. (B) In the region immediately caudal to the excision, notochord and floor plate (FP) are lacking and somites (So) are fused under the rounded neural tube. (C) In an intermediate region, notochord is absent but floor plate morphology is evident; somites are bilaterally disposed. (D) Caudally notochord and floor plate are present. (E-G) Transverse sections of embryos fixed 7 days after surgery (stage 35 HH). (E) The enlarged notochord has moved under the neural tube deprived of floor plate. It is surrounded by vertebral cartilage (V). (F) In the thoracic region, a very small non-polarized neural tube is surrounded by loose mesenchyme. Fused dorsal root ganglia (DRG) are present underneath. (G) In the lumbar region, normally developed neural tube, DRG and vertebra are present in spite of the absence of notochord. The notochord is present more caudally. Co, coelomic cavity; IM, intermediate mesoderm; M, muscles; MN, mesonephros; NA, neural arch; P, pelvic girdle; RG, Remak ganglion. Feulgen-Rossenbeck's staining. Bars, 20 μm (A), 50 μm (B-D), 100 μm (E, F), 200 μm (G).

The distribution of donor cells in this embryo demonstrates that, at the 6-somite stage, cells yielding both notochord and floor plate are located in the median pit. The fact that graft-derived floor plate begins more rostrally than the notochord indicates that the notochordal material is pushed caudally by the growth of its more rostral segment. This capacity to grow of the newly formed notochord is also manifested by the enlargement of its extremity in the experiments where the median pit is removed. Excision of the endoderm together with the median pit material seems to prevent the caudal progression of the notochord of the host embryo.

Experiment 2B. Grafting the quail Hensen's node with chick endoderm in place

In this design, the material located in the median pit and rostrally to it was removed as before but, in contrast to experiment 2A,

the endoderm was left in situ. The excised material was replaced by its quail counterpart. Three chimaeras of this type were analyzed. Two were killed at the 25-somite stage (E2.5, stage 15HH). The graft gave rise to the floor plate from the level of somite 22 and to notochord from the level of somite 25, these labelled tissues extending to the most caudal part of the embryo. In this series, no gap was observed between the host and the labelled notochord, showing that, in presence of the host intact endoderm, normal rostrocaudal growth of the preexisting notochord could take place. There were no indications of any morphological change marking the transition between primary and secondary neurulation. As in experiment 2A, quail cells were found to constitute the CNH located in the ventrorostral part of the tailbud where precursors of the caudal-most notochord have been localized at the 25-somite stage (Catala et al., 1995). In the later chimaera killed at E4, quail floor plate and
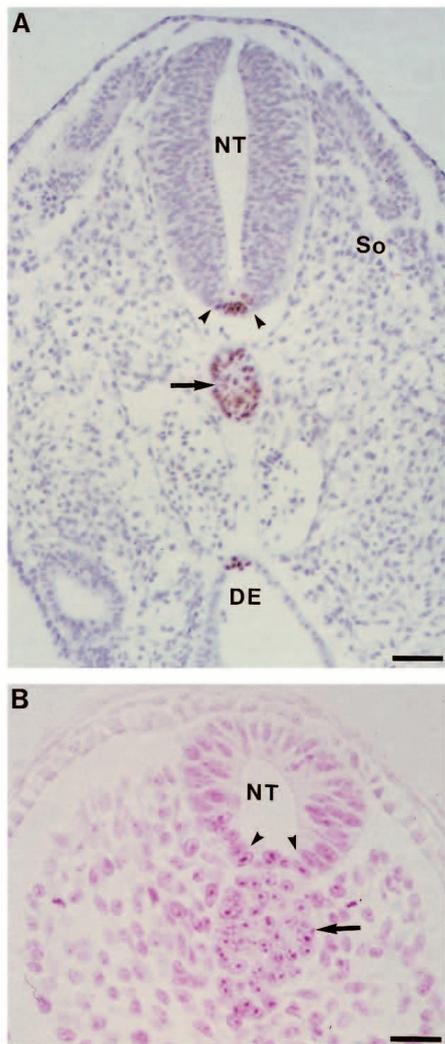
Fig. 4. Transverse sections of quail-chick chimaeras operated according to experiment 2A (A) or 2B (B) (see text) and stained using the QCPN mAb (A) or after Feulgen-Rossenbeck's reaction (B). If the graft involves the endoderm (2A), it gives rise to the floor plate (arrowheads), the notochord (arrow) and the dorsal part of the endoderm (DE) (A). In contrast in experiment 2B, the endoderm was excluded from the graft, which yields only notochord and floor plate. The section is performed in the extreme tip of the tail where notochord (arrow) and floor plate (arrowheads) were of graft origin. (B). Bars, 50 μm (A) and 20 μm (B). NT, neural tube; So, somitic derivatives.

notochord were found extending to the farthest tip of the spinal cord, namely the ventriculus terminalis (Fig. 4B).

This experiment suggests that, in presence of intact endoderm, rostrocaudal growth of the already formed notochord can occur and that common morphogenetic mechanisms function during primary and secondary neurulation.

Experiment 3. Graft of the basolateral spinal cord progenitors

Three chimaeras with quail replacement of the superficial layer immediately caudal to the median pit (Fig. 2, region 3) were analyzed. One of them, killed 6 hours after grafting (10-somite

stage) showed donor cells in the medial neural plate located caudally to the median pit and in mesenchymal tissue underneath (Fig. 5A). A few donor cells scattered in the unsegmented paraxial mesoderm were also detected in more rostral regions (not shown). The distance between the rostral limits of mesoderm and neural participations was evaluated to 250 μm at this stage. This result shows that a relative rostrocaudal displacement between neural plate and mesodermal cells takes place at this level. Importantly, the graft spanned the midline of the neural plate.

In a second chimaera, killed at 24-somite stage i.e. 1 day after the operation (E2.5) donor cells gave rise to the ventrolateral part of the neural tube, that is, the basal plate region. In this experiment, the floor plate as well as the notochord were derived from the chick host (data not shown but see Fig. 5B below). The rostrocaudal extension of the neural graft was about 300 μm (corresponding to the length of 2 or 3 somites). Furthermore, as in the younger chimaera, donor cells contributed to paraxial mesoderm situated more rostrally than the neural tissue. In the third chimaera killed at E3.5 (40-somite stage), the graft also yielded the ventrolateral tissues of the neural tube located between somites 30 and 33 (Fig. 5B). The floor plate and notochord were formed by host cells at all levels of the embryo.

This experiment demonstrates that the precursors of the floor plate and those of the lateral walls of the neural tube (caudal to somite 25), arise from different transverse levels of the embryo at 6-somite stage. The median pit corresponding to Hensen's node contains the material that generates the floor plate and the notochord. As the node regresses caudally, cells that will form the floor plate separate the basal plate into left and right halves (Fig. 5B).

Experiment 4. Graft of alar plate at the transverse level of Hensen's node

Four chimaeras that received the graft of the lateral part of the neural plate located at the level of the median pit (Fig. 2, region 4) were analyzed 1 day after surgery at 25-somite stage. The graft produced the dorsal part of the neural tube located at the level of somites 19 to 22 (Fig. 5C). Furthermore, although grafted unilaterally, the quail tissue contributed to neural crest cells on each side of the neural tube (Fig. 5C). Moreover, a few labelled cells were found contributing to somites 15 to 20. These cells are likely to have arisen from deep cells belonging to the paraxial mesoderm that were adherent to the transplanted neural plate fragment.

Experiment 5. Graft of regions of secondary neurulation

Four chimaeras that received a graft of the superficial layer of the laterocaudal part of the sinus rhomboidalis (Fig. 2, region 5) were killed at 25-somite stage, 1 day after surgery. Results show that this region is the zone of transition between primary and secondary neurulation. In the rostral part of the graft, the donor cells produced the ipsilateral alar plate of the neural tube and superficial ectodermal cells (not shown). At a more posterior level, corresponding to the region of the secondary neurulation, the graft contributed to the dorsal-most part of medullary cord. Interestingly, quail cells were distributed over both ipsilateral and contralateral sides at this level (Fig. 5D), pointing to a different characteristic in morphogenetic movements in the dorsal part of the spinal cord during secondary neurulation.
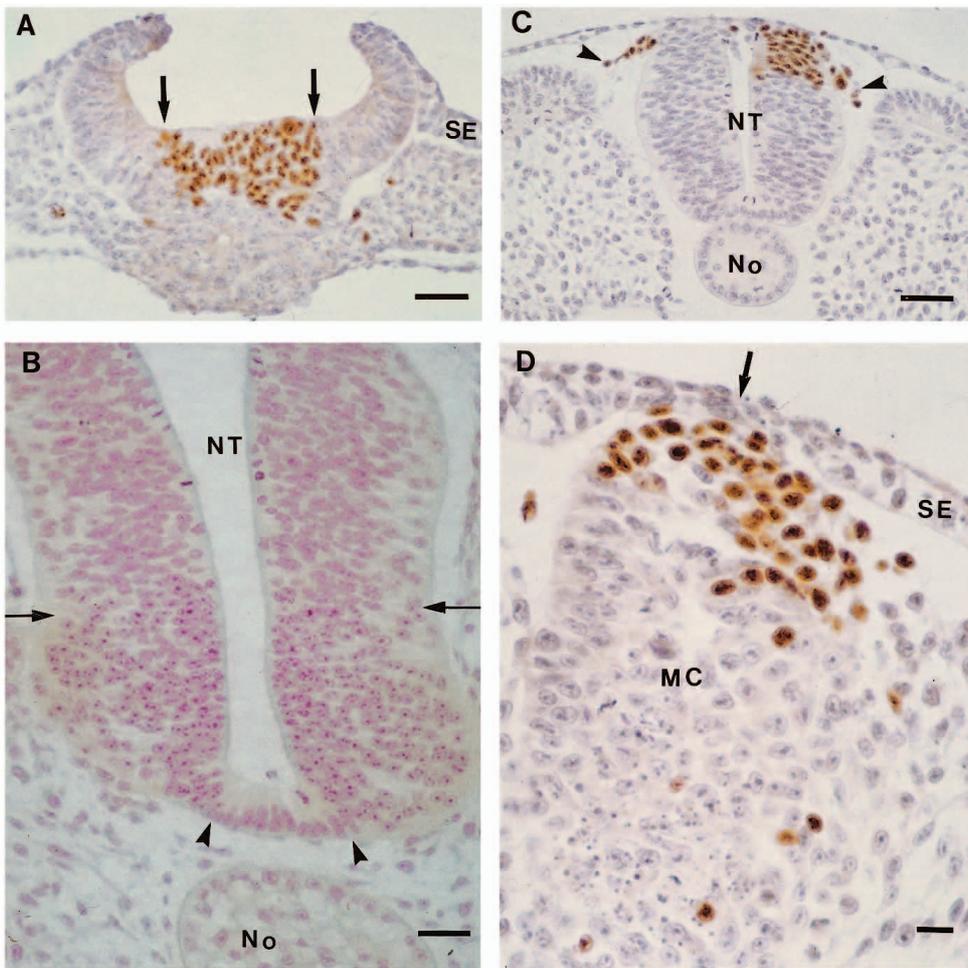
Fig. 5. Transverse sections of quail-chick chimaeras operated according experiments 3 (A,B), 4 (C) and 5 (D) (see Fig. 2) and stained with the QCPN mAb (A,C,D) or after Feulgen-Rossenbeck's reaction (B). In experiment 3, when the chimaera is killed a few hours after surgery (A), the grafted territory (arrows) corresponds to the superficial layer of the sinus rhomboidalis located immediately caudal to Hensen's node (compared with Fig. 1D). When the chimaera is observed at E4 (B), the graft gives rise to the ventral part of the neural tube (NT) (arrows) except for its floor plate (arrowheads), which is derived from the host Hensen's node. Note that the notochord (No) is also of host origin. Region 4 (C) gives rise to the unilateral dorsal moiety of the neural tube and to neural crest cells, which migrate bilaterally (arrowheads). Region 5 (D) participates in the formation of the medullary cord (MC) where neurulation proceeds by cavitation. Note that the graft contribution is bilaterally distributed in the medullary cord (arrow indicates the midline). Bars, 50 μm (A), 20 μm (B), 40 μm (C) and 10 μm (D). SE, surface ectoderm.

Experiment 6. Graft of the posterior sinus rhomboidalis

Five chimaeras involving grafts in the midline of the caudal part of the sinus rhomboidalis (Fig. 2, region 6) were analyzed at E4-E5. In all cases, the graft gave rise to cells dispersed in the somites located between the forelimbs and hindlimbs and their derivatives (Fig. 6A). Contributions of donor and host cells to the somitic derivatives (dermatome, myotome, sclerotome) were equivalent. These mesodermal precursor cells did not contribute to the notochord, indicating that the regression of the CNH splits trunk mesoderm precursors, laying down the notochord in its wake. We conclude that the surface layer of the posterior sinus rhomboidalis contains mesodermal, not neural, precursors.

Experiment 7. Graft of anterior primitive streak

Six chimaeras with quail grafts involving the rostral-most extremity of the primitive streak (Fig. 2, region 7) were observed. One was killed 1 hour after the graft. The tissue from the donor was found in the medial embryonic region in the rostral-most part of the primitive streak. In two chimaeras killed 1 day after surgery (25-somite stage), the graft gave rise mainly to intermediate mesoderm (Fig. 6B). A few cells coming from the donor were also found in somites and in the medial region of the lateral plate. No donor cells were found

in the tailbud. The three last chimaeras were killed at E5. Quail cells were found predominantly in the caudal part of the kidney. Furthermore, a few cells contributed to somitic structures. We conclude that the anterior primitive streak, just caudal to the sinus rhomboidalis at the 6-somite stage, gives rise predominantly to kidney precursor cells.

Experiment 8. Graft of central primitive streak

We have analyzed 7 chimaeras with grafts in the middle part of the primitive streak (Fig. 2, region 8). In one chimaera fixed 4.5 hours after grafting, donor cells were found both in the superficial layer of the primitive streak and in the underlying mesoderm with lateral migration underneath the host superficial layer (Fig. 6C). Two chimaeras were killed 1 day after surgery at 23- and 26-somite stages, respectively. In the younger one, quail cells were found in the caudal lateral plate mesoderm. In the one killed at 26-somite stage, caudal folding had begun and the quail cells were observed in the perianal mesoderm, which is a derivative of ventral mesoderm. Three chimaeras were killed at E3.5 (corresponding to 45-somite stage) and one at E5. In the four cases, quail cells participated in the formation of the mesoderm of the caudal digestive tract at the level of the cloaca (Fig. 6D). We conclude that the middle primitive streak gives rise to ventroposterior mesoderm at the 6-somite stage.
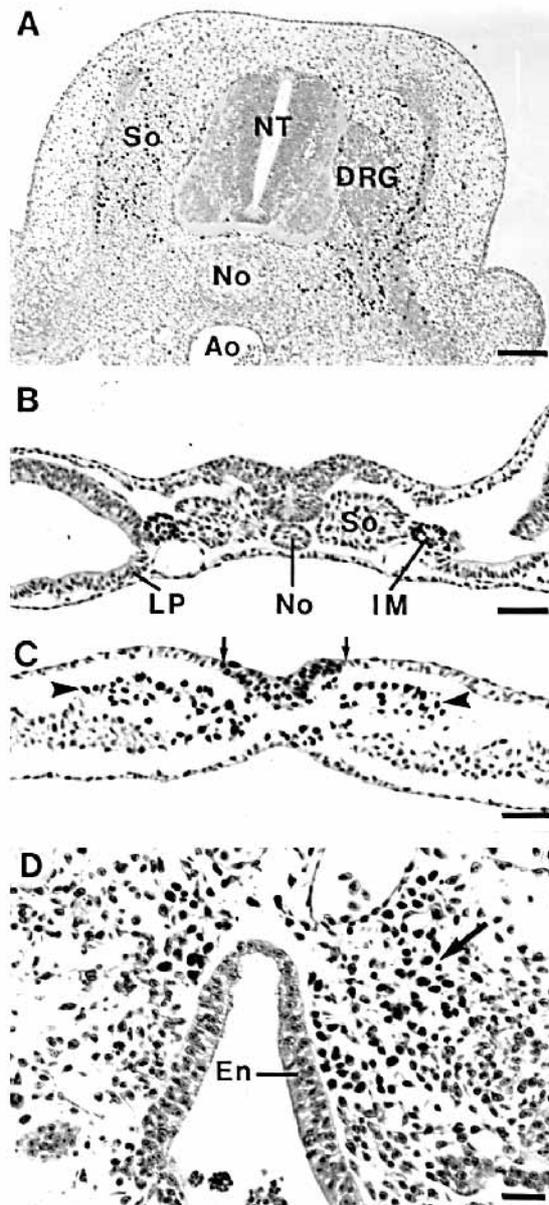
Fig. 6. Transverse sections of quail-chick chimaeras operated according to experiments 6 (A), 7 (B) and 8 (C,D) and stained with the QCPN mAb. Region 6 (A) yields somitic derivatives occupying both medial and lateral somitic domains. Region 7 (B) gives rise to the intermediate mesoderm (IM). When observed a few hours after surgery (C corresponding to the level figured on 1E), grafted cells from region 8 are still located in the midline (arrows). Note that migration has begun and some quail cells are located underneath the chick superficial layer (arrowheads). When observed at E4 (D), region 8 gives rise to cells forming the mesodermal lining (arrow) of the caudal endoderm (En), namely in the cloaca. Bars, 100 μm (A), 50 μm (B and C) and 25 μm (D). Ao, aorta; LP, lateral plate; NT, neural tube; No, notochord; So, somite or somitic derivatives.

DISCUSSION

How does the notochord elongate?

In the 6-somite-stage avian embryo, Hensen's node appears as

a median depression situated in the middle of the sinus rhomboidalis. We excised this structure together with the newly formed notochord and underlying endoderm. This results in the complete absence of notochord from the cervicothoracic region (level of somite 18-20) caudalwards. The notochord anterior to the excision enlarges at its caudal extremity. Incomplete excision at the posterior level of the median pit results in the presence of a notochordal fragment in the most caudal region of the embryo. Our results are in agreement with those obtained after excisions of Hensen's node performed at various developmental stages (from intermediate streak to 10-somite stages) (Wolff, 1935a,b; Grabowski, 1956; Smith and Schoenwolf, 1989; Hirano et al., 1991; Yuan et al., 1995).

When we replace the chick Hensen's node together with the underlying endoderm by their quail counterpart, the outcome of the host's notochord is the same as in the embryos that have been subjected to the excision alone, i.e. it ends in the upper thoracic region as a swollen stump. But, in the thoracic, lumbosacral and tail regions of the grafted embryo, the notochord and floor plate are generated by the graft while the rest of the neural tube is of chick origin. The quail floor plate begins right at the level of the graft (somite 18-20), corresponding to the cervicothoracic region (cervical (C) 13 to thoracic (T) 1), whereas the quail notochord appears only at the mid-thoracic level (somite 24-25, T5). It thus appears that the material yielding the notochord and floor plate in the thoracic and posterior levels of the body is located within the median pit at 6-somite stage. This material corresponds to the mass of medial tissues seen on Fig. 1C in a normal embryo. As shown in Fig. 1C and in more anterior and posterior transverse sections in the same figure (Fig. 1B,D), the CNH material becomes inserted into the overlying neural plate, forming dorsally the future floor plate and ventrally the notochord. At this stage, notochord and floor plate rudiments are intimately associated. Afterwards they become separated by a basement membrane and the notochord slides caudally in comparison with the floor plate, thus accounting for the different rostral levels of the graft-derived notochord and floor plate. This phenomenon is likely to account for the results obtained by Artinger and Bronner-Fraser (1993). After ablation of the newly formed notochord, these authors observed an absence of floor plate in 11 embryos killed 2 days after surgery. But, surprisingly, they found that the neural tube was normal in 20 among 21 embryos observed 4 days after surgery (HH stages 25-27). They suggested that formation of the floor plate at this level is 'delayed' and finally occurs from planar induction. We favor another interpretation of these results since, in our partial excisions, a floor plate is present rostrally to the caudal notochord as early as 24 hours after operation. We do believe that this difference in the levels of notochord and floor plate observed in our and other experiments can be accounted for by the differential growth and movement of the two structures, without the need for planar induction as an explanation.

Another important observation is that the notochord located rostrally to the excision continues to grow and forms a large bulb (Fig. 3A,E and similar experiments by van Straaten and Hekking, 1991). Therefore, rostrally to Hensen's node, the freshly laid down notochord is the site of intensive growth that presumably contributes to its elongation. The results suggest that the notochord is subjected to longitudinal tension, due to

the anteroposterior active migration of Hensen's node material. When the notochordal material is transected by the excision of the median pit, these tensions are removed and the already formed notochord continues to grow, namely by proliferation as reported by Sausedo and Schoenwolf (1993). On the contrary, when the anterior part of the median pit is removed but its posterior region left in situ, the latter is able to move posteriorly, forming a small fragment of notochord and floor plate in the caudal region. Therefore, it appears that both the movement of the median pit proper plus the growth of the newly formed notochord are responsible for the elongation of the notochord. This mechanism is similar to that already observed in the caudal region of the chick embryo as shown by our experiments on tail bud development (Catala et al., 1995). Interestingly, the observations made previously by Schoenwolf (1977, 1978), after either extirpation of the tail bud or grafts of tritiated thymidine-labeled tail buds on unlabeled hosts, can be accounted for by the rostrocaudal elongation of the notochordal primordium.

Therefore, notogenesis in the avian embryo follows a common morphogenetic pattern from the 6-somite stage onwards i.e. in primary and secondary neurulation. Two elementary mechanisms may account for notochordal elongation: addition of cells coming from Hensen's node as described by Sausedo and Schoenwolf (1993, 1994) and cellular proliferation and rearrangement in the caudal part of the already formed notochord. Our results are also in agreement with results obtained by Gont et al. (1993) in the *Xenopus laevis* embryo. From the early neurula stage onwards, cells located in the late blastoporal lip give rise to the CNH, which is fated to form the caudal part of the notochord and the ventral spinal cord.

A separate origin for floor plate and lateral walls of the spinal cord

We have found that Hensen's node gives rise to floor plate and notochordal cells, whereas the rest of the neural plate derives from differently located progenitors. This result is similar to what we have observed in secondary neurulation (Catala et al., 1995). Indeed, at this stage, prospective floor plate cells are located in the CNH whereas the rest of the neural tube arises from cells situated more caudally in the tailbud. We have shown that the same morphogenetic mechanism applies to primary neurulation, secondary neurulation and the transition zone.

In the classic graft experiments of Spemann (1938), the grafted dorsal lip of the blastopore differentiated into notochord and floor plate which, in the induced embryonic axis, is inserted into the neural tube and medial somites as clearly represented on Spemann's own figures in *Embryonic Development and Induction* (1938, Fig. 78 and Fig. 80). This, like our own experiments, shows that floor plate and notochord have a common embryonic origin. Recently, Leber and Sanes (1995), using a retroviral marking technique, found that the lineage of the floor plate and that of the rest of the neural tube are different since they usually did not observe clones containing the two types of cells. Furthermore, they noticed that the clones giving rise to floor plate cells are arranged in a rostrocaudal direction in contrast to the clones giving rise to more lateral neural regions, which are oriented in a radial direction. This result indicates that mitoses in these cells are directionally arranged allowing the

elongation of the floor plate that we have observed. This rostrocaudal elongation of the floor plate material may explain the results observed by Stern et al. (1991). They labeled one cell of the neural tube by the fluorescent marker LRD (lysinated rhodamine dextran). When the injection involved a lateral cell of the neural tube facing the segmented paraxial mesoderm, the progeny of this cell never crossed a boundary aligned with the middle of each somite. In contrast, when a floor plate cell was injected, its progeny spread extensively with a rostrocaudal extension of more than 5 somites. These results (Stern et al., 1991) and those presented here indicate that floor plate cells elongate and undergo differential displacement with respect to the rest of the neural tube.

Secondary neurulation

Primary and secondary neurulations proceed according to different mechanisms (e.g. Schoenwolf and Alvarez, 1992). Here, we were able to follow the fate and morphogenetic movements affecting the various territories of the embryo from the level of the graft corresponding to the area including the upper thoracic level (somite 22, corresponding to T3) down to the caudal end of the fully developed axis (somite 53). We have localized, at the 6-somite stage, the cells that later on form the tail bud: the notochord-floor plate anlage is located at the level of Hensen's node and moves from rostral to caudal. Spinal cord precursors are located on the surface layer of sinus rhomboidalis in regions lateral and posterior to Hensen's node. Mesodermal progenitors are in the rostral part of the primitive streak and in the caudal midline of the sinus rhomboidalis (Fig. 7). Formation of the secondary neural tube results from an infolding of the ectodermal cells forming the laterocaudal part of the sinus rhomboidalis. This process is similar to that observed in the teleost embryos and is responsible for the formation of the neural keel (Schmitz et al., 1993; Papan and Campos-Ortega, 1994). Besides the differences in the morphogenetic processes leading to the formation of the neural tube, one striking difference between primary and secondary neurulation, as shown by our experiments, is that, in chick, the contribution to the dorsal neural tube of the lateral neural plate is strictly unilateral for primary neurulation whereas it is bilateral and therefore involves extensive cell mixing in the secondary one (Fig. 4D). In zebrafish, precursors located on one side of the embryo were shown to contribute to descendants lying on the two sides of the neural tube (Kimmel et al., 1994; Woo and Fraser, 1995), pointing to another similarity between neurulation in fish and secondary neurulation in bird.

Regression of the chick primitive streak may be explained by two elementary mechanisms. First, the CNH physically regresses from rostral to caudal and separates the neural plate into two halves, which give rise to the ventrolateral parts of the neural tube (Fig. 7). Second, the cells located at the caudal part of the primitive streak do not participate in the formation of the tail bud (Nicolet, 1970) and diverge laterally explaining the shortening of the primitive streak. In the mouse, lineage tracing of the notochord and primitive streaks suggest that similar movements take place (Wilson and Beddington, 1996).

Neural crest cells migrate bilaterally

After unilateral graft of the lateral region of the neural plate together with the adjacent neural fold, we have observed that the neural crest cells originating from the graft migrate ipsi-
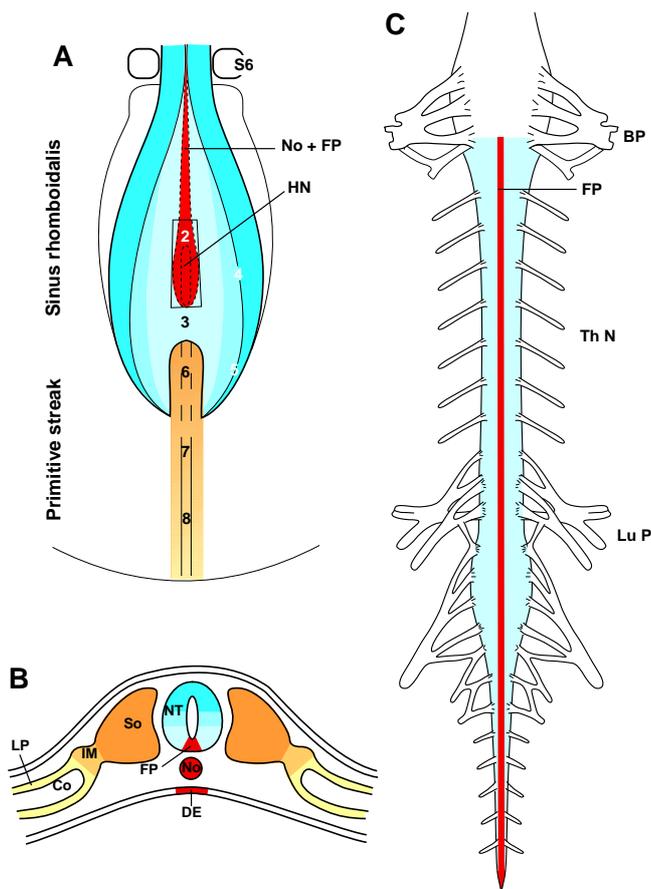
Fig. 7. Summary of the results: the grafted territories are represented in A by the numbers 2 to 8. Their derivatives are shown on a transverse section of an avian embryo after neurulation (B) and on a ventral view of the spinal cord (C). Hensen's node (HN) gives rise to both notochord (No) and floor plate (FP) from the upper thoracic level down to the extremity of the tail. The rest of the spinal cord originates from regions located caudally and laterally to the Hensen's node. Mesodermal precursors are arranged on the midline according to their future mediolateral disposition. BP, brachial plexus; Co, coelomic cavity; DE, dorsal endoderm; IM, intermediate mesoderm; LP, lateral plate; LuP, lumbar plexus; So, somite; ThN, thoracic nerves.

laterally and contralaterally to the sides of the grafted neural tube (Fig. 5C). It is interesting to note that both host and donor crest cells were found in the two sides of the embryo. This result indicates that the bilateral migration of the grafted cells cannot be explained by an 'aggressive' behavior of quail cells in the chick embryo. Moreover, the contribution of the graft to neural crest is not identical for the two sides of the embryo and the major stream of migration is the ipsilateral side. Such a bilateral migration of neural crest cells had been already observed in the amphibian embryo (Detwiler and Kehoe, 1939; Hörstadius, 1950 for a review). More recently, bilateral migration of avian neural crest cells has been observed after injecting lysinated-rhodamine-dextran into a single cell of the neural fold (Selleck and Bronner-Fraser, 1995).

Mesoderm precursors are located on the posterior midline

Our fate map of the 6-somite-stage avian embryo (Fig. 7)

shows that mesodermal precursors are still located in the midline of the sinus rhomboidalis and in the primitive streak at this stage. They migrate from the midline to their definite positions according to a movement of lateral divergence. Furthermore, we have demonstrated that the more caudal these precursors, the more lateral their fate. Thus, mesodermal precursors are arranged in a rostrocaudal organization which represents the future dorsoventral axis of the mesoderm. These observations are in agreement with the fate maps constructed in avian or mammalian embryos at earlier stages (Rosenquist, 1966; Nicolet, 1970; Tam and Beddington, 1987; Lawson et al., 1991; Bortier and Vakaet, 1992; Schoenwolf et al., 1992; Garcia-Martinez et al., 1993; Smith et al., 1994; Wilson and Beddington, 1996). We conclude that this mesodermal organization persists at the 6-somite (this work) and tailbud (Catala et al., 1995) stages, that is, to completion of gastrulation.

Cell fates in the chick spinal cord

Fig. 7 summarizes the fate map of the chick sinus rhomboidalis at the 6-somite stage. The various spinal cord components map to distinct regions. (1) The CNH region located in Hensen's node gives rise to floor plate, notochord and dorsalmost endoderm (Fig. 4A) throughout the thoracic, lumbar and caudal regions of the body. Comparing the results from our experiments 2A and 2B suggests that the endodermal lineage is already segregated from the CNH lineage, a result in agreement with the recent observations made in the mouse embryo by Wilson and Beddington (1996). (2) The basal plate region of the spinal cord is located posteriorly to the Hensen's node, spanning the midline. As the CNH regresses, it bisects the basal plate anlage laying down the floor plate in the midline (Fig. 5A,B). (3) The alar plate and neural crest of the primary neurulation region is located lateral to Hensen's node. (4) The posteriormost neural folds of the sinus rhomboidalis give rise to the dorsal part of the spinal cord in regions undergoing secondary neurulation and displays extensive bilateral mixing. (5) Finally, the midline of the posterior region of the sinus rhomboidalis does not give rise to spinal cord but rather to somitic mesoderm. These cell fate studies extend our understanding of the morphogenetic movements that generate the spinal cord of the thoracic, lumbar and caudal regions of the amniote embryo.

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