De novo induction of the organizer and formation of the primitive streak in an experimental model of notochord reconstitution in avian embryos

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SUMMARY

We have developed a model system for analyzing reconstitution of the notochord using cultured blastoderm isolates lacking Hensen's node and the primitive streak. Despite lacking normal notochordal precursor cells, the notochord still forms in these isolates during the 36 hours in culture. Reconstitution of the notochord involves an inducer, which acts upon a responder, thereby inducing a reconstituted notochord. To better understand the mechanism of notochord reconstitution, we asked whether formation of the notochord in the model system was preceded by reconstitution of Hensen's node, the organizer of the avian neuraxis. Our results show not only that a functional organizer is reconstituted, but that this organizer is induced from the responder. First, fate mapping reveals that the responder forms a density, morphologically similar to Hensen's node, during the first 10-12 hours in culture, and that this density expresses typical markers of Hensen's node. Second, the density, when fate mapped or when labeled and transplanted in place of Hensen's node, forms typical derivatives of Hensen's node such as endoderm, notochord and the floor plate of the neural tube. Third, the density, when transplanted to an ectopic site, induces a secondary neuraxis, identical to that induced by Hensen's node. And fourth, the density acts as a suppressor of notochord reconstitution, as does Hensen's node, when transplanted to other blastoderm isolates. Our results also reveal that the medial edge of the isolate forms a reconstituted primitive streak, which gives rise to the normal derivatives of the definitive primitive streak along its rostrocaudal extent and which expresses typical streak markers. Finally, our results demonstrate that the notochordal inducer also induces the reconstituted Hensen's node and, therefore, acts like a Nieuwkoop Center. These findings increase our understanding of the mechanism of notochord reconstitution, provide new information and a novel model system for studying the induction of the organizer and reveal the potential of the epiblast to regulate its cell fate and patterns of gene expression during late gastrula/early neurula stage in higher vertebrates.

Key words: Chick embryo, Floor plate, Gastrulation, Hensen's node, In situ hybridization, Mesoderm, Neurulation, Nieuwkoop Center, Regeneration, Regulation

INTRODUCTION

The embryonic axis is progressively formed during vertebrate development through a series of inductive and suppressive cell-cell interactions and cellular movements. A striking example of embryonic induction was discovered more than 70 years ago by Spemann and Mangold (1924), who demonstrated that transplantation of the dorsal lip of the blastopore to the ventral region of the amphibian embryo resulted in the formation of a new embryonic axis containing an induced neural tube. Because the transplanted lip recruited host cells and organized them into a secondary embryonic axis, it was called the organizer. Organizer activity has since been found in other vertebrates, where the equivalent structure is called the node (murine embryos: Beddington, 1994), Hensen's node (avian embryos: Waddington, 1932; Waddington and Schmidt, 1933; Gallera, 1971; Hara, 1978; Dias and Schoenwolf, 1990; Storey et al., 1992, 1995) and the embryonic shield (teleost embryos: Oppenheimer, 1959; Ho, 1992).

The cellular and molecular nature of organizer activity has long attracted the attention of developmental biologists. In the avian embryo, injection of cell markers and homotopic and isochronic grafting studies have provided detailed fate maps of Hensen's node, which show that several midline structures (notochord, floor plate and roof of gut) derive from it (Spratt, 1955; Rosenquist, 1966, 1983; Nicolet, 1971; Hara, 1978; Selleck and Stern, 1991, 1992; Schoenwolf et al., 1992; Garcia-Martinez et al., 1993; Psychoyos and Stern, 1996a). Moreover, heterotopic grafting studies (Gallera, 1974; Garcia-Martinez and Schoenwolf, 1992; Storey et al., 1992; Inagaki and Schoenwolf, 1993) have shown that prospective notochordal cells of Hensen's node are committed to a notochordal fate by the late gastrula/early neurula stage. Finally, several different transcription factors and secreted signaling molecules are expressed in Hensen's node and its

derivatives. The localized expression of such genes can be used to identify the organizer, although the role of these genes in organizer specification and function are largely unknown.

Surprisingly in view of its importance in formation and patterning of the neuraxis, ablation of Hensen's node at the late gastrula/early neurula stage, the stage at which prospective notochordal cells are already committed to a notochordal fate, often results in normal embryos containing a notochord (Waddington, 1932; Waterman, 1936; Grabowski, 1956; Butros, 1967; Gallera and Nicolet, 1974; Smith and Schoenwolf, 1989; Darnell et al., 1992; Schoenwolf and Yuan, 1995; Yuan et al., 1995a, b; Psychoyos and Stern, 1996b). Furthermore, using cultured whole embryos, Psychoyos and Stern (1996b) reported that removal of Hensen's node and the cranial 40% of the primitive streak leads to the formation of a new functional organizer. Collectively, the above experiments in combination with others using heterotopic grafting (Alvarez and Schoenwolf, 1991; Schoenwolf and Alvarez, 1991; Garcia-Martinez and Schoenwolf, 1992; Storey et al., 1992; Garcia-Martinez et al., 1997) have revealed that the avian blastoderm has considerable ability to regulate at the late gastrula/early neurula stage, and that this ability to regulate is controlled by cell-cell interactions.

Utilizing blastoderm isolates, we have investigated the cell-cell interactions that regulate reconstitution of the notochord and have shown that notochord reconstitution after removal of notochordal precursor cells requires an inducer, which acts upon a localized region of epiblast called the responder; in the presence of Hensen's node, inducer/responder activity is suppressed, preventing formation of supernumerary notochords (Yuan et al., 1995a, b). In the present study, we continue by asking whether a functional organizer is reconstituted in blastoderm isolates and if so, what is its tissue of origin and does it self-differentiate, or does it require induction (and if so, from what tissue does the inducer originate)? Our results demonstrate that an organizer is reconstituted in this model system and that it derives from the same responder required for notochord reconstitution. Moreover, our results show that reconstitution of the organizer requires an inducer – the same inducer required for reconstitution of the notochord. Thus this inducer is functionally equivalent to the Nieuwkoop Center of amphibian embryos, which is required for formation of the dorsal lip of the blastopore, that is, Spemann's organizer.

MATERIALS AND METHODS

Whole embryo culture

Fertile White Leghorn chicken eggs were incubated until embryos reached stages 3d-4 (Hamburger and Hamilton, 1951, with stage 3 substages as described by Schoenwolf et al., 1992). Culture dishes and embryos were prepared as previously described (Yuan et al., 1995a) for modified New (1955) culture.
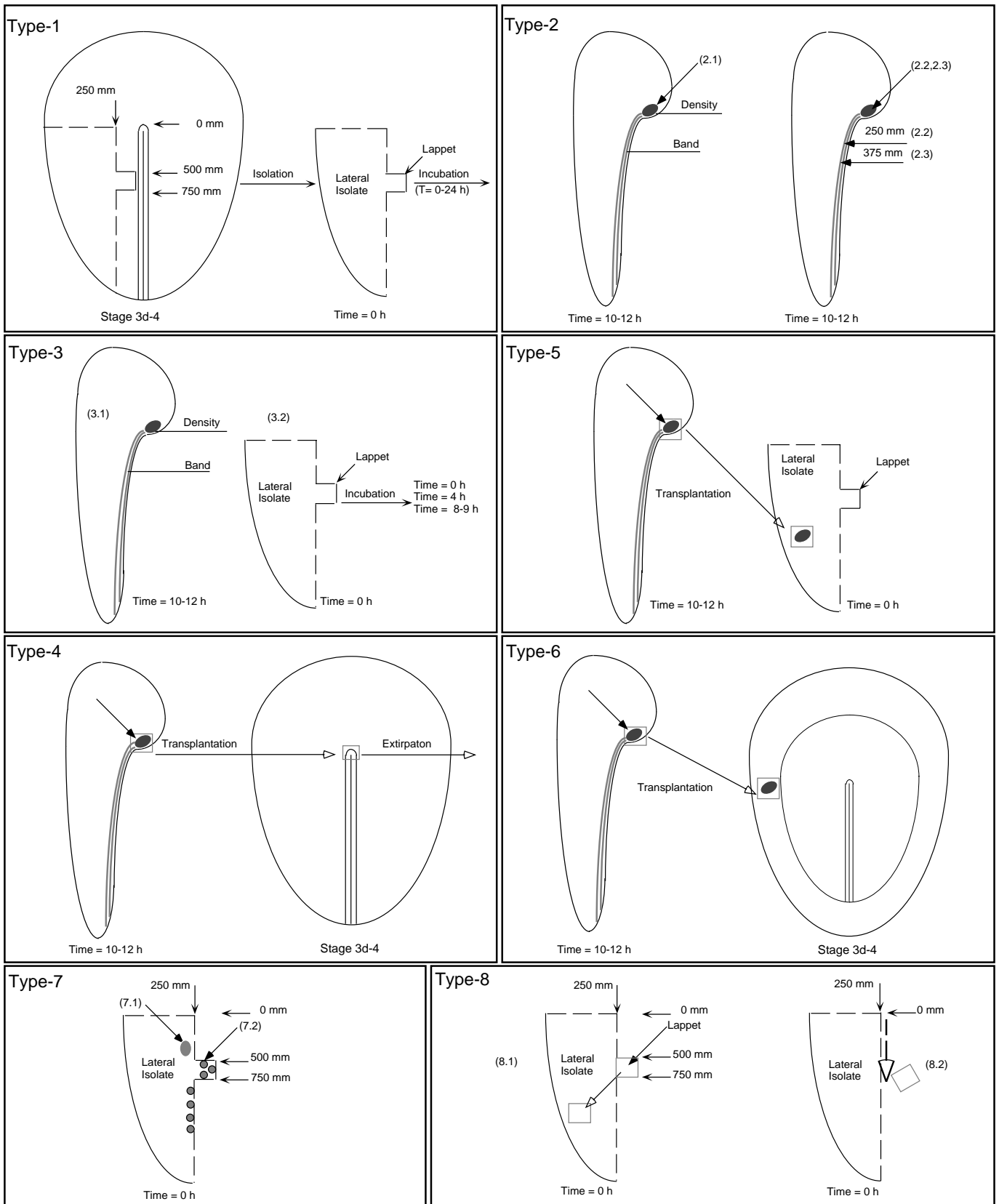
Experimental model and design

As an experimental model, so-called lateral isolates were prepared from blastoderms cultured on vitelline membranes (Figs 1A, 2A; Yuan et al., 1995b). Isolates lacked Hensen's node and primitive streak and they contained all three layers of the blastoderm. Each isolate consisted of a right or left laterocaudal piece containing a lappet, which is the area of the epiblast containing the strongest ability to

Fig. 1. Scheme showing the eight types of experiments conducted. Right and left lateral isolates from chick blastoderms at stages 3d-4 were used as the experimental model. The isolates consisted of all three layers of the blastoderm and contained both the inducer (i.e., lappet) and responder (see Fig. 2A) identified earlier (Yuan et al., 1995 b). In type-1 experiments, lateral isolates were cultured for selected periods between 0-24 hours and then collected for morphological study/Not-1 labeling, or were viewed continuously with time-lapse video microscopy. In type-2 experiments, lateral isolates were cultured for 10-12 hours until a rostromedial density and a medial band formed. In type-2.1 experiments, the rostromedial density was fate mapped by injecting CRSE and culturing isolates for an additional 24 hours. In type-2.2 and 2.3 experiments, the density was injected with CRSE as for type-2.1 experiments and the medial band was also fate mapped (by injection of CFSE to provide a second fluorescent color) at the 250 μm (type-2.2 experiments) or 375 μm (type-2.3 experiments) level. In type-3 experiments, lateral isolates were cultured for 10-12 hours (type-3.1 experiments) or for 0, 4 or 8 hours (type-3.2 experiments) and processed for in situ hybridization with one of several riboprobes. In type-4 experiments, we asked whether the density was capable of substituting for Hensen's node. Lateral isolates were cultured for 10-12 hours until a rostromedial density and medial band formed. The density was then labeled by injecting it with CRSE, as in type-2.1 experiments, and grafted in place of Hensen's node in cultured chick blastoderms at stages 3d-4, which were then cultured for an additional 24 hours. Type-5 experiments were identical to type-4 experiments except that the labeled density was grafted to another lateral isolate to test its ability to prevent notochord reconstitution in the lateral isolate. Type-6 experiments were identical to type-4 and -5 experiments, except that the labeled density was grafted to the area opaca in cultured intact chick blastoderms at stages 3d-4 to test its ability to induce and organize an ectopic neuraxis. In type-7 experiments, the responder region (type-7.1 experiments), the perimeter of the inducer lappet (type-7.2 experiments) or the caudomedial edge of a lateral isolate was fate mapped by injecting CFSE and culturing isolates for 10-12 hours. In type-8 experiments, lateral isolates lacking the inducer lappet were prepared. In type-8.1 experiments, a CRSE-labeled inducer lappet was grafted into the lateral isolate, which was then cultured for 10-12 hours. In type-8.2 experiments, the lateral isolate was cultured for 10-12 hours without further manipulation.

induce notochord reconstitution, and each isolate contained a localized region of epiblast, called the responder, which forms the notochord and floor plate during notochord reconstitution. More specifically, in the intact embryo, Hensen's node (defined operationally as the rostral end of the primitive streak, extending transversely the width of the streak and longitudinally 125 μm) suppresses the activity of the inducer and responder, but its immediate derivative (i.e., the head process) lacks suppressive activity. The lappet, containing the strongest ability to induce notochord (and, therefore, is the area called the inducer), extends lateral to the primitive streak for 250 μm and is located from 500-750 μm caudal to the rostral end of Hensen's node. Finally, the localized area of epiblast 250-500 μm lateral to the primitive streak and 250-500 μm caudal to the rostral end of Hensen's node is the chief contributor of cells to the notochord and floor plate during reconstitution (and, therefore, is the area called the responder). Usually, each blastoderm yielded two isolates, a right and left, which were both cultured on the same vitelline membrane. In cases where subsequent manipulations were performed (see below), one of the two isolates was left undisturbed and served as a control. In the present study, we used lateral isolates for 8 types of experiments (Fig. 1).

After operations were completed, cultures were placed into humidified chambers in incubators at 38°C for 4-36 hours depending on the experiment. Embryos from most experiments were examined

Type-1

250 mm

0 mm

500 mm

750 mm

Isolation

Lateral Isolate

Lappet

Incubation (T= 0-24 h)

Stage 3d-4

Time = 0 h

Type-2

(2.1)

Density

Band

(2.2,2.3)

250 mm (2.2)

375 mm (2.3)

Time = 10-12 h

Time = 10-12 h

Type-3

(3.1)

Density

Band

(3.2)

Lateral Isolate

Lappet

Incubation

Time = 0 h
Time = 4 h
Time = 8-9 h

Time = 10-12 h

Time = 0 h

Type-5

Transplantation

Lateral Isolate

Lappet

Time = 10-12 h

Time = 0 h

Type-4

Transplantation

Extirpaton

Time = 10-12 h

Stage 3d-4

Type-6

Transplantation

Time = 10-12 h

Stage 3d-4

Type-7

250 mm

(7.1)

0 mm

(7.2)

Lateral Isolate

500 mm

750 mm

Time = 0 h

Type-8

250 mm

0 mm

Lappet

(8.1)

Lateral Isolate

500 mm

750 mm

250 mm

0 mm

Lateral Isolate

(8.2)

Time = 0 h

Time = 0 h

at 6 to 12 hours intervals, and were re-detached from and recentered on the vitelline membranes as necessary. In cultures that contained both manipulated isolates and control isolates, both isolates were discarded when the control isolate failed to form recognizable structures.

For virtually all experiments, a video recording was made of both

donor and host embryos (and some were also photographed) at the time of the operation to provide permanent documentation of each experimental case. A few embryos were subjected to time-lapse video microscopy after surgery, using a system described previously (Smith and Schoenwolf, 1989). Most were processed for histology using standard techniques.

Following cells in blastoderm isolates

In some experiments, grafting was used to follow the fates and distribution of labeled cells. Donor tissues obtained from chick embryos were labeled with 5-carboxytetramethylrhodamine, succinimidyl ester (CRSE, Catalog no. C-2211; Molecular Probes, Inc., Eugene, OR) or 5-[and-6]carboxyfluorescein diacetate, succinimidyl ester (CFSE, Catalog no. C-1157; Molecular Probes, Inc.) and transplanted to unlabeled chick embryos, which served as hosts. Dyes were injected using full-strength solutions (10 mg in 1 ml DMSO) with the aid of a Picrospritzer II (General Valve, Corp., Fairfield, NJ) and a micropipette (tip diameter, about 10 µm for the localized injections) mounted on a hydraulic micromanipulator. For other experiments not involving grafting, regions of isolates were labeled by microinjecting a small bolus of CRSE or CFSE. In a subset of these latter experiments, embryos were double-labeled with separate injections of CRSE and CFSE.

CRSE- or CFSE-labeled cells can be distinguished from host cells by using epifluorescence microscopy or anti-rhodamine or anti-fluorescein primary antibodies followed by peroxidase immunocytochemistry (see below). Both groups of labeled cells could be readily detected and discriminated from one another (at time 0 and at any selected interval during the course of the experiment up to the time cultures were terminated) using an appropriate filter set.

Immunocytochemistry

Indirect, whole-embryo immunocytochemistry was done as described previously (Patel et al., 1989) with two modifications: embryos were fixed with 4% paraformaldehyde in PBS; and in many cases, the peroxidase reaction product was intensified by adding 25 µl 2% $CoCl_2$ and 20 µl 2% $Ni(NH_4)_2(SO_4)$ per ml of DAB-PBT. For immunocytochemical detection of two labels, we used two primary antibodies and a single secondary antibody. To do this, the entire procedure was run twice, once for each primary antibody. After the first run with the first primary antibody, the peroxidase reaction product was intensified, yielding a black reaction product, but in the second run with the second primary antibody, intensification was omitted, yielding a brown reaction product.

Six primary antibodies were used in this study: (1) Not-1, a monoclonal antibody that recognizes a notochord epitope in the chick and quail (Yamada et al., 1991); (2) 4D9 (Patel et al., 1989), a monoclonal antibody that recognizes the En-2 protein in the chick and quail in the nuclei of the roof and lateral walls of the neural tube at the caudal mesencephalon and rostral metencephalon levels (Darnell et al., 1992); (3) anti-rhodamine (Molecular Probes, Inc., Catalog no. A-6397); and (4) anti-fluorescein (Molecular Probes, Inc., Catalog no. A-889; Garton and Schoenwolf, 1996), antibodies that bind, respectively, to the rhodamine groups of CRSE and the fluorescein groups of CFSE, thereby marking the cytoplasm of labeled cells; (5) L5, a monoclonal against the L5 carbohydrate epitope, which recognizes several cell-surface glycoproteins in neural tissue from early chick embryos and can be used as a neuroepithelium-specific marker during early stages of chick development (Streit et al., 1990; Roberts et al., 1991; Storey et al., 1995); (6) anti-HNF-3β, a monoclonal antibody that recognizes the HNF-3β protein in the chick and quail in the nuclei of the floor plate of the neural tube (Ericson et al., 1996).

Whole-mount in situ hybridization

In situ hybridization was performed essentially as described by Wilkinson (1992), with two modifications: (1) proteinase K was

eliminated; and (2) BM Purple (Boehringer Mannheim, Indianapolis, IN) replaced NBT/BCIP in the final reaction solution. After in situ hybridization, embryos were dehydrated using isopropanol and processed for paraffin embedment; serial transverse sections were cut at 15 µm. In some experiments, embryos were labeled immunocytochemically as described above after in situ hybridization.

Eleven riboprobes were used as specific markers: *paraxis* (Garcia-Martinez et al., 1997); *gsc* (*goosecoid*; Izpisúa-Belmonte et al., 1993); *c-Otx2* (Bally-Cuif et al., 1995); *cNot-1* (Stein and Kessel, 1995); *Shh* (*Sonic hedgehog*; Riddle et al., 1993; Roelink et al., 1994); *HNF-3β* (Ruiz i Altaba et al., 1995); *cNR-1* (Levin et al., 1995); *Brachyury* (Kispert et al., 1995); *Hoxb-1* (Maden et al., 1991); *fgf8* (Crossley and Martin, 1996); and *ch-TBX6L* (Knezevic et al., 1997).

RESULTS

In previous studies, we developed an experimental model (Figs 1, 2A; Yuan et al., 1995b) for studying notochord reconstitution using lateral blastoderm isolates lacking the suppressor (i.e., Hensen's node) but containing both the inducer lappet (i.e., a flap located 0-250 µm lateral to the primitive streak and 500-750 µm caudal to the rostral end of Hensen's node) and the responder (i.e., the localized area of epiblast 250-500 µm lateral to the primitive streak and 250-500 µm caudal to the rostral end of Hensen's node). In the present study, we use these isolates to demonstrate that notochord reconstitution is preceded by the induction of a organizer, and that the medial edge of the isolate forms a reconstituted primitive streak. All experiments are diagrammed in Figure 1 (details of each experiment are given in the legend); major results are summarized in Table 1.

Type-1 experiments: formation of the rostromedial density and medial band

Previous studies using lateral blastoderm isolates have shown that a notochord is reconstituted within 36 hours in culture (Yuan et al., 1995b). To gain further insight into the process of notochord reconstitution we examined in type-1 experiments the time course of reconstitution in lateral blastoderm isolates (Fig. 1, type-1 experiments; Fig. 2A). Some embryos were examined continuously with time-lapse videomicroscopy and others were collected at selected intervals during the 24 hours in culture and examined with immunocytochemical labeling with Not-1 antibody. Not-1 expression was not detectable during the first 20 hours in culture but by 24 hours, light Not-1 expression was present along the medial side of the isolate (Fig. 2B). Despite the lack of Not-1 staining prior to 24 hours, extensive tissue movements, indicative of gastrulation, were detected with time-lapse video microscopy. By 10-12 hours in culture, a density formed near the rostromedial edge of each isolate (Fig. 2C). During subsequent culture, the density exhibited regression movements along the medial edge of the isolate, and a longitudinal rod of tissues was laid down in its wake.

Sections of isolates examined after 10-12 hours in culture confirmed the presence of a rostromedial density and showed it consisted of a mass of cells continuous with one another dorsoventrally, without the presence of distinct cell layers, and marked dorsally by a funnel-shaped depression (Fig. 2D). Rostral to the density distinct cell layers were present (Fig. 2E), but more caudally the density was continuous with a medial

**Table 1. Summary of the eight types of experiments conducted**

Types of experiments	Markers used	Total number of cases	Structures labeled with markers used ()[1]
1	Not-1 antibody	4[2], 6[3], 10[4]	notochord (0, 0, 10, respectively)
2.1	Not-1; anti-HNF-3β[5,6]	8[7]	notochord; floor plate of neural tube (8; 8, respectively)
	CRSE/anti-rhodamine[8]	6[7]	notochord and floor plate of neural tube (6)
2.2	CRSE[8]; CFSE[9]; *paraxis*; Not-1[10]	10[7]	notochord; somites; somites; notochord (10; 10; 10; 10, respectively)
	CRSE/anti-rhodamine[8]; CFSE/anti-fluorescein[9]	6[7]	notochord and floor plate of neural tube; principally paraxial mesoderm including somites (6; 6, respectively)
2.3	CRSE/anti-rhodamine[8]; CRSE/anti-fluorescein[11]	3[7]	notochord and floor plate of neural tube; principally lateral plate mesoderm (3; 3, respectively)
3.1[12]	*gsc*	12[2]	rostromedial density (12)
	cOtx-2	9[2]	rostromedial density (9)
	cNot-1	6[2]	rostromedial density (6)
	Shh	3[2]	rostromedial density (3)
	HNF-3β	24[2]	rostromedial density (24)
	cNR-1	6[2]	none
	cNR-1	6[3]	area adjacent to rostromedial density and lateral plate mesoderm (6)
	brachyury	6[2]	rostromedial density and medial band (6)
	Hoxb-1	6[2]	medial band (6)
	fgf8	4[2]	medial band (4)
	ch-TBX6L	8[2]	medial band (8)
3.2[12]	*gsc*	6[13]	none
	HNF-3β	12[13]	none
	HNF-3β	6[14]	none
	HNF-3β	7[15]	rostromedial density (7)
	brachyury	11[13]	medial edge of inducer lappet and caudomedial edge of isolate (11)
4	CRSE/anti-rhodamine[8]	9[4]	midline endoderm, notochord and floor plate of neural tube (9)
	CRSE/anti-rhodamine[8]; anti-HNF-3β[5]	10[4]	midline endoderm, notochord and floor plate of neural tube; floor plate of neural tube (10; 10, respectively)
5	CRSE/anti-rhodamine[8]; Not-1[5]	6[7]	notochord and adjacent mesoderm and endoderm (?); notochord (8)[16]
6	CRSE/anti-rhodamine[8]; 4D9[5]	3[7]	endoderm, mesenchyme and notochord (3); midbrain-hindbrain junction (3)[17]
	CRSE/anti-rhodamine[8]; *c-Otx2*[10]	47	endoderm, mesenchyme and notochord (4); forebrain-midbrain (4)[17]
	CRSE/anti-rhodamine[8]; *fgf8*[10]	3[7]	endoderm, mesenchyme and notochord (3); mainly forebrain and hindbrain (3)[17]
	CRSE/anti-rhodamine[8]; L5[5]	3[7]	endoderm, mesenchyme and notochord (3); neural tube (3)[17]
7.1	CFSE/anti-fluorescein[18]	8[2]	rostromedial density and rostrolateral mesoderm, endoderm (8)
7.2	CFSE/anti-fluorescein[19]	3[2]	medial band and lateral mesoderm, endoderm (3)
8.1	CRSE/anti-rhodamine[20]; *HNF-3β*[10]	6[3]	rostrolateral mesoderm, endoderm (6); rostromedial density (6)
8.2	*HNF-3β*	8[2]	none
	brachyury	4[2]	medial band (4)

[1]Number of labeled cases.
[2]10-12 hours of culture.
[3]20 hours of culture.
[4]24 hours of culture.
[5]Double-labeled: immunocytochemistry.
[6]The rostromedial density was not injected with CRSE.
[7]36 hours of culture.
[8]Rostromedial density injected with CRSE.
[9]Medial band injected at the 250 μm level with CFSE.
[10]Double-labeled: in situ hybridization followed by immunocytochemistry.
[11]Medial band injected at the 375 μm level with CFSE.
[12]In situ hybridization.
[13]0 hours of culture.
[14]4 hours of culture.
[15]8-9 hours of culture.
[16]The notochord derived from the graft was labeled with anti-rhodamine, which masked possible labeling with Not-1; in 2 cases the host also formed a partial notochord, which labeled with Not-1 only.
[17]Labeled both host and ectopic neuraxes.
[18]Responder injected with CFSE.
[19]Perimeter of inducer lappet and caudomedial edge injected with CFSE.
[20]Inducer lappet injected with CRSE.

band that extended along the entire length of the medial edge of the isolate and like the density, was not segregated into separate cell layers (Fig. 2F). Based on these morphological features, we conclude that structurally the rostromedial density resembled Hensen's node (with its primitive pit) and the medial band resembled the remainder of the primitive streak. To

Fig. 2. In type-1 experiments, the time course of notochord reconstitution was determined revealing the formation of the rostromedial density and medial band. (A) The lateral isolate at time 0 showing the locations of the responder (r) and inducer (i; lappet). (B) The lateral isolate labeled with Not-1 after 24 hours in culture. (C) The lateral isolate after 12 hours in culture. A rostromedial density (red arrow; unstained but darker appearing in transillumination because of greater cell density) and a medial band (arrowheads) formed. Dashed lines indicate planes of sections shown in D-E. (D) Transverse section through the rostromedial density (middle dashed line in C). Note the continuity between layers at the medial edge of the isolate, the funnel-shaped depression (fsd) within this region of continuity and the presence of densely packed cells within the area of continuity; lateral to the area of continuity, three distinct layers were present: ectoderm (ect), mesoderm (mes) and endoderm (e). (E) Transverse section rostral to the density (top dashed line in C). Note the presence of three distinct layers: ectoderm (ect), mesoderm (mes) and endoderm (e). (F) Transverse section through the medial band caudal to the density (bottom dashed line in C). Note the similarity in structure of the medial band to that of the density; lateral to the medial band, three distinct layers were present: ectoderm (ect), mesoderm (mes) and endoderm (e). In type-2 experiments, the rostromedial density and medial band were fate mapped. As a control for type-2.1 experiments, lateral isolates were double labeled after 36 hours in culture with Not-1 without intensification and with anti-HNF-3β with intensification. This revealed that the notochord (red arrow) and floor plate of the neural tube (blue arrow) formed at the medial edge of the isolate (G,H). e, endoderm; ee, epidermal ectoderm; ne, neuroepithelium; som, somite. In type-2.1 experiments, the rostromedial density was injected with CRSE; isolates were then cultured for an additional 24 hours and immunolabeled with anti-rhodamine antibody. (I) Transverse section through the trunk showing that labeled cells from the density were localized in the notochord and floor plate of the 'half' neural tube. e, endoderm; ee, epidermal ectoderm; fp, floor plate; ne, neuroepithelium; nc, notochord; som, somite. (J) In type-2.2 experiments, the rostromedial density was injected with CRSE, and the medial band at the 250 µm level was injected with CFSE. After an additional 24 hours in culture, the cells labeled with CRSE contributed to the notochord/floor plate of the 'half' neural tube (orange color; arrowhead), whereas the cells labeled with CFSE contributed mainly to rostral somites and much less so to the lateral plate mesoderm (green color, arrow). (K) Type-2.3 experiments were identical to type-2.2 experiments except that the medial band at the 375 µm level was injected. After an additional 24 hours in culture, the cells labeled with CRSE again formed notochord/floor plate of the 'half' neural tube (orange color; arrowhead), whereas cells labeled with CFSE contributed mainly to lateral plate mesoderm and much less so to the caudal somites (green color; arrow). (L) As a control for type-2.2 and 2.3 experiments, isolates were double labeled with *paraxis* and Not-1 after a total of 36 hours in culture. This revealed that the notochord (arrowhead) and segmented somites (arrow) formed near the medial edge of the isolate. Bars, 200 µm (A-C,G,J,K,L); 100 µm (L); 50 µm (D-F,H,I).
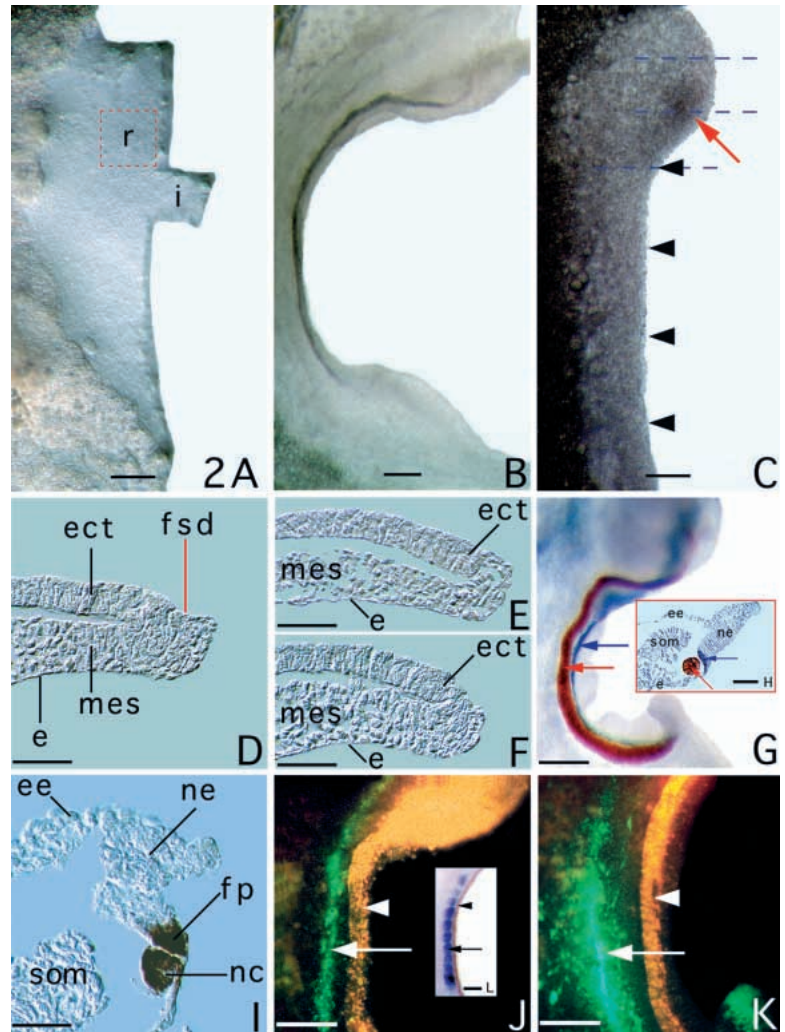
determine whether this resemblance was coincidental or meaningful, type-2 through type-6 experiments were conducted.

Type-2 experiments: fates of the rostromedial density and medial band

In type-2 experiments, we asked whether the fates of the cells within the rostromedial density and medial band were consistent with these structures being, respectively, reconstituted Hensen's node and reconstituted primitive streak. All experiments were done on isolates cultured for 10-12 hours. As a control for type-2.1 experiments, we double-labeled lateral isolates immunocytochemically with Not-1 and anti-HNF-3β after a total of 36 hours in culture. This

confirmed our previous morphological evidence (Yuan et al., 1995b) that the isolate formed notochord and overlying floor plate of the neural tube at its medial edge (Fig. 2G,H). In type-2.1 experiments, we labeled the density with a localized injection of CRSE. Labeled cells were detected 24 hours later in the endoderm (not shown), notochord and overlying floor plate of the 'half' neural tube (Fig. 2I). In type-2.2 and 2.3 experiments, we labeled the rostromedial density with a localized injection of CRSE and the medial band with a localized injection of CFSE at one of two levels: 250 µm caudal to the density, or 375 µm caudal to the density. Twenty-four hours later, CRSE-labeled cells contributed to medial structures (Fig. 2J,K), which were confirmed histologically after double-immunocytochemistry to be
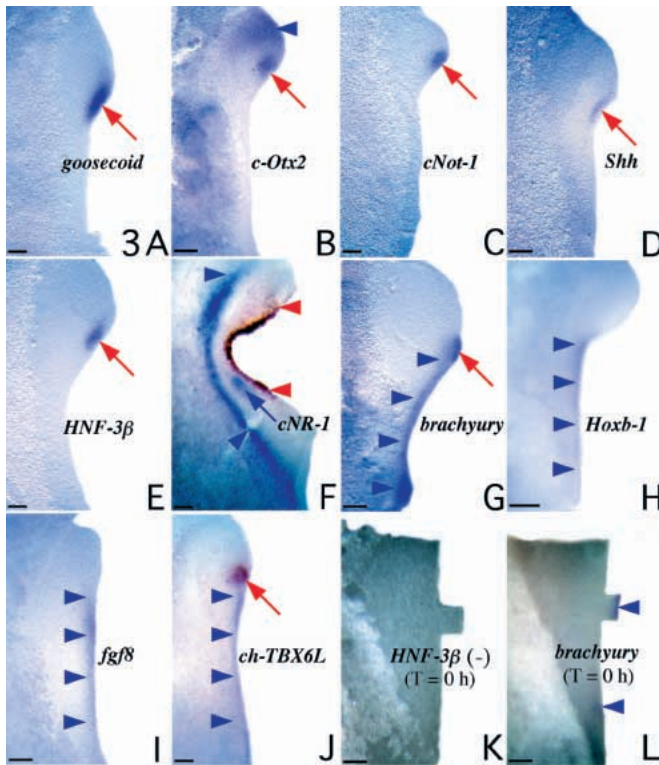
Fig. 3. In type-3.1 experiments, the patterns of the gene expression in the rostromedial density and medial band of isolates cultured for 10-12 hours were examined with in situ hybridization (A-E). All views are of the ventral side of the isolates. Markers specific for Hensen's node were expressed by the rostromedial density (arrows: *gsc*, A; *c-Otx2*, B; *cNot-1*, C; *Shh*, D; *HNF-3β*, E; arrowhead in B indicates rostral neural plate also expressed *c-Otx2*). *cNR-1*, however, was not expressed in isolates at 10-12 hours in culture, so an additional group of isolates were cultured for 10-12 hours until the rostromedial density formed. Then the density was labeled with CRSE, isolates were cultured for an additional 8-10 hours and embryos were processed for in situ hybridization using the *cNR-1* riboprobe and finally for immunocytochemistry with anti-rhodamine antibody (F). *cNR-1* was expressed in two patches: just lateral to the density (blue arrow) and within the lateral plate mesoderm (blue arrowheads). Anti-rhodamine labeling revealed that the rostromedial density formed notochord/floor plate of the 'half' neural tube and regressed caudally as it did so (red arrowheads). Markers specific for the primitive streak were also examined in type-3.1 experiments in isolates cultured for 10-12 h. Such markers were expressed within the medial band (arrowheads: *brachyury*, G; *Hoxb-1*, H; *fgf8*, I; *ch-TBX6L*, J). *Brachyury* was also expressed by the rostromedial density (arrow, G), but the other three markers of primitive streak were expressed caudal to the density (H-J; arrow in J, rostromedial density marked prior to in situ hybridization with CRSE and after in situ hybridization with anti-rhodamine antibody). In type-3.2 experiments, the expression of two markers of Hensen's node and one marker of primitive streak were examined at earlier times. Markers of Hensen's node were not expressed at time 0 (K, *HNF-3β* but *brachyury* was weakly expressed (L, arrowheads). Bars, 200 μm.

endoderm, notochord and floor plate of the 'half' neural tube (not shown), whereas CFSE-labeled cells contributed to more lateral structures, with those from type-2.3 injections (Fig. 2K) being more laterally displaced than those from type-2.2 injections (Fig. 2J); confirmed histologically (not shown) to
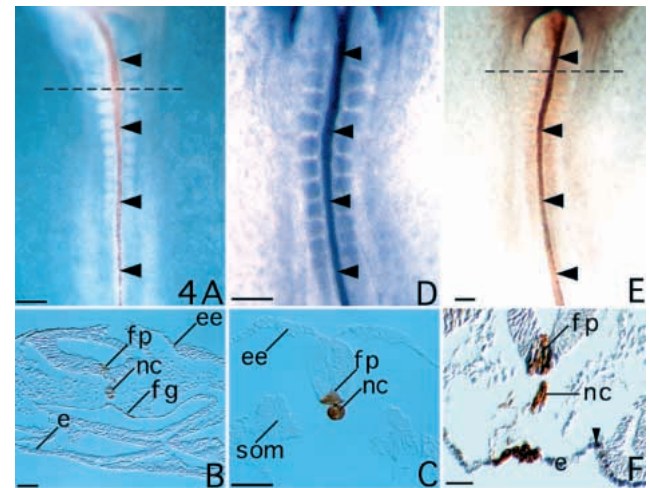

Fig. 4. In type-4 experiments, Hensen's node from chick embryos at stages 3d-4 was replaced with a labeled rostromedial density, and the grafted embryos were cultured for 24 hours. (A) The rostromedial density contributed cells to the midline of the embryo. (B,C) Transverse sections at two levels (B, level more rostral to that shown at the top of the photograph in A; C, level indicated by the dashed line in A) revealed that the rostromedial density contributed cells to the floor plate of the neural tube (fp), notochord (nc) and endoderm of the midline roof of the foregut (fg). e, endoderm; ee, epidermal ectoderm; som, somite. Some grafted embryos were double labeled, first with anti-HNF-3β without intensification (D) and then with anti-rhodamine with intensification (E). (F) Transverse sections (dashed line in E) revealed cells labeled with anti-rhodamine (brown cytoplasmic labeling) in the floor plate of the neural tube (fp), notochord (nc) and midline endoderm (e). Double labeled cells were present in the floor plate of the neural tube and in the midline endoderm (note especially cells in the floor plate with brown cytoplasm and blue nuclei; considerable overlap occurs in labeling in the plane of the section). Arrowheads indicates a cell nucleus in the endoderm labeled with HNF-3β only. Bars, 200 μm (A, D); 100 μm (E); 50 μm (B, C, F).

be principally paraxial mesoderm including well formed somites (type-2.2 injections) and lateral plate mesoderm (type-2.3 injections). As a control for these experiments, we double-labeled lateral isolates after a total of 36 hours in culture, first by in situ hybridization with *paraxis* (a somitic marker) and subsequently by immunocytochemistry with Not-1. This confirmed our previous morphological evidence (Yuan et al., 1995b) that notochord formed at the medial edge of the isolate and that somites formed just lateral to the notochord (Fig. 2L). Our current results compare favorably with fate maps of normal avian embryos, which show that at stages 3d-4 Hensen's node contributes chiefly to the midline endoderm, notochord and floor plate of the neural tube and that the mesoderm within the primitive streak has a rostrocaudal order that translates into mediolateral position within the ingressed mesoderm, namely, that more rostral prospective mesoderm within the primitive streak ingresses to a more medial position than does more caudal streak mesoderm, which ingresses more laterally (Selleck and Stern, 1991; Schoenwolf et al., 1992; Garcia-Martinez et al., 1993). Thus fate mapping studies strengthen the suggestion obtained from morphology that the rostrocaudal density is a

reconstituted Hensen's node and the medial band is a reconstituted primitive streak.

Type-3 experiments: patterns of gene expression during reconstitution

In type-3 experiments, we used whole-mount in situ hybridization to ask whether the rostromedial density and the medial band express markers that are specific for, respectively, Hensen's node and primitive streak. Six markers of Hensen's node were used: *gsc, c-Otx2, cNot-1, Shh, HNF-3β* and *cNR-1*. In type-3.1 experiments, we used cultured isolates of 10-12 hours old; that is, at the time the rostromedial density became detectable morphologically. All of the above markers of Hensen's node were expressed by the density at this time (Fig. 3A-E), with the exception of *cNR-1* (not shown). In additional lateral isolates cultured for 10-12 h, we injected the rostromedial density with CRSE to track the movement of its cells, cultured these labeled isolates for an additional 8-10 h, processed them for in situ hybridization using the *cNR-1* riboprobe and then processed them for immunocytochemistry using anti-rhodamine antibody. This experiment revealed that (1) the rostromedial density underwent regression during this additional period in culture, leaving behind cells along the medial edge of the isolate, and (2) *cNR-1* was expressed in two patches in relation to the regressing density and the lateral plate mesoderm (Fig. 3F). Both right and left isolates were routinely used for all type-3 (and type-1, 2 and 4-8 as well) experiments; no side-related differences in gene expression were noted, even for *cNR-1*, which was expressed in the right isolate (Fig. 3F) as well as in the left (not shown).

Four markers of primitive streak, *brachyury* (also a node marker), *Hoxb-1, fgf8* and *ch-TBX6L*, were also used in type-3.1 experiments on isolates cultured for 10-12 hours; that is, at the time the medial band became detectable morphologically. All of these markers of primitive streak were expressed by the medial band (Fig. 3G-J). *Brachyury*, in addition, was expressed by the rostromedial density (Fig. 3G), but the other three markers were expressed along the medial edge of the isolate, beginning just caudal to the rostromedial density (Fig. 3H-J; note in J, the rostromedial density was labeled with CRSE prior to in situ hybridization to mark its localization, and subsequently labeled immunocytochemically with anti-rhodamine/peroxidase).

Collectively, the results from type-3.1 experiments show that the rostromedial density expresses all of the available markers of Hensen's node, and the medial band expresses all of the available markers of primitive streak. Thus the use of specific markers further strengthens the idea obtained from morphology and fate mapping that the rostrocaudal density is a reconstituted Hensen's node and the medial band is a reconstituted primitive streak.

In type-3.2 experiments, we further probed lateral isolates with three selected markers: two markers of Hensen's node and one marker of primitive streak. When examined at time 0, both markers of Hensen's node (i.e., *gsc* and *HNF-3β*) were negative (Fig. 3K), but the marker of primitive streak (i.e., *brachyury*) was weakly positive on the medial edge of the inducer lappet and on the caudomedial edge of the isolate (Fig. 3L). The expression of *HNF-3β* was further examined. After 4 hours in culture, *HNF-3β* expression was not detectable. However, after 8-9 hours in culture, *HNF-3β* was readily detectable within the rostromedial density (not shown).

Type-4 experiments: the rostromedial density can substitute for Hensen's node

During late gastrula/early neurula stages, Hensen's node gives rise to several structures residing on or spanning the midline: the notochord/head process, prechordal mesoderm, definitive (gut) endoderm and floor plate of the neural tube. To determine whether the rostromedial density can form the normal derivatives of Hensen's node in grafted embryos, in type-4 experiments we replaced Hensen's node with a CRSE-labeled rostromedial density in embryos at stages 3d-4. After an additional 24 hours in culture, the rostromedial density contributed cells to midline host structures, namely, notochord, endoderm and floor plate of the neural tube (Fig. 4A-C). To determine whether the grafted cells that contributed to the floor plate of the neural tube, expressed floor-plate markers, we double labeled grafted embryos with anti-rhodamine without intensification and with anti-HNF-3β with intensification. In control chick embryos (not shown), anti-HNF-3β labeled only the floor plate of the neural tube and not the notochord (also see Ericson et al., 1996). Double labeling showed that grafted cells of the rostromedial density contributing to the floor plate of the neural tube expressed HNF-3β (Fig. 4D-F), whereas grafted cells contributing to the notochord did not. In conclusion, the rostromedial density is capable of replacing Hensen's node in grafted embryos and giving rise to normal derivatives of Hensen's node, providing further evidence that the rostromedial density is a reconstituted Hensen's node.

Type-5 experiments: the rostromedial density has the ability to suppress notochord reconstitution

During normal embryonic development, Hensen's node suppresses the formation of supernumerary, lateral notochords (Yuan et al., 1995b). If the rostromedial density is identical to Hensen's node, then it should also have this ability to suppress. To test this possibility, in type-5 experiments we grafted a labeled rostromedial density to lateral isolates and cultured these grafted isolates for an additional 36 hours. Then, isolates were double labeled immunocytochemically with anti-rhodamine antibody and intensification followed by Not-1 antibody without intensification. In all cases the graft self-differentiated a notochord (based on morphology; because Not-1 and anti-rhodamine antibodies both label cytoplasm, the brown color resulting from Not-1 labeling without intensification was undetectable owing to the previous labeling of the same cells with anti-rhodamine and intensification, which colors them black). In 4 of these 6 cases, the host failed to reconstitute a notochord (determined by the lack of brown-colored cells medial to the black, self-differentiating graft cells; Fig. 5A). In 2 of these 6 cases, a small notochordal remnant (i.e., partial notochord) was formed by the host (i.e., brown-colored cells medial to the black-colored self-differentiating graft cells; Fig. 5B), suggesting that the host's ability to form a reconstituted notochord was largely suppressed. The results of type-4 and -5 experiments in combination suggest that the rostromedial density has the ability to suppress the formation of supernumerary notochords, providing further support that the rostromedial density is a reconstituted Hensen's node.

Type-6 experiments: the rostromedial density can organize an ectopic embryo and induce a patterned neural tube

Hensen's node possesses the ability to organize an ectopic embryo and induce a patterned neural tube. If the rostromedial density is identical to Hensen's node, then it should also have this ability to organize and induce. To test this possibility, in type-6 experiments we grafted a CRSE-labeled rostromedial density to the extraembryonic region of unlabeled chick host embryos at stages 3d-4 and cultured grafted embryos for an additional 24 h. The results showed that the density, which contributed cells to the endoderm, mesenchyme and notochord of the ectopic embryo, was capable of organizing an ectopic embryo and that such embryos contained induced neural tubes (i.e., derived from the host), which expressed a generic neural plate marker, L5 (not shown), and all three regional markers tested: 4D9 (antibody for En-2 protein; Fig. 6A,B), *c-Otx2* (Fig. 6C,D); *fgf8* (Fig. 6E,F). Thus the results of type-6 experiments provide further support that the rostromedial density is a reconstituted Hensen's node.

To summarize, the results from type-1 through type-6 experiments collectively and consistently provide evidence that the rostromedial density is a reconstituted Hensen's node. Moreover, the results from type-1 through type-3 experiments collectively and consistently provide evidence that the medial band is a reconstituted primitive streak. In type-7 and -8 experiments, we asked, respectively, what are the origins of the rostromedial density and the medial band, and are these structures self-differentiated or do they require induction?

Type-7 experiments: the origins of the rostromedial density and medial band

Type-2 (fate mapping) experiments revealed that the rostromedial density is the source of the reconstituted notochord in lateral isolates. In previous studies (Yuan et al., 1995b), we demonstrated that the region 250 μm to 500 μm caudal to the rostral end of Hensen's node and 250 μm to 500 μm lateral to the primitive streak is a responder region for notochord reconstitution; that is, this region responds to a signal generated by the inducer lappet. In type-7.1 experiments we asked whether the responder is the source of the rostromedial density. CFSE was injected into the responder (location based on measurements) in lateral isolates at time 0, which were subsequently cultured for 10-12 hours. Labeled cells contributed to the rostromedial density as well as spreading rostrolaterally toward the area opaca where they contributed to the mesoderm and endoderm (Fig. 7A). We conclude that the rostromedial density, like the reconstituted notochord, derives from the responder.

In type-7.2 experiments, we labeled the perimeter of the inducer lappet and the caudomedial edge of the lateral isolate with CFSE at time 0, and cultured labeled isolates for 10-12 hours. Labeled cells contributed to the medial band as well as spreading laterally toward the area opaca where they contributed to the mesoderm and endoderm (Fig. 7B). We conclude that the medial band derives from the caudomedial edge of the lateral isolate and the perimeter of the inducer lappet.

Type-8 experiments: the inducer is required for reconstitution of the organizer

Previously, we showed that reconstitution of the notochord requires the action of an inducer upon a responder (Yuan et al., 1995b). In type-8 experiments, we asked whether the notochord inducer was required for: (1) formation of the rostromedial density – the reconstituted Hensen's node (organizer); (2) formation of the medial band – the reconstituted primitive streak. In type-8.1 experiments, inducer lappets from lateral isolates were labeled with CRSE and grafted into other lateral isolates from which the inducer lappet was extirpated; the grafted isolates were then cultured for 20 hours. Isolates were then processed for in situ hybridization with *HNF-3β* riboprobe and were subsequently labeled with anti-rhodamine antibody. As expected from previous experiments (Yuan et al., 1995b), a rostromedial density formed from host cells after grafting of the inducer. Moreover as expected from the present experiments, it expressed *HNF-3β* (Fig. 8A). Graft cells migrated principally rostrolaterally, in close proximity to the rostromedial density, but did not contribute to it. In type-8.2 experiments, the inducer lappet was extirpated from lateral isolates, which were subsequently cultured for 10-12 hours and then processed for in situ hybridization with the *HNF-3β* or *brachyury* riboprobe. In the absence of the inducer lappet, a rostromedial density did not form, and *HNF-3β* expression was not detected. In contrast, a medial band formed and *brachyury* was heavily expressed within it. These results, in conjunction with those of type-1 through type-7 experiments, suggest that formation of a reconstituted organizer requires the same inducer that is required for reconstitution of the notochord, but that reconstitution of the streak might occur independently of this inducer.

DISCUSSION

Notochord reconstitution in lateral blastoderm isolates is preceded by reconstitution of the organizer and primitive streak

During normal development, the notochord arises from committed precursor cells contained within Hensen's node at the rostral tip of the primitive streak at late gastrula/early neurula stage (stages 3d-4). Since the experiments of Waddington and co-workers (reviewed by Waddington, 1952), Hensen's node has been considered to be the avian equivalent of Spemann's organizer; that is, the structure responsible for inducing the neuroectoderm and organizing the body axis during gastrulation and neurulation. Surprisingly, extirpation of Hensen's node just prior to the formation of the notochord often results in essentially normal embryos in which a notochord develops at least in part (Waddington, 1932; Waterman, 1936; Butros, 1967; Grabowski, 1956; Gallera and Nicolet, 1974; Smith and Schoenwolf, 1989; Darnell et al., 1992; Schoenwolf and Yuan, 1995; Yuan et al., 1995a, b; Psychoyos and Stern, 1996b). Recently, Psychoyos and Stern (1996b) showed, using whole cultured blastoderms in which Hensen's node and the rostral 40% of the primitive streak were removed, that notochord reconstitution is preceded by formation of a reconstituted organizer. Here we present 6 lines of evidence that in lateral isolates an organizer is also reconstituted: within 10-12 hours in culture after removal of the notochordal precursor cells, a Hensen's node-like structure forms based on (1) morphology (type-1 experiments) and (2)
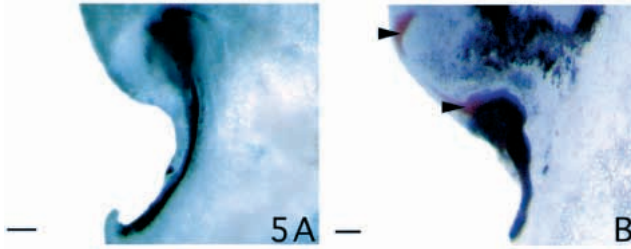
Fig. 5. In type-5 experiments, the ability of the rostromedial density to suppress notochord reconstitution was tested. (A) Embryo double immunolabeled with anti-rhodamine and Not-1. Brown cells, indicative of cells forming notochord but not derived from the graft (which has been labeled with anti-rhodamine and intensification), were absent. Thus the grafted rostromedial density suppressed notochord reconstitution. (B) Embryo double immunolabeled with anti-rhodamine and Not-1. Arrowheads indicate a partial notochord, indicating that suppression was not complete in this embryo. Bars, 200 μm.

gene expression (type-3 experiments); the Hensen's node-like structure or rostromedial density gives rise to normal Hensen's-node derivatives, either (3) when fate mapped (type-2 experiments) or (4) when grafted in place of Hensen's node in host embryos (type-4 experiments); (5) the rostromedial density can suppress notochord reconstitution in isolates (type-5 experiments) as can Hensen's node (Yuan et al., 1995b); and (6) the rostromedial density can organize an ectopic embryo with an induced and patterned neural tube (type-6) experiments.

Two additional comments about patterns of gene expression in lateral isolates during reconstitution are warranted. First, at 10-12 hours in culture all genes that are expressed within the rostromedial density are expressed symmetrically within the organizer. This could suggest that the stage of the organizer at the time of its formation was equivalent to no later than stages 3d-4, because genes such as *Sonic hedgehog* become asymmetrically expressed thereafter (Levin et al., 1995). Alternatively, it might mean that factors that establish asymmetry are no longer present in lateral isolates. These two possibilities are not mutually exclusive and evidence exists to support both of them. For example, in support of the first possibility, Hensen's node is a potent inducer of ectopic embryos at stages 3d-4 (and earlier), but looses its potency at later stages (Dias and Schoenwolf, 1990; Storey et al., 1992). The density is also a potent inducer, suggesting a maximal age of about stage 4. In support of the second possibility, the *nodal*-related gene (*cNR-1*), which is not detectable at 10-12 hours in culture, becomes detectable 8-10 hours later, and is then expressed by both the right and left lateral isolates (rather than being restricted to the left side; Levin et al., 1995). Thus the expression of this gene, which also shows sidedness in the intact embryo, loses its sidedness in lateral isolates. A second comment about patterns of gene expression in lateral isolates

is that *goosecoid*, an organizer-specific molecule (Cho et al., 1991), was expressed in rostromedial density of lateral isolates cultured 10-12 h. In the study by Psychoyos and Stern (1996b) it was reported that *goosecoid* was not expressed during reconstitution. We believe that the use of lateral isolates, rather than their whole-embryo paradigm, provided better temporal and spatial resolution, allowing us to detect such a transiently expressed gene.

We also present 3 lines of evidence that in lateral isolates a primitive streak is reconstituted: within 10-12 hours in culture after removal of Hensen's node and the entire primitive streak, a primitive streak-like structure forms. This is based on (1) morphology (type-1 experiments), (2) gene expression (type-3 experiments); and (3) the primitive streak-like structure or medial band gives rise to normal primitive-streak derivatives when fate mapped and it does so in proper rostrocaudal order (type-2 experiments). Furthermore, fate mapping suggests that the primitive streak at the time of its formation is at stages 3d-4 (or later), because at earlier stages (i.e., stages 3a-b) the primitive streak contains prospective heart cells (Garcia-Martinez and Schoenwolf, 1993), not prospective somitic cells, which are still lateral to the primitive streak in the epiblast (Garcia-Martinez, Lopez-Sarchez and Schoenwolf, unpublished data).
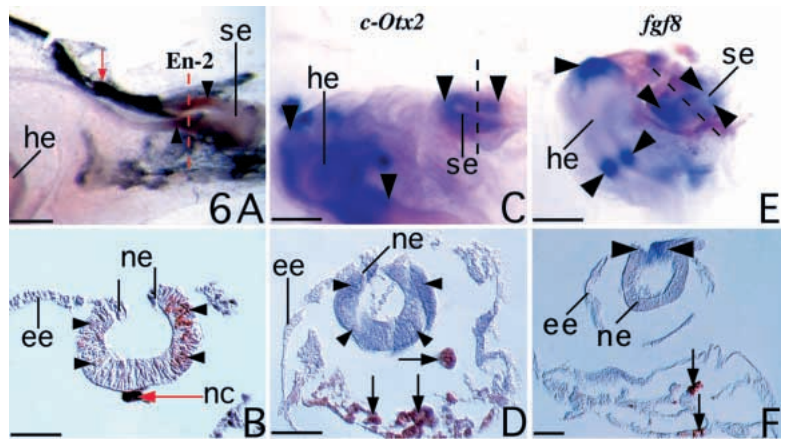

Fig. 6. In type-6 experiments, the ability of the rostromedial density to organize an ectopic embryo with an induced and patterned neural tube was tested. Graft cells were labeled with anti-rhodamine and intensification and, therefore, have a black color. (A) The secondary embryo (se) and host embryo (he) both labeled with 4D9 (antibody for En-2 protein; arrowheads, brown color; only 4D9 expression in the secondary embryo is shown). (B) Transverse section at the level indicated by the dashed line in A. The notochord (nc) derived from graft cells and, therefore, was colored black. The epidermal ectoderm (ee) and neuroectoderm (ne) were both host derived, and the latter expressed 4D9 (arrowheads, brown color). (C) The secondary embryo (se) and host embryo (he) both labeled with *cOtx-2* (arrowheads, purple color). (D) Transverse section at the level indicated by the dashed line in C. Much of the head mesenchyme (arrows) beneath the neural tube derived from graft cells and, therefore, has a brown color (i.e., immunocytochemistry is not intensified after in situ hybridization). The epidermal ectoderm (ee) and neuroectoderm (ne) were both host derived, and the latter expressed *cOtx-2* (arrowheads, purple color). (E) The secondary embryo (se) and host embryo (he) both labeled with *fgf8* (arrowheads, purple color). (F) Transverse section at the level indicated by the dashed line in C. A few of the cells of the head mesenchyme and endoderm (arrows) derived from the graft are shown at this section level (brown color). The epidermal ectoderm (ee) and neuroectoderm (ne) were both graft derived, and the latter expressed *fgf8* in its roof at the level shown (arrowhead, purple color). Bars, 200 μm (A,C,E); 50 μm (B,D,F).

Fig. 7. In type-7 experiments, the origin of the rostromedial density and the medial band were identified. (A) A lateral isolate 10-12 hours after injection of the responder with CFSE. The labeled cells have contributed to the rostromedial density (arrow) as well as to the rostrolateral mesoderm, endoderm (arrowhead). (B) A lateral isolate 10-12 hours after injection of the perimeter of the inducer lappet and the caudomedial edge of the isolate with CFSE. The labeled cells have contributed to the medial band (arrows) as well as to the lateral mesoderm, endoderm (arrowheads) Bars, 200 μm.
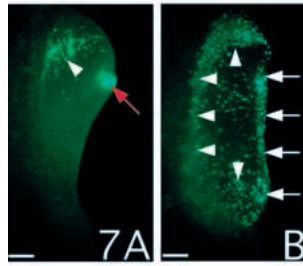
The organizer forms from the responder through the action of an inducer

Recently, using lateral blastoderm isolates, we showed that reconstitution of the notochord involves an inducer, which acts upon a responder (Yuan et al., 1995a, b). In the present study we show, using fate mapping (type-7 experiments), that the reconstituted organizer originates from the same responder required for formation of a reconstituted notochord; that is, an area located 250-500 μm caudal to the rostral end of Hensen's node and 250-500 μm lateral to the lateral edge of the primitive streak. Furthermore, we show that formation of the reconstituted organizer requires the action of an inducer (type-8 experiments) – the same inducer required for formation of a reconstituted notochord. In the absence of the inducer lappet, neither Hensen's-node morphology or organizer-specific gene expression occurs. Thus this inducer seems to be functionally equivalent to the Nieuwkoop center of amphibians (Nieuwkoop, 1973).

In contrast, the primitive streak seems to be reconstituted in the absence of the inducer lappet, as determined by the expression of *brachyury*, a mesoderm-specific marker (Kispert et al., 1995). Examination of lateral isolates at time 0 revealed
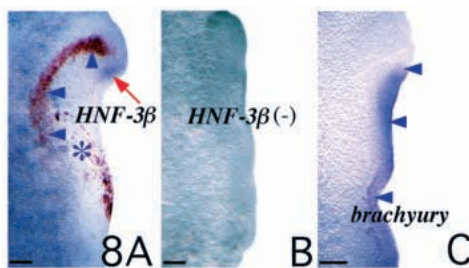

Fig. 8. In type-8 experiments, the role of the inducer lappet in formation of the reconstituted organizer and primitive streak was examined. (A) Lateral isolate 20 hours after its inducer lappet was extirpated and another inducer lappet, labeled with CRSE, was grafted into it. The approximate site of grafting is indicated by the asterisk. A reconstituted organizer formed, which labeled with *HNF-3β* (arrow), and graft cells migrated principally rostromedially in close proximity to the reconstituted organizer (arrowheads). (B,C) Lateral isolates 10-12 hours after extirpation of their inducer lappets. In the absence of an inducer lappet, a rostromedial density does not form and *HNF-3β* is not expressed (B), but a medial band does form and *brachyury* is expressed (C, arrowheads). Bars, 200 μm.

that the medial edge of the inducer lappet weakly expressed *brachyury*, as did the caudomedial edge of the isolate. Because the latter expressing region would still be present in lateral isolates lacking inducer lappets, it is possible that *brachyury* is auto-upregulated in these isolates. Further studies are underway to determine in more detail the requirements for reconstitution of the primitive streak and whether *brachyury* expression is indicative of the formation of a functional primitive streak.

Although the full repertoire of molecules that cause mesoderm induction *in vivo* is not known, our understanding of this process has progressed greatly in the past few years. In amphibians, the use of mesoderm-induction assays has implicated growth factors such as activin, *Vg1*, *Wnt-8* and *noggin* as important in directing the formation of dorsal mesoderm (i.e., Spemann's organizer; Watabe et al., 1995). In chick, as in other vertebrates where mesoderm induction has been studied (reviewed by Beddington and Smith, 1993; Slack, 1993; Kessler and Melton, 1994), peptide growth factors of the fibroblast growth factor (FGF) and transforming growth factor-β families (TGF-β) play roles in the induction of axial mesoderm in the normal embryo. Interestingly, recent experiments from our laboratory using lateral blastoderm isolates have shown that the growth factors FGF2, FGF4, activin and TGF-β can mimic the activity of the inducer (Yuan and Schoenwolf, unpublished data). Attempts to identify endogenous molecules involved in avian organizer formation and function are currently underway in our laboratory.

Regulative ability of the blastoderm at the late gastrula/early neurula stage

This study, in conjunction with several previous studies (Waddington, 1932; Waterman, 1936; Grabowski, 1956; Butros, 1967; Gallera and Nicolet, 1974; Smith and Schoenwolf, 1989; Alvarez and Schoenwolf, 1991; Schoenwolf and Alvarez, 1991; Darnell et al., 1992; Garcia-Martinez and Schoenwolf, 1992; Storey et al., 1992; Schoenwolf and Yuan, 1995; Yuan et al., 1995a, b; Psychoyos and Stern, 1996b; Garcia-Martinez et al., 1997), reveals that the avian blastoderm at the late gastrula/early neurula stage has a remarkable potential for regulation, which is lost by about the mid-neurula stage (stages 6-7; Psychoyos and Stern, 1996b; Garcia-Martinez et al., 1997). Heterotopic grafting studies show that for most cell types, cell fate is determined locally by cell-cell interactions rather than by a cell's ancestry. Prospective notochordal cells are an exception to this rule, being already committed by the late gastrula/early neurula stage (Gallera, 1974; Garcia-Martinez and Schoenwolf, 1992; Storey et al., 1992; Inagaki and Schoenwolf, 1993).

The results of the present study demonstrate that the responder region, a localized area of epiblast that is destined during normal development to form principally the caudal end of the neural plate and is the same area that is required for reconstitution of the notochord in lateral isolates (Yuan et al., 1995b), acquires organizer activity in lateral isolates within 10-12 hours in culture. Acquisition of this activity occurs after removal of a suppressor (Hensen's node; Yuan et al., 1995b) and results from inductive interactions with an inducer, the same area required for induction of the reconstituted notochord. When grafted extraembryonically, the reconstituted organizer self-differentiates notochord as well as organizes an

ectopic embryo, demonstrating that cells of the organizer are committed to a notochordal fate by 10-12 hours in culture. Thus our current experiments have begun to reveal the timing of both organizer induction and notochordal cell commitment, as well as identifying for the first time the cell populations that undergo interaction during formation of the avian organizer.

Our current results also reveal that a new primitive streak can be reconstituted from the medial edge of the isolate within 12 hours of ablation of the primitive streak. The medial edge of the isolate consists of prospective mesoderm (paraxial and lateral plate; Garcia-Martinez et al., 1993), which reorganizes into a band-like structure and expresses *brachyury*. Further experiments are underway to determine the extent of the blastoderm that is capable of reconstituting primitive streak, as well as the cell interactions that regulate this process.

Lateral blastoderm isolates: a unique model for analyzing the cellular and molecular mechanisms of organizer induction and activity in higher vertebrates

Our past (Yuan et al., 1995b) and present experiments reveal the power of lateral blastoderm isolates to unravel the cell interactions involved in reconstitution of the body axis in avian embryos. Recent experiments on fish (Shih and Fraser, 1997) and mouse embryos (Schoenwolf et al., unpublished) suggest that the ability to reconstitute the body axis is a common feature of vertebrates. Thus it is likely that cellular and molecular mechanisms of reconstitution are similar among species and may represent a recapitulation of events occurring in formation of the body axis during normal development. Because of the temporal and spatial resolution available with the use of chick lateral blastoderm isolates, we believe that they will provide an important experimental tool for elucidating mechanisms underlying formation of the vertebrate body axis.

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