Defining subregions of Hensen's node essential for caudalward movement, midline development and cell survival

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SUMMARY

Hensen’s node, also called the chordoneural hinge in the tail bud, is a group of cells that constitutes the organizer of the avian embryo and that expresses the gene HNF-3β. During gastrulation and neurulation, it undergoes a rostral-to-caudal movement as the embryo elongates. Labeling of Hensen’s node by the quail-chick chimera system has shown that, while moving caudally, Hensen’s node leaves in its wake not only the notochord but also the floor plate and a longitudinal strand of dorsal endodermal cells. In this work, we demonstrate that the node can be divided into functionally distinct subregions. Caudalward migration of the node depends on the presence of the most posterior region, which is closely apposed to the anterior portion of the primitive streak as defined by expression of the T-box gene Ch-Tbx6L. We call this region the axial-paraxial hinge because it corresponds to the junction of the presumptive midline axial structures (notochord and floor plate) and the paraxial mesoderm. We propose that the axial-paraxial hinge is the equivalent of the neurenteric canal of other vertebrates such as Xenopus. Blocking the caudal movement of Hensen’s node at the 5- to 6-somite stage by removing the axial-paraxial hinge deprives the embryo of midline structures caudal to the brachial level, but does not prevent formation of the neural tube and mesoderm located posteriorly. However, the whole embryonic region generated posterior to the level of Hensen’s node arrest undergoes widespread apoptosis within the next 24 hours. Hensen’s node-derived structures (notochord and floor plate) thus appear to produce maintenance factor(s) that ensures the survival and further development of adjacent tissues.

Key words: Floor plate, Notochord, Microsurgery, Quail-chick labeling, Apoptosis, HNF-3β/Ch-Tbx6L interface, Gastrulation, Neurulation, Neurenteric canal

INTRODUCTION

In amniote vertebrates, the organizer, analogous to dorsal blastoporal lip of the amphibian embryo, consists of a mass of cells located at the anterior end of the primitive streak, designated as Hensen’s node (HN) in the chick embryo. HN is also referred to as the chordoneural hinge (CNH) during neurulation process (see Catala et al., 1995, 1996; Teillet et al., 1998a). The primitive streak, the equivalent of the lateral blastoporal lips of the amphibian embryo, is the site of invagination of paraxial and lateral mesoderm.

Several fate-mapping studies have been carried out in the chick embryo during gastrulation and neurulation. These utilized a variety of labeling procedures: application of carbon particles (Spratt, 1946; Spratt and Condon, 1947) or colored marks (Pasteels, 1937; Spratt, 1955, 1957), incorporation of tritiated thymidine (Rosenquist, 1966; Nicolet, 1970), quail-chick tissue transplantations (Vakaet, 1984; Schoenwolf et al., 1992; Garcia-Martinez et al., 1993; Catala et al., 1995, 1996) or vital fluorescent dye injections (Schoenwolf and Sheard, 1990; Selleck and Stern, 1991). These studies (see Teillet et al., 1998a and references therein) have convincingly established that, during its caudal movement, HN-CNHi yields the cells constituting the notochord. The labeling of floor plate cells has been noticed in some of these experiments (Rosenquist, 1966; Nicolet, 1970; Schoenwolf and Sheard, 1990; Selleck and Stern, 1991), which were generally (but not always) carried out on chick embryos cultivated in vitro between the primitive streak and early somitic stages (stages 3 to 9 of Hamburger and Hamilton (HH), 1951). In fact, it has been recently demonstrated that HN-CNHi is at the origin not only of the notochord but of all the cells forming the midline structures in the three germ layers (floor plate, notochord and dorsal endoderm) (Catala et al., 1995, 1996; Le Douarin et al., 1996, 1997, 1998; Teillet et al., 1998a).

It thus appears that elongation of the avian embryo and deposition of its axial structures depend on the multiplication and the rostral-to-caudal migration of cells contained in the HN-CNHi. Interestingly, the node or ‘Knoten’ was firstly described by V. Hensen in mammals in 1876 as a “Zellenmass […] in welchem alle drei Blätter verschmolzen sind”, (a mass of cells […] in which the three germ layers are fused), thus awarding to it a primordial role in the gastrulation process.
Fig. 1. Scheme of the microsurgical experiments performed on Hensen’s node at the 5- to 6-somite stage chicken embryos.
(A) Experiment 1: the mass of cells located at the level of the median pit, in the center of the sinus rhomboidalis, is excised.
(B) Experiment 2: the limit of the excision is extended either 50 μm (a) or 100 μm (b) caudal to the posterior lip of the median pit.
(C) Experiment 3: tissues 100 μm caudal to the median pit are selectively excised. (D) Experiment 4: tissues 100 μm caudal to the median pit are replaced by their stage-matched quail counterpart. S6, somite 6; D, dorsal; V, ventral.

Fig. 2. (A) Morphological observations and (B-H) molecular analysis of the sinus rhomboidalis of 5- to 6-somite-stage chicken embryos. The different panels represents (a) dorsal view, (b) sagittal section, (c) transverse sections at the median pit level and (d) transverse section immediately caudal to the node (about 60 μm). (Aa) A non-treated embryo, photographed on a black dish, showing the floor plate/notochord complex on the left, the node area in the middle, and the primitive streak on the right. Double arrows indicate the respective rostrocaudal levels of the transverse sections c and d. (Ab-d) Semithin (1 μm) sections showing the cell arrangement of the tissues in the sagittal (b) and transverse (c,d) planes. (B-H) Expression patterns of several genes in dorsal view of whole mounts (a) and sections (b-d) as previously established in A: (B) Shh, (C) HNF-3β, (D) Chordin, (E) CNOT2, (F) Ch-Tbx6L, (G) cSox2 and (H) BMP-4 probes. Descriptions and explanations are in the text. (Ca,Cb) Arrows indicate the anterior and posterior lips of the median pit. (Ea,Ed) Arrows correspond to the lateral expression of CNOT2 in the posterior neural plate. (Fa) Arrow indicates the axial anteriormost level of Ch-Tbx6L expression. Bars: a, 200 μm; b, 100 μm; c,d, 50 μm.
Neurulation in vertebrates has long been considered as arising from the transformation of the ectodermal layer into a neural plate via vertical induction from the dorsal axial mesoderm, the notochord. A further role for the notochord was proposed in the induction of the floor plate, a specialization of the ventral neural tube that plays a role in the guidance of axons crossing the midline during neurogenesis (van Straaten et al., 1985a,b; Placzek et al., 1990; Yamada et al., 1991). According to this model, the midline cells of the neural plate receive a signal from the notochord, mediated by the product of the gene Sonic Hedgehog (Shh) (Roelink et al., 1994, 1995). This activates HNF-3β, a gene of the forkhead family of transcription factors, in presumptive floor plate cells (Ericson et al., 1996). After HNF-3β induction, the floor plate cells then express Shh (Roelink et al., 1994, 1995). HNF-3β was actually shown to be critical for the development of axial structures in the vertebrate embryo since neither notochord nor floor plate develop in mice in which this gene was inactivated (Ang and Rossant, 1994; Weinstein et al., 1994). However, HNF-3β is expressed in a continuous manner in HN-CNH and in the three structures (dorsal endoderm, notochord and floor plate) that they yield (Teillet et al., 1998a), ruling out that HNF-3β expression in the floor plate occurs as a result of the inductive influence of the notochord. Furthermore, the interpretation of the lack of floor plate which is observed when the notochord is removed in the chick embryo (van Straaten and Hekking, 1991; Placzek et al., 1990; Yamada et al., 1991) has been reconsidered. Rather than indicating an inductive relationship of the notochord to floor plate, the absence of floor plate is due to the removal of the anlage of the floor plate itself (Teillet et al., 1998b). This possibility had been looked at before by other authors (Jessel et al., 1989; van Straaten and Hekking, 1991).

In the present work, we analyze the molecular, morphological and behavioral characteristics of HN-CNH from the 5- to 6-somite stage onward. At this stage, HN is deprived of floor plate (FP) and where the No is lacking. (E) The NT presents a FP at a level where the No is absent. (F) FP and No are both present in the caudal part of the embryo. En, endoderm.
located in the center of the sinus rhomboidalis in a medial depression called the median pit. We distinguish three subregions distributed along the anteroposterior (AP) axis: (i) a rostral region, where the floor plate and notochord are recognizable but closely associated, (ii) the bulk of the node, where cellular arrangement and molecular specificities prefigure the future floor plate and notochord, and (iii) the caudal node, where cells are randomly arranged and are in close contact with the anterior end of the primitive streak. As a whole, this latter domain constitutes what we designate as the axial-paraxial hinge (APH), a region that plays a critical role in the caudalward movement of the midline cells. We show that removal of the APH, although blocking the formation of the midline structures, does not prevent either the caudal extension of the embryo or the formation of the posterior neural tube. However, in the absence of the floor plate, notochord and dorsal endoderm, the posterior part of the embryo rapidly undergoes massive cell death by apoptosis.

MATERIALS AND METHODS

Quail (Coturnix coturnix japonica) and chick (Gallus gallus domesticus) eggs from commercial sources were incubated at 38°C in a humidified atmosphere. Embryos were staged according to Hamburger and Hamilton (HH) (1951), or the number of somites or embryonic day (E). All experiments were carried out on 5- to 6-somite-stage embryos.

Microsurgery experiments

Type 1 (Fig. 1A): The node material located in the median pit was excised microsurgically as described in Catala et al. (1996).

Type 2 (Fig. 1B): Excision of the node material was extended caudally, behind the posterior limit of the median pit, by approximately 50 μm (type 2a) or 100 μm in length (type 2b).

Type 3 (Fig. 1C): Tissues situated caudal to the median pit (about 100 μm long), including underlying endoderm, were selectively excised. In this experiment, the bulk of HN material was left in situ. The superficial ectoderm corresponding to the excised region was either removed or left intact.

Type 4 (Fig. 1D): A region (100 μm long) caudal to the median pit was excised as in experiment 3 and replaced by its quail counterpart.

Type 5 (see Fig. 10A): The caudal part of the node was excised and replaced by the equivalent volume of tissue taken from a more rostral position of a stage-matched quail. The rostrocaudal orientation of the graft was maintained.

Semithin sections

Chick and quail control embryos (5- to 6-somite stage) were fixed in 2.5% glutaraldehyde, 2% paraformaldehyde in 0.05 M sodium cacodylate buffer pH 7.6, postfixed in 1% osmic acid (same buffer) and embedded in Araldite. Semithin (1 μm) transverse and sagittal sections were stained with methylene blue.

In situ hybridizations

Nine chicken riboprobes were used as markers: HNF-3β (Ruiz i Altaba et al., 1995), Shh (Riddell et al., 1993), CNOT2 (Stein et al., 1996) and Chordin (see Sasai et al., 1994) for HN and midline structures derived from the node, C-SOX2 (Rex et al., 1997) for neural ectoderm, Pax-3 and Pax-6 (Goulding et al., 1993) for the dorsalventral polarity of the neural tube, Slug (Nieto et al., 1994) and BMP-4 (Francis et al., 1994) for the neural folds, neural crest and dorsal neural tube, and Ch-Tbx5L (Knezevic et al., 1997) for the paraxial mesoderm.

For whole-mount in situ hybridization, we used the method of Henrique et al. (1995). Treated embryos were photographed, embedded in 7.5% albumin-15% gelatin and sectioned (30-45 μm thick) using a vibratome (Leica VT 1000 E). Mounted sections were photographed using Nomarski optics (Leica).

RESULTS

Morphological and molecular characteristics of Hensen’s node

Regional differences in gene expression patterns are observed in HN region at the 6-somite stage (Fig. 2). Shh is strongly expressed in the rostral half of HN both dorsally and ventrally, in future floor plate and notochord cells (Fig. 2Ba,b,c). In the caudal node, Shh transcripts become progressively less abundant and are located essentially in the most ventral cells, except for endodermal cells (Fig. 2Bb). In contrast, HNF-3β is expressed in the entire mass of cells situated within the median pit and extending about 70 μm posteriorly (Fig. 2Ca,b,c). Both Shh and HNF-3β transcripts are found in the notochord and the floor plate rostral to the node, and they are completely absent in the lateral and caudal neural plate and the primitive streak (Fig. 2Ba,d,Ca,d). In the node proper, the chordin expression pattern is very similar to that of HNF-3β (Fig. 2D), but more rostrally, chordin is no longer expressed in the floor plate (not shown). CNOT2, as described by Stein et al. (1996), is predominantly expressed in the ventral part of the node (Fig. 2Eb,c) and only slightly in its dorsal part, in continuity with two lateral ectodermal areas of the sinus rhomboidalis (Fig. 2Ea, c, d). Rostral to the median pit level, expression of CNOT2 is only present in the notochord (Fig. 2Eb). Chordin, HNF-3β and CNOT2, which are expressed in cells situated more caudally than Shh, are not expressed at the anteriormost midline level of expression of Ch-Tbx5L (arrow in Fig. 2Fa). This gene is expressed very strongly in the non-segmented paraxial mesoderm and in the primitive streak cells in an increasing dorsoventral gradient (Fig. 2F). As observed by Rex et al. (1997), C-SOX2, a marker of early neural ectoderm, is also strongly expressed in the extensive area of the sinus rhomboidalis. In the node and the caudal floor plate, the transcripts are much less abundant (Fig. 2G). Caudal to the median pit, C-SOX2 is expressed in the superficial layer, which will become basal plates of the caudal neural tube (Catala et al., 1996). A few transcripts are also present in the rostralmost part of the primitive streak, which underlies it (Fig. 2Gb, d). BMP-4, as already shown by Watanabe and Le Douarin (1996),
is expressed in the neural folds at the lateral limits of the area of the sinus rhomboidalis (Fig. 2H). Interestingly, BMP-4-expressing cells are also seen ventrally in the caudalmost part of the node as well as within the superficial layer, caudal to the median pit (Fig. 2Hb-d).

Comparison of the expression patterns of these different genes and of the cellular arrangement in the node region (Figs 2Ab-d, 3A) led us to a better appreciation of the limits and structure of HN and to define three zones in this region (Fig. 3D). Anteriorly (zone a), the derivatives of the node that express HNF-3β and Shh (notochord and floor plate) are separated by forming basement membrane but are closely associated. In the area of the median pit (zone b), the future floor plate can be distinguished by a columnar arrangement of its cells (Fig. 3A). Underneath this forming epithelial layer, the presumptive notochordal cells are randomly and loosely arranged. HNF-3β and Shh are both expressed in this region, which constitutes the bulk of the node. Caudal to the border of the median pit, the cells of the node that express HNF-3β but not Shh (zone c; compare Fig. 2Bb and 2Cb) are closely packed without exhibiting any epithelial arrangement. Caudal to the HNF-3β/Shh− territory, cells located axially express a gene of the Brachyury family, Ch-Tbx6L (Fig. 3B,C). Interestingly, the HNF-3β− and Ch-Tbx6L-expressing areas, forming respectively the caudal HN and the tip of the primitive streak (TPS), do not overlap.

Excision of zone b does not prevent caudalward movement of zone c

The node material in the median pit (zone b) was removed as indicated in Fig. 1A. In this experiment, zones a and c were left intact. As observed in a previous study (see Fig. 3 in Catala et al., 1996), midline cells were interrupted, but not totally absent caudal to the excision level (Fig. 4A,B). This finding confirmed that a caudal remnant of the node material was left in situ after ablation of zone b. In fact, the neural tube was formed along the entire length of the neuraxis, but the floor plate and notochord were absent throughout most of the trunk, as confirmed by in situ hybridization with Shh (not shown) and HNF-3β probes at E2.5 (n=6) (Fig. 4). In the region where the notochord and floor plate were lacking, the somites were fused on the midline and failed to form vertebrae in older embryos (E5 to E7.5, n=5). The myotomes differentiated into muscles that joined medially underneath the neural tube, as did the dorsal root ganglia (see Fig. 5D).

Caudally, midline cells derived from the residual posterior part of the node material formed a floor plate and a notochord. In this region, the neural tube and the derivatives of the paraxial mesoderm were normal. Interestingly, the anterior limit of the floor plate that differentiated under these conditions was always more rostral than that of the notochord by several somites’ length (Figs 4, 5).

In situ hybridizations performed at E6.5 (n=3) (Fig. 5) showed that, in the affected region (Fig. 5A-E), Pax-6 and Pax-3 were expressed throughout the neural tube with a decreasing ventral-to-dorsal gradient. As in control embryos, these transcripts were always absent from the dorsalmost part of the neural tube, the roof plate, where BMP-4 and Slug (not shown) were expressed. Thus, compared to a more caudal control region, ventrally HNF-3β and Shh expression was lacking, whereas dorsal patterning was essentially normal (Fig. 5K-O).

We conclude that the caudal region of HN (zone c) can move by itself, even when most of the node material has been removed. In absence of the bulk of the node, the residual HN yields the midline structures (notochord and floor plate) of the lumbo-sacro-caudal part of the body but does not completely compensate for the ablation of the node material corresponding to the median pit. The neural tube, which forms in the region lacking midline structures, shows abnormal dorsoventral patterning with genes expressed dorsolaterally extending to a ventral position. Interestingly, at the level where the floor plate has developed in the absence of the notochord, the neural tube is normally patterned (Fig. 5F-J).

A neural tube lacking midline cells develops caudally after excision of zone b plus zone c

In experiment 2a, excision of the node material was extended by 50 μm caudal to the median pit border. Only a small region of the posterior HNF-3β+ cells was left intact (Figs 1Ba, 6A). At E2.5, the midline cells that formed posteriorly were few in number relative to the result of excision of zone b alone (type 1 experiment). A short segment of Shh+ notochord was present in the tailbud (n=2) (Fig. 6B). In older embryos (E3.5-E8.5, n=5), the notochord and floor plate were found only at the most caudal levels (Fig. 6C). The most posterior region of HN is thus able to move caudally, even when it is reduced to a small number of cells that lack connections to the rostral material.

In extreme cases (type 2b experiment), when all of the node material including the TPS was removed (excision extending 100 μm caudal to the median pit limit; Figs 1Bb, 6D), a neural tube developed caudal to the ablation but it was completely devoid of midline cells (Fig. 6E). Later on (E5.5), the operated embryos were truncated beyond the forelimbs (n=6) (Fig. 6F).

Excision of zone c prevents midline cell formation and leads to massive cell death in posterior tissues

We next performed the complementary experiment to examine the behavior and fate of zone b when the most posterior region of the node (zone c) was selectively removed (Figs 1C, 7B,C). Due to the close spatial relationship between the posterior end of HN and the rostral region of the primitive streak, this type of excision included not only the caudal node, but also the TPS, together with the underlying endodermal layer. The superficial ectoderm was either removed or left in place. Out of 144 operated embryos, 89 survived to E2.5 (62%). Among those, 72% (n=64) developed a neural tube posterior to the thoracic region, which morphologically lacked floor plate and notochord (Fig. 7D,E). Some of the remaining embryos presented a normal morphology, indicating that the excision was not sufficient to stop the caudalward movement of HN.

Whole-mount in situ hybridization performed on 49 of the affected embryos showed that the node material left in situ yielded the thoracic floor plate and notochord, to which a mass of Shh+ cells (Fig. 8A) was appended ventrally. Pax-3 (Fig. 8B,J) and Pax-6 (Fig. 8C,K) were expressed throughout the caudal neural tube except dorsally, where transcripts of Slug (Fig. 8D,L) and BMP-4 (not shown) were restricted as in type 1 experiment.

From E2.5 on, whether or not superficial ectoderm was left
in situ, the region of the embryo located posterior to the midline cell arrest underwent extensive cell death of all tissues (i.e. neural tube, ectoderm, somites and somatopleural mesoderm) except the endoderm and splanchnopleural mesoderm (n=3; Fig. 7G, H).

By E4, the embryos (n=8) were truncated. The morphology
of these embryos was similar to that of embryos from which all node material had been removed (type 2b experiment) (compare Fig. 7I,J to Fig. 6F).

**Fate of zone c**

The important role of zone c in the regression of HN and deposition of midline cells prompted us to explore its own fate during normal development. This was achieved by constructing chimeras in which this precise region, together with TPS and the overlying presumptive neural plate (see Catala et al., 1996) of a chick embryo was replaced by its counterpart from a stage-matched quail (Fig. 1D, type 4 experiment). The chimeras (n=5) were analyzed with the mAb QCPN between E2 and E4.5. Quail cells were found (i) bilaterally distributed through

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**Fig. 6.** Expression patterns of HNF-3β (A,D) and Shh (B,C,E) by whole-mount in situ hybridization after type 2a (A-C) and type 2b (D,E) experiments, in which the posterior limit of excision of the node is extended respectively 50 and 100 μm caudal to the posterior lip of the median pit. (A) Immediately after an excision of type 2a, only a small population of HNF-3β+ cells remains in situ. (B) One day after this operation (E2.5), the notochord (No) is interrupted at the level of somite 19-20. Caudal movement of the remaining node material occurred so that a short segment of Shh+ No is present in the tailbud (arrowhead). (C) At E4, this material is still visible in the tail (arrowhead). Note that a long portion of neural tube (NT) developed without midline cells. (D) After an excision extended 100 μm caudal to the median pit, there are no HNF-3β transcripts caudal to the ablation. (E) The No is interrupted at the thoracic level at E2.5. There are no Shh+ cells in the caudal part of the embryo excluding the endoderm (En). (F) At E5, surviving embryos are truncated at forelimbs level.

**Fig. 7.** Consequences of the excision of the tissues located within 100 μm caudal to the posterior lip of the median pit (MP) at the 5- to 6-somite stage (type 3 experiment). (A) Control embryo and (B) operated embryo in ventral view of the sinus rhomboidalis, after in situ hybridization with the HNF-3β probe. Rostral and caudal limits of the MP and of the excision (Exc) are indicated (arrows). (C) Dorsal view of a non-hybridized operated embryo immediately following operation. (D) Dorsal and (E) ventral views of a non-hybridized operated embryo, 1 day after excision (E2.5). The notochord (No) is interrupted at the thoracic level. Posterior to this level, the neural tube (NT) developed in absence of No. (F-H) Apoptosis revealed by the TUNEL technique in a normal (E2.5) chick embryo (F) and in a chick embryo of the same age deprived of No and floor plate (FP) by excision of zone c (G,H). There is abundant cell apoptosis in the NT, the somites (S) and the somatopleural mesoderm (So), but not the splanchnopleure (Sp). Note that aorta (Ao) roots are present. (I) Progressive degeneration of caudal tissues by cell death results in (J) truncation of the embryos 3 days after the excision (E4.5). (F-H) Bar, 100 μm
the basal plate of the thoracolumbar spinal cord, (ii) in the medial part of bilaterally paired somites from somites 19-20 into the tail and (iii) in the most caudal region of the floor plate and in the CNH and (iv) in what remained of the primitive streak within the tail bud (Fig. 9). It was striking to see that cells of graft origin contributed to only a small proportion of the total number of midline cells in the very caudal region of the neural tube that develops posterior to the level of the node at operation time. Thus, *HNF-3β* cells of *zone c* are able to produce many more axial derivatives when challenged by removal of *zone b*.

**Zone b cannot substitute for zone c activity**

One possible cause of the arrest of caudal migration of the node in the absence of its caudal part might be the physical consequence of a hole in the blastoderm. In order to test this hypothesis, the caudal part of the node was removed and replaced by an equivalent volume of tissue taken from *zone a* or *zone b* from a stage-matched quail embryo (Fig. 10A-B). Thus a size-matched *Shh*, *HNF-3β* piece of tissue was substituted for a group of cells that were *Shh*, *HNF-3β* (Fig. 10C).

Chimeras examined at E2.5 (*n*=8) showed that the chick host notochord and floor plate were interrupted at the level of the forelimbs as expected (level of somites 20). In five embryos (see Fig. 10D), the grafted quail node material yielded a segment of midline cells. The floor plate was normally integrated in the host neural tube over a certain length (Fig. 10D,E). Respective rostral and caudal limits of the floor plate and notochord were displaced one from the other (Fig. 10D,F). More caudally, the neural tube developed in the absence of all midline cells (Fig. 10D,G). In the three other embryos, the midline cells formed a cord in which floor plate and notochord were joined together and separated from the host neural tube. In all cases, the most posterior region that was deprived of midline cells underwent extensive cell death (not shown). Thus, the heterotopic (*zone b* into *zone c*) graft showed a limited capacity of caudal extension and never gave rise to normal midline structures along the whole body.

We conclude therefore that, in HN, the capacity to extend to the caudalmost tail end of the embryo is restricted to *zone c*, which plays a crucial role in the mechanisms of laying down the axial structures.

**DISCUSSION**

We have analyzed the molecular, morphological and behavioral characteristics of HN at the 5- to 6-somite stage. The role of defined subregions of the node, during its rostral-to-caudal migration, was tested by examining the consequences of their selective removal or heterotopic replacement in quail-chick chimeras.

**Molecular and morphological heterogeneity of Hensen’s node**

At the molecular level (see Fig. 2), we have confirmed that the entire avian node uniformly expresses *HNF-3β* and that this expression is continuous in the node derivatives: floor plate, notochord and dorsal endoderm. In contrast, *CNOT2* transcripts are predominately, but not exclusively, present in the ventral part of HN, and *Shh* expression extends more posteriorly (but less than *HNF-3β*) in the ventral part than in the dorsal part, thus revealing both dorsoventral and a rostrocaudal molecular heterogeneity of the node at that stage. An important point of this study is that the node and the primitive streak are closely juxtaposed (see Fig. 3), as revealed by the discrete patterns of expression of *HNF-3β* and *Ch-Tbx6L* (respective markers of the node and of the primitive streak cells). The expression domain of *cSox2*, a gene switched on as the ectoderm becomes neuralized (Rex et al., 1997), was shown to coincide strikingly with the presumptive neural ectoderm in the sinus rhomboidalis that was previously defined by quail-chick transplantations (Catala et al., 1996, and Fig. 11).

Morphologically, we have also observed an anteroposterior heterogeneity in the node. One can distinguish three zones (Fig. 3 and refer to Fig. 11): *zone a*, which is located cranial to the median pit, where the three derivatives of the node (floor plate, notochord and dorsal endoderm) are clearly distinguishable and separated by forming basement membrane (see Duband and Thiery, 1982) but remain closely associated, *zone b*, which corresponds to the median pit, where the superficial cells are arranged in a columnar epithelium (the future floor plate) overlying a tissue that exhibits a loose cellular arrangement in continuity with the notochord while the endoderm is a separate cell layer underneath, and *zone c*, which is found caudal to the median pit, and covered by a sheet of neuralized (*cSox2*) ectoderm. In *zone c*, node cells are densely packed down to the endodermal layer and closely associated with TPS cells, which are more loosely distributed beneath the ectodermal layer. Apposition of *zone c* and TPS constitutes the APH.

**Role of the different regions of the node in midline development and caudal extension**

Replacement of the node region of a chick embryo by its quail counterpart results in the labeling of the floor plate, notochord and dorsal endoderm extending along the whole length of the embryo from the thoracic level to the tail (see Fig. 7 of Catala et al., 1996).

In the present work, we have excised the node material precisely at the level of the median pit corresponding to *zone b*. In this case, we observed that notochord and floor plate were lacking for several somites’ length in the thoracolumbar region.

Previously, several authors had performed HN ablations at earlier developmental stages. The excision was generally followed by regeneration of the node when the excision was carried out before extension of the head process at stage HH4. Regulation potential (i.e. the capacity to regenerate the node) may correspond to the persistence of cell invagination from the epiblast to the node. However, as soon as the head process is formed, the capacity to regenerate has completely disappeared (Abercombie and Waddington, 1937; Gallera and Nicolet, 1974; Yuan et al., 1995a,b; Psychoyos and Stern, 1996; Yuan and Schoenwolf, 1998, 1999). In agreement with the previous studies, we did not observe any regeneration when we performed complete excision of the node (*zone b + zone c*) as late as the 5- to 6-somite stage (HH8). At this stage, all the midline precursor cells are present within HN, and grafting of
quail cells in the perinodal area confirms that ingestion of epiblast cells within the node no longer occurs (Catala et al., 1996).

When the excision of HN is limited to zone b, the remaining HNF-3β+ Shh+ caudal region moves posteriorly by itself while the embryo elongates. The caudal node (zone c) gives rise to a segment of notochord and floor plate posterior to the excision. Interestingly, at the anterior limit of the disrupted midline cells, the notochord ends as a mass of cells that extends more caudal than the floor plate. In contrast, posterior to the interruption, floor plate cells positive for Shh and HNF-3β are regularly present over a length of about 2 to 5 somites more rostral than the notochord. This dissociation of the rostral and caudal limits of the floor plate and notochord indicates that these structures extend independently with respect to one another after they are laid down by HN.

The presence of a posterior notochord and floor plate after excision of zone b suggests that the caudal part of HN, which normally produces a limited number of midline cells (as seen in quail-chick transplant of zone c in type 4 experiments), is able to regulate to a certain extent for the lack of zone b material. This regulation may correspond to the recruitment of precursors or pluripotential cells from zone c. This hypothesis agrees with recent studies of node excision in late gastrulation stage mouse, in which persistence of ‘residual’ node, possibly corresponding to chick zone c, could account for restoration of midline structures (Davidson et al., 1999). In chicken embryos, as long as HNF-3β+ HN material remains in situ, even if reduced to a few cells, it is still able to move caudally. The more material excised, the wider the interruption in the midline structures. The complete and reproducible absence of midline cells in the lumbosacral and caudal neural tube is obtained by excision either of all node material (zone b+c), or of its most caudal part (zone c), associated in both cases with the TPS. Moreover, the fact that the rostral-to-caudal movement of the node is linked to a particular property of its posterior end is supported by type 5 experiments, in which zone c is replaced by an equivalent amount of zone b tissue. The caudalward movement of the node is then arrested, as it is after a simple excision. However, since zone c excision involves the TPS, it is impossible to distinguish whether the capacity of HN to move resides exclusively in its posterior region, or if it also implicates the TPS.

Relationship between axial and paraxial mesodermal progenitors: the axial-paraxial hinge

HNF-3β and Ch-Tbx6L territories are closely juxtaposed but do not overlap to form the APH. In certain vertebrates, such as amphibians, and also in certain mammals, a structure called the neurenteric canal establishes a link between the neural tube and the gut. At the tail bud stage, the neurenteric canal separates the tail-organizer where the axial mesoderm originates, from the prospective paraxial mesoderm (Gont et al., 1993). In the avian embryo, the neurenteric canal has never been described. We propose that it might correspond to APH.

As shown in Xenopus (Gont et al., 1993; De Robertis et al., 1994), zebrafish (Shih and Fraser, 1996; Kanki and Ho, 1997), chick (Catala et al., 1995, 1996; Knezevic et al., 1998) and mouse (Wilson and Beddington, 1996), the movements and role of this region in vertebrate embryos are similar during axial development. Notochord elongation and lateral divergence of mesoderm take place on each side of this interface, which disappears when extension of the AP axis is completed. Thus, the neurenteric canal in Xenopus (Gont et al., 1993), the APH in the chick embryo (our observations) and Kupffer’s vesicle (KV) in the zebrafish embryo (Kanki and Ho, 1997) seem to be equivalent structures.

Similarities between the forerunner cell population, which forms the roof of the KV, and the CNH of amphibia have already been underlined (Cooper and D’Amico, 1996) and KV appears as an evolutionary remnent of the neurenteric canal (D’Amico and Cooper, 1997). Zebrafish KV and chicken APH can also be directly compared since the former was shown to arise, at least in part, from the dorsal margin of the blastoderm, where the fish embryo organizer is located (Cooper and D’Amico, 1996). Moreover, it expresses transcripts specific to both axial and paraxial progenitors like floating head (flh; the zebrafish homologue of CNOT) and no tail (ntl, the zebrafish homologue of Brachyury) (Melby et al., 1996). KV contains cells with unrestricted axial/paraxial potential, since they later give rise to tail notochord and trunk muscles (Melby et al., 1996). The quail-chick transplantations of the APH (type 4 experiment) also gave rise to axial and paraxial derivatives.

The midline structures are not necessary for neural tube formation, but are essential for survival of the neural tube and surrounding tissues

Our experiments also demonstrate that the presence of the floor plate and the notochord is not required for the formation of a neural tube. However, the caudal part of the body, which is formed in the absence of midline structures, undergoes cell death, leading to the truncation of the embryo by E4. Before exhibiting signs of cell death, Pax-3 and Pax-6 are expressed throughout the neural tube except for the roof plate. This abnormal dorsoventral patterning is similar to that observed when midline cells are absent of the neural tube (Goulding et al., 1993; Chiang et al., 1996; see also Tanabe and Jessell, 1996, for references).

In addition, we observed that the caudal neural tube, which developed in the absence of the caudalward movement of HN (type 3 experiment), degenerated more rapidly than the neural tube secondarily deprived of its midline cells (type 1 experiment). After removal of zone c, the part of the body lying posterior to the excision is never in contact with the structures derived from the node that produce the SHH protein. Strikingly, less than 20 hours after zone c excision, the posterior part of the body (i.e. neural tube, paraxial mesoderm and somatopleura) is being eliminated by apoptosis.

We have shown previously that removal of notochord and floor plate at later stages of development results in apoptosis and complete destruction of the somitic derivatives, dermomyotome and sclerotome, which can be prevented by the graft of SHH-producing cells (Teillet et al., 1998b). Moreover, SHH has been implicated in neural tube survival (Miao et al., 1997; Dahmane and Ruiz-i-Altaba, 1999; Wechsler-Reya and Scott, 1999; Wallace, 1999). Experiments to test the hypothesis that the truncation of the embryos observed after ablation of the entire node or of the APH alone may result directly from the lack of SHH, or from an indirect effect via the paraxial mesoderm, are currently in progress.
Hensen’s node structure and function

In view of the present experimental results, we propose that at the 5- to 6-somite stage, corresponding to the mid-extension of the body axis, the different subregions of the node may present different states of cell commitment (Fig. 11). In the most rostral region, corresponding to zone a, floor plate cells, which are in contact with the two lateral primordia of the neural plate, and notochord cells to which they are closely associated, are committed to their respective fates. In zone b, cells have a high proliferation potential since they will give rise to all the midline cells from the brachial level down to the tail (Catala et al., 1996). Superficial cells, the future floor plate cells, are being inserted into the neural plate. Cells located more ventrally are continuous with the notochord. Our morphological and molecular data suggest that, within this region, cell commitment, if not complete, is in progress. In zone c, we hypothesize that the caudalmost HN cells may still be uncommitted and then could be subjected to differential signals leading them to either a notochord or a floor plate fate. Another possibility could be that different progenitors already coexist and interact in this area. When labeled at the 5- to 6-somite stage, only a few of these cells contribute to midline structures (experiment type 4). In contrast, when challenged by the extirpation of the pool...
of highly proliferative precursors of zone b, more zone c cells are recruited than normal and regulation of the midline deficiency takes place (see results of experiment type 1 and 2a). Thus, zone c cells could possess the self-renewal potentialities of stem cells.

In conclusion, zone c has the potential to compensate for zone b function in the midline construction, as well as to mediate caudalward extension of HN and assure the survival of the embryonic structures.
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REFERENCES


