Reconstitution of the organizer is both sufficient and required to re-establish a fully patterned body plan in avian embryos

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SUMMARY

Lateral blastoderm isolates (LBIs) at the late gastrula/early neurula stage (i.e., stage 3d/4) that lack Hensen's node (organizer) and primitive streak can reconstitute a functional organizer and primitive streak within 10-12 hours in culture. We used LBIs to study the initiation and regionalization of the body plan. A complete body plan forms in each LBI by 36 hours in culture, and normal craniocaudal, dorsoventral, and mediolateral axes are re-established. Thus, reconstitution of the organizer is *sufficient* to re-establish a fully patterned body plan. LBIs can be modified so that reconstitution of the organizer does not occur. In such modified LBIs, tissue-type specific differentiation (with the exception of heart differentiation) and reconstitution of the body plan fail to occur. Thus, the reconstitution of the organizer is not only sufficient to re-establish a fully patterned body plan, it is also *required*. Finally, our results show that formation and patterning of the heart is under the control of the organizer, and that such control is exerted during the early to mid-gastrula stages (i.e., stages 2-3a), prior to formation of the fully elongated primitive streak.

Key words: Cardiac mesoderm, Chick embryos, Gastrulation, Gene expression, Heart, In situ hybridization, Neurulation, Nieuwkoop center, Regionalization

INTRODUCTION

Our understanding of the complex process involved in the formation of the vertebrate body plan is founded on the results of Spemann and Mangold (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 secondary embryo containing an induced (host-derived) neural tube. Because the transplanted lip recruited host cells and organized them into this secondary embryo, the dorsal lip of the blastopore constitutes an organizer (commonly called Spemann's organizer or the organizer). The structure in other vertebrates that is functionally equivalent to the amphibian organizer is the embryonic shield in fish (Oppenheimer, 1959; Ho, 1992; Shih and Fraser, 1996), Hensen's node in birds (Gallera, 1971; Dias and Schoenwolf, 1990; Storey et al., 1992, 1995; and references therein), and the node in mammals (Beddington, 1994). Although the main interest of late has been focused on the identification of the cellular and molecular underpinnings of organizer activity in different vertebrates (reviewed by Lemaire and Kodjabachian, 1996), much progress has been made in understanding the formation of the organizer, a critical event in vertebrate development.

In amphibians, the mechanisms underlying the formation of the dorsal lip of the blastopore have been studied intensively by using *Xenopus* as a model system (reviewed by Sive, 1993;

Kessler and Melton, 1994). It is now clear that the organizer is formed on the dorsal side of the gastrula embryo in the equatorial region as a result of inductive interactions between the marginal zone and the dorsal vegetal (endodermal) region, also known as the Nieuwkoop center (Nieuwkoop, 1969, 1973; Gimlich and Gerhart, 1984; Dale and Slack, 1987).

In the avian embryo, formation of Hensen's node has been examined recently by using an experimental model in which the notochord is reconstituted in lateral blastoderm isolates (LBIs) lacking Hensen's node and primitive streak (Yuan et al., 1995a,b; Yuan and Schoenwolf, 1998). The use of LBIs has revealed that the avian organizer is also formed by a process of induction, specifically through the interaction of two areas of the blastoderm, an inducer and a responder. In this model in which isolates are made at the late gastrula/early neurula stage (i.e., stage 3d/4), the responder (epiblast that includes caudal prospective neuroectoderm) is induced by the inducer (epiblast and primitive streak that include prospective somitic and lateral plate mesoderm) to form a fully functional organizer. Thus, the inducer in this avian model system can be considered to be the functional equivalent of the Nieuwkoop center of amphibians, that is, it induces the organizer. During normal development, Hensen's node acts as a suppressor at the late gastrula/early neurula stage to block further potential inductive interactions; thus, only one body axis typically forms.

The use of LBIs offers several important advantages for studying initiation and patterning of the vertebrate body plan.

First, the interacting responding and inducing tissues that are required for formation of the organizer in this model system are easily identified using readily discernible landmarks. Second, each of these tissues has sufficient size and can be physically separated from the other, allowing them to be manipulated in a variety of ways for a series of detailed studies. Finally, reconstitution of the organizer in this model system can be studied at the late gastrula/early neurula stage (i.e., stage 3d/4), a stage at which detailed fate mapping data exist (e.g., Rosenquist, 1966; Nicolet, 1971; Bortier and Vakaet, 1992; Schoenwolf et al., 1992; Garcia-Martinez et al., 1993; Hatada and Stern, 1994; Yuan et al., 1995b). Consequently, this experimental paradigm provides an accessible, convenient, well-defined model system in which to investigate the patterning of the body plan in a higher vertebrate as it is initiated, beginning with the induction of the organizer and primitive streak.

We have used LBIs, other types of blastoderm isolates, and molecular markers to examine the role of the organizer in initiating and regionalizing the body plan and in establishing the body axes. Our results suggest that a structurally and molecularly complete body plan is reconstituted in LBIs, and that normal craniocaudal, dorsoventral, and mediolateral axes are re-established. Reconstitution of the organizer is both sufficient and required for formation of this fully patterned body plan. Moreover, the formation and patterning of the heart is under control of the organizer, and such control is exerted during the early to mid-gastrula stages (i.e., stages 2-3a), prior to formation of the fully elongated primitive streak. Collectively, these results provide further information about how the body plan is progressively and hierarchically established during vertebrate development through a cascade of inductive and suppressive cell-cell interactions and cell movements.

MATERIALS AND METHODS

Whole-embryo culture

Fertile White Leghorn chicken eggs or quail eggs were incubated until embryos reached stage 3d/4 (type-1, -2, and -7 experiments; Hamburger and Hamilton, 1951, with stage 3 substages as described by Schoenwolf et al., 1992), 3a/b (type-3 experiments), and 3a (type-4 through -7 experiments). Culture dishes and embryos were prepared as previously described (Yuan et al., 1995a) for modified New culture (ventral-side up, with the blastoderm isolate cultured on the vitelline membrane; New, 1955).

Blastoderm isolates

Lateral blastoderm isolates (LBIs) were used in some of our experiments as models, based on their successful use in previous studies (Yuan et al., 1995b; Yuan and Schoenwolf, 1998). LBIs lacking Hensen's node and primitive streak were prepared at the late gastrula/early neurula stage (stage 3d/4; Fig. 1A,B), with each LBI containing all three layers of the blastoderm. Usually, each blastoderm yielded two LBIs, a 'right' and 'left' (here, referring to the *apparent* right and left sides of the isolates cultured ventral-side up), which were both cultured on the same vitelline membrane. In type-1 experiments, each LBI consisted of a right or left laterocaudal piece containing a inducer lappet (Fig. 1A; i, inducer) and responder region (Fig. 1A; r, responder). The inducer lappet is the area of the epiblast (and underlying tissues) containing the strongest ability to induce another area of the epiblast, called the responder, to form a reconstituted organizer; the reconstituted organizer gives rise to the

reconstituted notochord and floor plate of the neural tube during subsequent development (Yuan and Schoenwolf, 1998). The inducer lappet extends lateral to the primitive streak for 250 μm and is localized mainly from 500-750 μm caudal to the cranial end of Hensen's node. The responder is localized in the epiblast mainly 250-500 μm lateral to the primitive streak and 250-500 μm caudal to the cranial end of Hensen's node. In type-2 experiments, inducer lappets were removed (Fig. 1B; i, inducer), but the responder was left intact (Fig. 1B; r, responder). In six type-2 experiments, the endoderm covering of the LBI was removed. In type-3 experiments, modified LBIs were used. These were constructed at stage 3a/b and the two LBIs obtained from each blastoderm were left interconnected cranially (Fig. 1C). The blastoderm 250 μm adjacent to the primitive streak was removed on the 'left' side, but it was kept undisturbed on the 'right' side. Because the adjacent epiblast contains the inducer, the 'left' side served as the experimental side and the right side as the control side. In type-4 through -6 experiments, transverse blastoderm isolates (TBIs) were constructed at stage 3a. Each blastoderm was transected at a level 250 μm (type-4 and -6) or 375 μm (type-5) caudal to the cranial end of the primitive streak, and in type-5 and -6 experiments the transection plane was staggered to cross the midline either at the cranial end of Hensen's node (type-5 experiments) or 750 μm caudal to the cranial end of the primitive streak (type-6). In type-5 and -6 experiments, selected populations of cells were labeled with a fluorescent dye as described previously (Yuan and Schoenwolf, 1998). In type-7 experiments, the prospective cardiac mesodermal cells within the primitive streak of stage 3a embryos were grafted to germ cell crescent isolates (GCIs) of stage 3d/4 embryos. In 6 cases, quail embryos were used as donors and chick embryos as hosts. In 4 other cases, chick embryos were used as donors, and grafted cells were labeled with a fluorescent dye, as above.

After operations were completed, cultures were placed into humidified chambers in incubators at 38°C for up to 36 hours. 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, permanent documentation on videotape was obtained at time 0.

Immunocytochemistry and whole-mount in situ hybridization

Indirect, whole-embryo immunocytochemistry was done as described previously (Patel et al., 1989). In some cases, immunocytochemical detection of two labels was also done as described previously (Yuan and Schoenwolf, 1998). In situ hybridization was performed essentially as described by Nieto (Nieto et al., 1996), except that the use of proteinase K, hydrogen peroxide and RNase A were omitted. In some cases, embryos were labeled immunochemically as described above after in situ hybridization. Following immunocytochemistry and/or in situ hybridization, embryos were dehydrated and then embedded in Paraplast X-tra paraffin; serial transverse sections were subsequently cut at 10-15 μm. The antibody QCPN was used to identify grafted quail cells in chick host embryos.

Markers defining regionalization of the body plan

Neuroectoderm and craniocaudal levels of the neural tube were identified using the following riboprobes: the pan-neural marker *Sox-2* (Rex et al., 1997) and the regional markers *fgf8* (marks forebrain, the isthmus of the midbrain/hindbrain, otocysts, and the newly formed caudal neural tube and the tail bud; Crossley et al., 1996; Storey et al., 1998); *Six-3* (marks the optic vesicles and the cranioventral forebrain; Bovolenta et al., 1998); *Otx2* (marks the forebrain through the midbrain; Bally-Cuif and Wassef, 1995); *Pax2* (marks the isthmus of the midbrain/hindbrain, the otocysts, and the tail bud; Torres et al., 1996; Herbrand et al., 1998); *Krox-20* (rhombomeres 3 and 5 of the hindbrain; Wilkinson, 1989); *Hoxb9* (marks caudal spinal cord; Storey et al., 1998). Additionally, En-2 antibody (marks the isthmus of the midbrain/hindbrain; Patel et al., 1989), HNK-1 antibody (a pan-neural
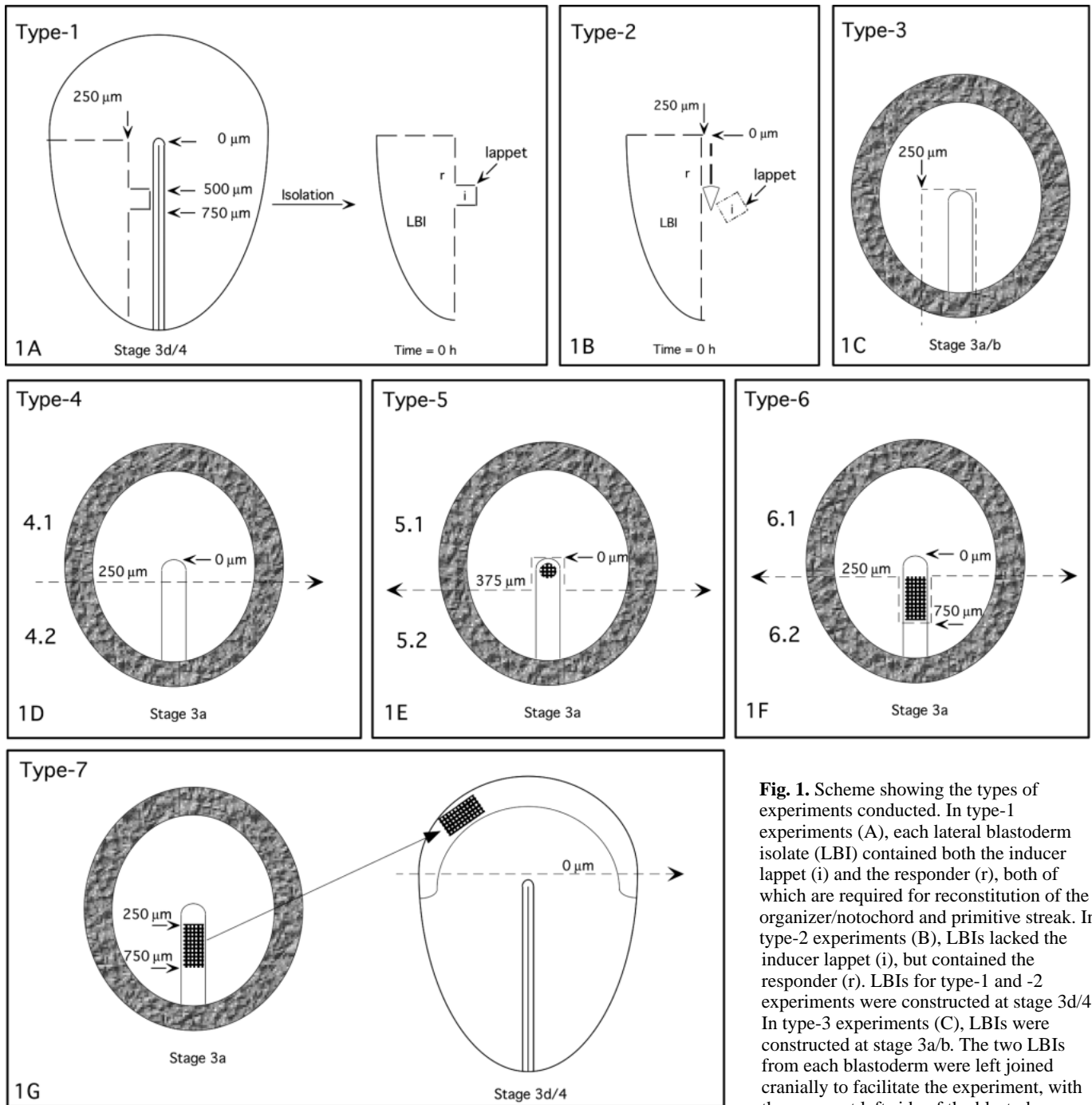
Fig. 1. Scheme showing the types of experiments conducted. In type-1 experiments (A), each lateral blastoderm isolate (LBI) contained both the inducer lappet (i) and the responder (r), both of which are required for reconstitution of the organizer/notochord and primitive streak. In type-2 experiments (B), LBIs lacked the inducer lappet (i), but contained the responder (r). LBIs for type-1 and -2 experiments were constructed at stage 3d/4. In type-3 experiments (C), LBIs were constructed at stage 3a/b. The two LBIs from each blastoderm were left joined cranially to facilitate the experiment, with the apparent left side of the blastoderm serving as the experimental side (i.e., lacking inducer) and the apparent right side serving as the control side (i.e., containing both inducer and responder); the primitive streak and 250 μm of adjacent blastoderm on the 'left' side were extirpated. In type-4 (D), -5 (E), and -6 (F) experiments, transverse blastoderm isolates (TBIs) were used; these were constructed at stage 3a. The blastoderm was transected 250 μm (type-4 and -6 experiments) or 375 μm (type-5 experiments) caudal to the cranial end of the node, and the transection plane was staggered in the midline in type-5 (crossed the cranial end of the streak) and -6 (crossed 750 μm caudal to the cranial end of the streak) experiments. Cranial and caudal blastoderm isolates from type-4, -5, and -6 experiments were designated .1 or .2, respectively (e.g., 4.1 indicates the cranial isolate from type-4 experiments). In type-7 experiments, the prospective cardiac mesodermal cells of the primitive streak from stage 3a donors (250-750 μm caudal to the cranial end of the primitive streak) were grafted to germ cell crescent isolates (i.e., germ cell crescents separated from the organizer by transection; dashed line) of stage 3d/4 host embryos. Cross hatching in E-G indicates populations of cells labeled with a fluorescent dye at time 0. Isolates for each experiment consisted of all three layers of the blastoderm and were cultured in modified New culture (ventral-side up) for selected periods of up to 36 hours, and then processed for in situ hybridization and/or immunocytochemistry, using various markers.

crest marker; Bronner-Fraser, 1986), and anti-neurofilament antibody (3A10) were used, respectively, as another craniocaudal marker; as a dorsal neural tube marker, partially defining the dorsoventral axis of the embryo; and as a marker of neuronal differentiation (defining intermediate zones of the neural tube between the roof and floor plates).

Midline structures – notochord and floor plate of the neural tube – were identified, respectively, using Not-1 antibody (for an epitope present in both the chick and quail notochord; Yamada et al., 1991) and HNF-3β antibody (for HNF-3β protein present in both the chick and quail in nuclei of the floor plate of the neural tube; Ericson et al., 1996; Yuan and Schoenwolf, 1998); *Sonic hedgehog* riboprobe (*Shh*; Riddle et al., 1993; Roelink et al., 1994) was also used as a marker of both notochord and floor plate of the neural tube in single embryos. Not-1 antibody, HNF-3β antibody, and *Shh* riboprobe also served as ventral neuraxial markers, partially defining the dorsoventral axis in the midline of the embryo. Finally, *lmx-1* riboprobe was used as a marker of both the dorsal and ventral neural tube (roof and floor plates; Giraldez, 1998).

Mesodermal subdivisions lateral to the notochord – somites, intermediate mesoderm, and lateral plate mesoderm – were identified, respectively, using the following riboprobes: *paraxis* (Bernes et al., 1997; Garcia-Martinez et al., 1997), *Pax2* (Torres et al., 1995; Herbrand et al., 1998), and *cytokeratin* (Charlebois et al., 1990; Tonegawa et al., 1997). Additionally, *nodal* riboprobe was used as a second lateral plate mesodermal marker (on the embryo's left side; Levin et al., 1995).

The heart mesoderm and its craniocaudal levels were identified using MF-20 antibody (a pan-heart marker; Han et al., 1992) and two riboprobes *VMHC1* (marker of the entire prospective ventricle and cranial prospective atria; Yutzey et al., 1994) and *AMHC1* (marker of the prospective atria; Yutzey et al., 1994).

RESULTS

All experiments are diagrammed in Fig. 1 and summarized in Table 1 (for details, see figure legends).

Type-1 experiments: reconstitution of the organizer in lateral blastoderm isolates is sufficient for reconstitution of a fully patterned body plan

A variety of region-specific markers were used to determine to

what extent the body plan is reconstituted following reconstitution of the organizer. Our results show that the craniocaudal, dorsoventral and mediolateral axes of the body are reconstituted, demonstrating that reconstitution of the organizer is sufficient for reconstitution of a fully patterned body plan.

Lateral blastoderm isolates (LBIs) containing both the inducer lappet and responder (Fig. 1A) were cultured for 36 hours and labeled subsequently with appropriate markers to assess the extent to which reconstitution of the craniocaudal, dorsoventral, and mediolateral axes occurred following reconstitution of the organizer. First, the pan-neural marker, *Sox-2*, was used (Fig. 2A,B). LBIs expressed *Sox-2* at the medial edge of the isolate along their entire craniocaudal extent. As in control embryos, labeling expanded cranially, indicating the brain level of the neuraxis, and narrowed caudally, indicating the spinal cord level. Next, *fgf8* was used, which is expressed in controls (Fig. 2C) at multiple, but highly localized, levels of the neuraxis. *Fgf8* was expressed in LBIs at similar localized patches that marked, in cranial-to-caudal sequence, forebrain, isthmus (midbrain/hindbrain junction), otic placode (level of the 5th and 6th rhombomeres of the hindbrain; confirmed with *Pax2* labeling, see Fig. 4A-D), and tail bud (Fig. 2D). To provide a finer determination of the degree to which neuraxial patterning occurred, several additional neuroectodermal markers were used that are expressed within a single level (or couple of levels) of the neuraxis. *Six-3* (cranial forebrain; Fig. 2E,F), *Otx2* (forebrain, midbrain and isthmus; Fig. 2G,H), En-2 (isthmus; not shown), *Krox-20* (3rd and 5th rhombomeres of the hindbrain; Fig. 2I,J), and *Hoxb9* (caudal spinal cord; Fig. 2K,L) were all expressed in LBIs in patterns very similar to those of control embryos. Collectively, these results provide strong evidence that all craniocaudal regions of the neuraxis were reconstituted in LBIs.

Next, early dorsoventral patterning of the neuraxis was examined to see whether it occurred normally. Previously, we showed that notochord and floor plate of the neural tube form in LBIs (Yuan and Schoenwolf, 1998). This was confirmed

Fig. 2. Type-1 (T1) experiments. LBIs cultured 36 hours and unoperated control embryos (C) examined with in situ hybridization and/or immunocytochemistry. Control embryo (A) and LBI (B) labeled with the pan-neural marker *Sox2,* showing the craniocaudal extent of the labeled neuroectoderm (arrowheads). Controls embryos (C,E,G,I,K) and LBIs (2D,F,H,J,L) double-labeled, respectively, with Not-1 antibody (C-L, arrows) and *fgf8* (C,D, arrowheads), *Six-3* (E,F, arrowheads), *Otx2* (G,H, arrowheads), *Krox-20* (2I, J, arrowheads), or *Hoxb9* (K,L, arrowheads). When compared to control embryos, it is clear that the LBIs regenerated the full craniocaudal pattern of the neuroectoderm. Bars, 200 μm (A,C,E,G,K); 100 μm (2B,D,F,H,I,J,L).
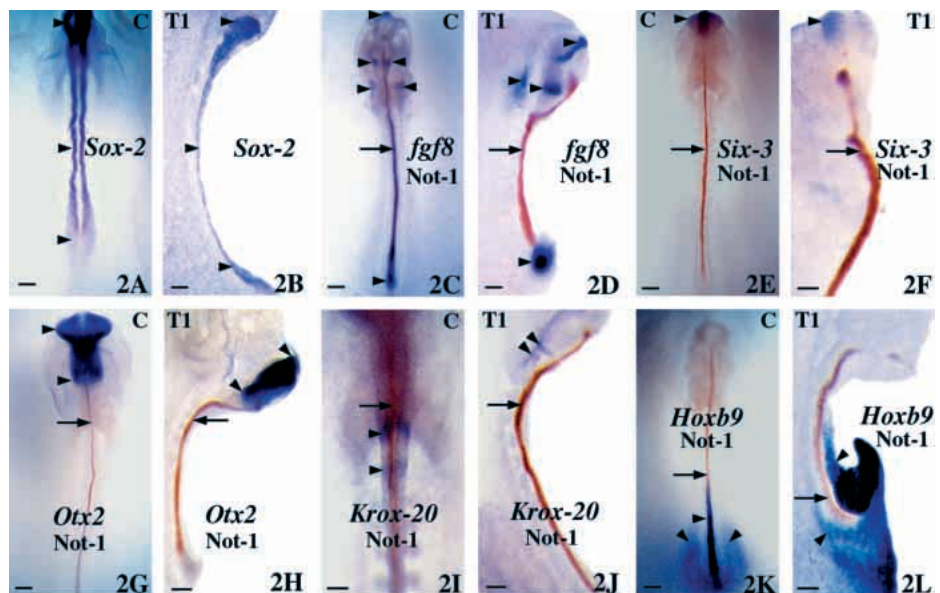
Table 1. Summary of the experiments conducted

Experiment type	Hours in culture	Markers used[a]	Number labeled with marker/total number of cases
Type-1			
	36	*Sox-2*	11/11
	36	*fgf8*	8/8
	36	*Six-3*	4/4
	36	*Otx2*	6/6
	36	En-2	6/6
	36	*Krox-20*	5/5
	36	*Hoxb9*	6/6
	36	HNF-3β	8/8
	36	*Shh*	8/8
	36	HNK-1	12/12
	36	*lmx-1*	6/6
	36	3A10	10/10
	36	Not-1[b]	20/20
	36	*paraxis*	10/10
	36	*Pax2*	8/8
	36	*cytokeratin*	6/6
	20	*Nodal*	6/6
	36	MF-20[c]	12/12
	36	*VMHC1*	6/6
	36	*AMHC1*	6/6
Type-2			
	36	Not-1[b]	0/24
	0	*Sox-2*	6/6
	36	*Sox-2*	0/12
	36	*fgf8*	0/8
	0	*Otx2*	6/6
	36	*Otx2*	0/6
	36	*Hoxb9*	0/6
	36	*paraxis*	0/8
	36	*Pax2*	0/6
	20	*Nodal*	0/6
	36	MF-20	6/6
	36	MF-20[d]	0/6
Type-3			
	36	MF-20/Not-1	17/17[e]
	36	MF-20/Not-1	3/17[f]
Type-4			
4.1	36	MF-20	0/10[g]
4.2	36	MF-20	7/10[h]
4.1	36	Not-1	10/10[g]
4.2	36	Not-1	5/10[h]
Type-5			
5.1	36	MF-20	0/8[g]
5.2	36	MF-20	8/8[h]
5.1	36	Not-1	6/8[g]
5.2	36	Not-1	0/8[h]
Type-6			
6.1	36	MF-20	5/23[g,i]
6.2	36	MF-20	2/23[h]
6.1	36	Not-1	5/23[g,i]
6.2	36	Not-1	0/23[h]
Type-7			
	36	MF-20/Not-1	0/6[j]
	36	MF-20/Not-1/QCPN	2/2[k]

[a]Italics indicates riboprobe; roman text indicates antibody.
[b]For all type-1 experiments and some type-2 experiments, Not-1 labeling was done after in situ hybridization with one of the listed riboprobes. For other type-2 experiments, Not-l labeling was done in conjunction with MF-20 labeling.
[c]MF-20 labeling was done after in situ hybridization with *AMHC1*.
[d]The endoderm, in addition to the inducer lappet, was removed.
[e]Stage 3a and 3b control (i.e., "right" side) isolates.
[f]Stage 3a and 3b experimental (i.e., "left" side) isolates. The three cases that labeled did so only with MF-20 (not Not-1) and were from

stage 3b embryos (i.e., 3 of 8 stage 3b embryos labeled). None of the embryos at stage 3a (i.e., 0 of 9) labeled with MF-20 (nor Not-1).
[g]Stage 3a cranial isolates.
[h]Stage 3a caudal isolates.
[i]Migration of cells from the primitive streak into the cranial isolate occurred in only the 5 cases in which labeling with MF-20 and Not-1 was positive.
[j]Germ cell crescent isolates containing stage 3a grafts of prospective cardiac mesodermal cells of the primitive streak. An ectopic embryo was not detectable in these isolates.
[k]Germ cell crescent isolates containing stage 3a grafts of prospective cardiac mesodermal cells of the primitive streak. An ectopic embryo formed in these and two other isolates (a total of 4 cases). A sample of two of these was triple labeled. All three markers were expressed in both ectopic embryos. Partial co-localization of MF-20 and QCPN (i.e., anti-quail antibody) labeling occurred, indicating that the ectopic heart was induced from *donor* tissue; co-localization of Not-1 and QCPN did not occur, indicating that the ectopic notochord was derived from an organizer induced by the graft from the *host* epiblast.

using Not-1 antibody to mark notochord (always in combination with other regional markers to provide spatial perspective; Fig. 2D,F,H,J,L), HNF-3β antibody to mark floor plate of the neural tube (data not shown, but see Yuan and Schoenwolf, 1998), and *Shh* to mark both notochord and floor plate of the neural tube (Fig. 3A). HNK-1 antibody (a neural crest marker; Fig. 3B) was used to determine that dorsalization of the neural tube and generation of migratory neural crest cells occurred in LBIs (Fig. 3C). Additionally, two more dorsoventral markers were used: *lmx-1*, which labels the full length of the neural tube in both controls (Fig. 3D) and LBIs (Fig. 3E) and is expressed in both the roof and floor plates (Fig. 3F); and anti-neurofilament antibody, which labels young neurons in the wall of the neural tube between the roof and floor plates (Fig. 3G,H). Collectively, these results provide strong evidence that normal dorsoventral patterning of the neuraxis occurred in LBIs.

Finally, it was determined whether mesodermal patterning occurred normally in LBIs. Labeling with Not-1 and *Shh* as described above and previously (Yuan and Schoenwolf, 1998) showed that axial mesoderm (notochord) formed in LBIs. Also as shown previously (Yuan and Schoenwolf, 1998) and confirmed here (data not shown), paraxial mesoderm (somites) that expressed *paraxis* formed in LBIs. Additionally, it was shown that LBIs expressed markers of intermediate mesoderm (*Pax2;* Fig. 4A,B,E,F) and lateral plate mesoderm (*cytokeratin*, Fig. 4G; and *nodal*, not shown, but see Yuan and Schoenwolf, 1998). Moreover, LBIs (like controls; Fig. 5A) expressed a pan-heart marker, MF-20, as well as the two available regional heart markers, *VMHC1* (not shown) and *AMHC1* (Fig. 5B). Collectively, these results provide strong evidence that mesodermal patterning occurred normally in LBIs, with all mediolateral subdivisions of the mesoderm forming at the appropriate craniocaudal level. We conclude from type-1 experiments that LBIs cultured for 36 hours reconstitute a fully patterned body plan.

Type-2 experiments: reconstitution of the organizer in lateral blastoderm isolates is required for formation of a fully patterned body plan

In type-1 experiments, a fully patterned body plan forms in LBIs after 36 hours in culture. Such patterning could arise de novo, from previously uncommitted cells in LBIs.
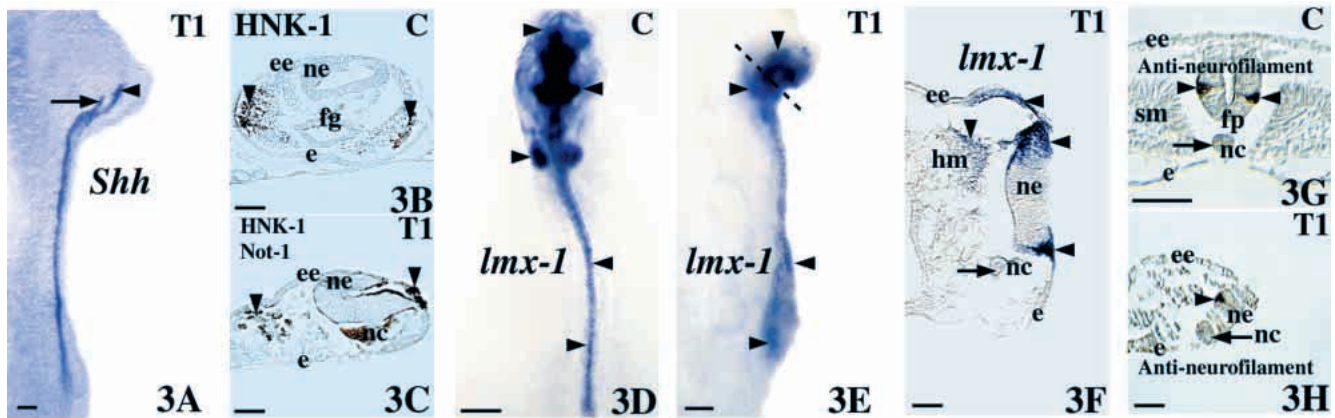
Fig. 3. Type-1 (T1) experiments. LBIs cultured 36 hours and unoperated control embryos (C) examined with in situ hybridization and/or immunocytochemistry. (A) LBI cultured 36 hours and labeled with *Shh* to confirm our previous study (using another marker; Yuan and Schoenwolf, 1998) that the ventral midline of the axis (notochord, arrow; floor plate of neural tube, arrowhead) formed following reconstitution of the organizer. (B) Transverse section of a control embryo (C), showing the expression of the dorsal neuraxial marker HNK-1 in neural crest cells, recently emigrated from the roof of the incipient neural tube (arrowheads). (C) Transverse section of an LBI cultured for 36 hours, showing the expression of HNK-1 in neural crest cells (arrowheads), recently emigrated from the roof of the half neural tube. The notochord is labeled with Not-1. Control embryo (D) and LBI (E) labeled with *lmx-1* (arrowheads). (F) Transverse section of the LBI shown in E at the level indicated by the black dashed line. Arrowheads indicate patches of *lmx-1* labeling nc, notochord (labeled with Not-1 in C; arrow in F-H). Transverse sections of a control embryo (G) and an LBI cultured 36 hours (H) showing labeling with anti-neurofilament antibody (arrowheads). e, endoderm; ee, epidermal ectoderm; fp, floor plate of neural tube; hm, head mesenchyme; nc, notochord (arrow); ne, neural ectoderm; sm, somite. Bars, 100 µm (3A, E); 50 µm (3B, C, F-H); 200 µm (3D).

Alternatively, some pre-patterning of LBIs could exist that occurs independently of reconstitution of the organizer. We conducted type-2 experiments to distinguish between these possibilities and to ask whether reconstitution of the organizer, which is shown in type-1 experiments to be *sufficient* for formation of a fully patterned body plan, is also *required* for its formation.

In type-2 experiments, LBIs were constructed that lacked inducer lappets (Fig. 1B). Such LBIs never reconstitute an organizer (Yuan and Schoenwolf, 1998). As shown previously and confirmed here, such LBIs also never reconstitute a notochord and, consequently, fail to label with notochordal markers such as Not-1 (Fig. 6A,B). Several other markers were examined to determine to what extent regionalization occurred in the absence of reconstitution of the organizer. At the time LBIs were constructed (time 0; stage 3d/4), LBIs weakly expressed the pan-neural marker *Sox-2*, as well as the regional marker *Otx2*, but these markers were no longer expressed by 36 hours in culture (Table 1). Additional neuraxial and mesodermal markers were examined after 36 hours in culture of LBIs lacking inducer lappets. None of the four neuroectodermal markers surveyed (*Sox-2*, *fgf8*, *Otx2* and
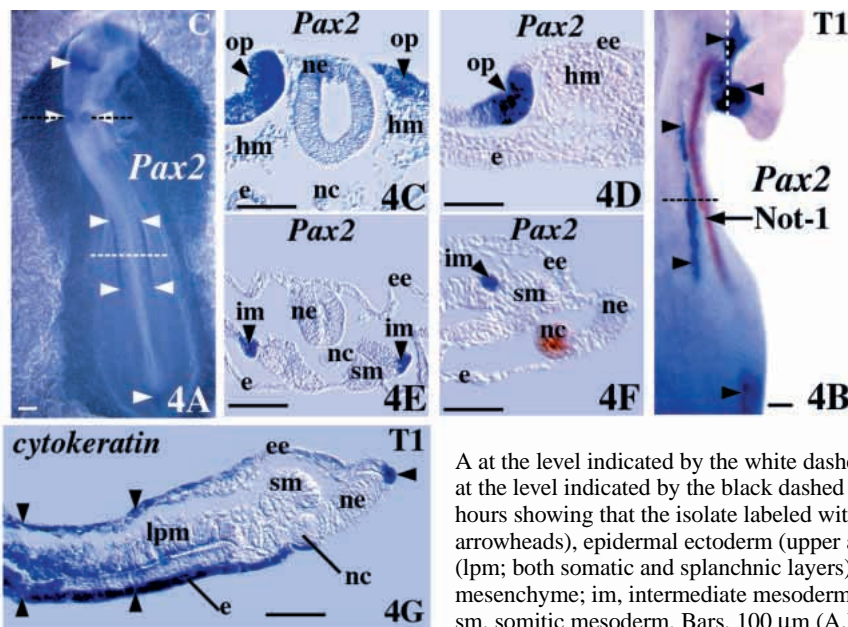
Fig. 4. Type-1 (T1) experiments. To determine whether the intermediate mesoderm and lateral plate mesoderm formed in LBIs we used markers specific for these two rudiments: *Pax2* and *cytokeratin*, respectively. (A) Control embryo (C) labeled with *Pax2* (arrowheads). (B) LBI cultured 36 hours and double-labeled with Not-1 (arrow) and *Pax2* (arrowheads). (C) Transverse section of the control embryo shown in A at the level indicated by the black dashed line. (D) Transverse section of the LBI shown in B at the level indicated by the white dashed line. (E) Transverse section of the control shown in A at the level indicated by the white dashed line. (F) Transverse section of the LBI shown in 4B at the level indicated by the black dashed line. (G) Transverse section of an LBI cultured 36 hours showing that the isolate labeled with *cytokeratin*, a marker of endoderm (lower arrowheads), epidermal ectoderm (upper arrowheads), and especially of lateral plate mesoderm (lpm; both somatic and splanchnic layers). e, endoderm; ee, epidermal ectoderm; hm, head mesenchyme; im, intermediate mesoderm; op, otic placode; nc, notochord; ne neuroectoderm; sm, somitic mesoderm. Bars, 100 µm (A,B); 50 µm (C-G).
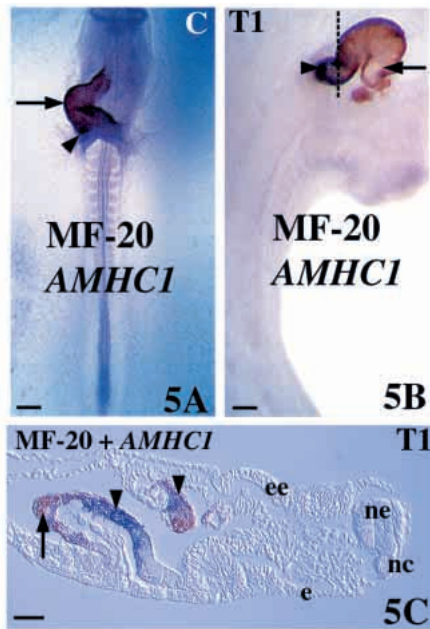
Fig. 5. Type-1 (T1) experiments. (A) Control embryo (C) double-labeled with MF-20 (brown; arrow) and *AMHC1* (purple overlapping brown; arrowhead). (B) LBI cultured 36 hours and double-labeled with MF-20 (brown; arrow) and *AMHC1* (purple overlapping brown; arrowhead). (C) Transverse section at the level indicated by the dashed line in B. *AMHC1* and MF-20 (arrowheads); MF-20 only (arrow). e, endoderm; ee, epidermal ectoderm; nc, notochord; ne, neuroectoderm. Bars, 200 μm (A); 100 μm (B); 50 μm (C).
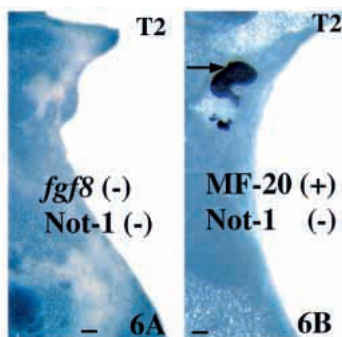
Fig. 6. Type-2 (T2) experiments to determine whether the organizer is required for the reconstitution of the body plan. (A) LBI cultured 36 hours and double-labeled with *fgf8* and Not-1. In the absence of the inducer lappet and, consequently, in the absence of reconstitution of the organizer, labeling with both markers is negative. (B) LBI cultured 36 hours and double-labeled with MF-20 and Not-1. In the absence of the inducer lappet and, consequently, in the absence of reconstitution of the organizer, Not-1 labeling is negative, but MF-20 labeling is positive (arrow). Bars, 100 μm.

Hoxb9) were expressed (Table 1), nor were any of several mesodermal markers (Not-1, *paraxis*, *Pax2*, and *Nodal*; Fig. 6A), except a heart marker, MF-20 (Fig. 6B). Because rostral endoderm (but not caudal endoderm) is capable of inducing heart (Antin et al., 1994; Sugi and Lough, 1994), type-2 experiments were repeated with 6 LBIs at stage 3d/4 that lacked endoderm as well as the inducer lappet. All 6 failed to

express MF-20 after 36 hours in culture, suggesting that the organizer may be acting indirectly by patterning the endoderm. This possibility is being explored elsewhere.

Collectively, the results from type-2 experiments provide strong evidence that reconstitution of the organizer is required for the formation of a fully patterned body plan in LBIs, and they also suggest that formation and patterning of the heart either (1) requires the organizer at an earlier stage than that at which LBIs were made (i.e., prior to the late gastrula/early neurula stage) or (2) occurs independently of the organizer. Regardless of which alternative is correct, the results from type-2 experiments show clearly that the ingressed cardiac mesoderm, or the ingressed cardiac mesoderm plus the surrounding microenvironment, is already sufficient to form a heart at the time (stage 3d/4) that LBIs lacking inducer lappets were constructed.

Type-3 experiments: LBIs from early mid-gastrula stage embryos (stage 3a) do not express a heart marker in the absence of reconstitution of the organizer

To attempt to distinguish between the two possibilities above, we modified our experiments by making LBIs at the mid-gastrula stage (stage 3a/b; Fig. 1C). The primitive streak and 250 μm of adjacent lateral blastoderm on the 'left' side of the blastoderm (when viewed ventrally in New culture) was extirpated. The 'right' side of the blastoderm then served as a control, containing both the inducing and responding tissues, whereas the 'left' (experimental) side lacked inducing tissue. After 36 hours in culture, blastoderms were double-labeled with MF-20 and Not-1. All of the control LBIs expressed both MF-20 and Not-1, indicating, respectively, that a heart formed and a new axis was reconstituted (Fig. 7, right side of figure [R]). However, only 3 of 17 experimental LBIs expressed MF-20 (Table 1), and none of these expressed Not-1 (Fig. 7, left side of figure). Examination of videotapes taken of type-3 embryos at the time of isolation revealed that all 3 embryos that formed heart in experimental LBIs were at stage 3b. This result shows that the cardiac mesoderm, or the cardiac mesoderm plus the surrounding microenvironment, is becoming sufficient to form a heart at stage 3b. The failure of the heart to form in the remainder of the experimental LBIs (at stages 3a and 3b), correlated with the lack of axis reconstitution, suggests that formation of the heart requires reconstitution of the organizer, and that this requirement occurs prior to the formation of the fully elongated primitive streak (i.e., prior to stage 3d/4).

However, there is another interpretation of this experiment that needs to be considered. The prospective cardiac mesodermal cells are located within the primitive streak and flanking epiblast at stage 3a/b (from 125-750 μm caudal to the cranial end of the primitive streak) and are undergoing ingression through the primitive streak; their ingression is virtually complete by stage 3d/4 (Garcia-Martinez and Schoenwolf, 1993; Garcia-Martinez et al., 1993; data in preparation). But the stage at which their ingression is initiated is not known, nor is the minimum number of ingressed cells required to form a heart. Thus, it is possible that the heart fails to form in type-3 experimental LBIs because the precursor cells required to form heart have not yet ingressed into the interior of the blastoderm or that an insufficient number of

Fig. 7. Type-3 (T3) experiments to determine whether the heart can form in the absence of reconstitution of the organizer at the mid-gastrula stage (stage 3a/b). LBIs cultured 36 hours and double-labeled with MF-20 and Not-1. On the control (apparent right; R) side, which contained the inducer for organizer/notochord reconstitution, both a heart (MF-20 [+], arrowhead) and notochord (Not-1 [+], arrow) formed following reconstitution. On the experimental (apparent left; L) side, which lacked the inducer for organizer/notochord reconstitution, neither a heart (MF-20 [−]) nor a notochord (Not-1 [−]) formed in the absence of reconstitution of the organizer/notochord. Bar, 100 μm.
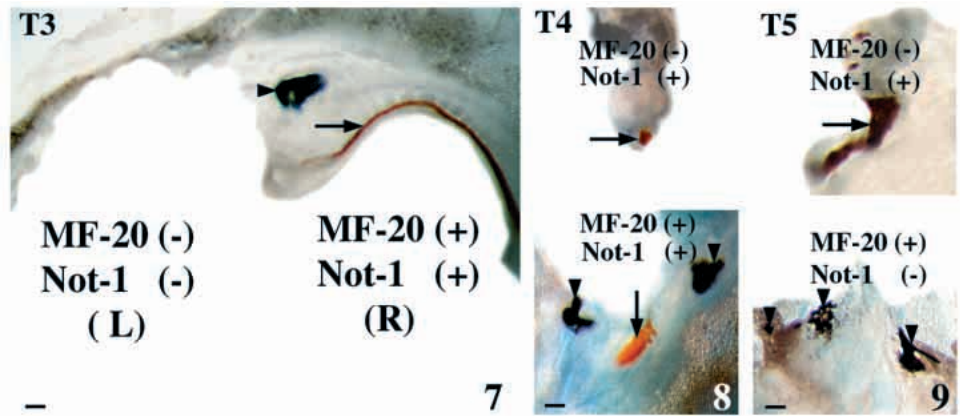
Fig. 8. Type-4 (T4) experiments. Cranial transverse blastoderm isolates (top panel) that contained an organizer formed a notochord (Not-1 [+]; arrow), but not a heart (MF-20 [−]), suggesting that an insufficient number of ingressed cardiac mesodermal cells were present. Caudal transverse blastoderm isolates (bottom panel) that were capable of reconstituting an organizer and had a sufficient number of ingressed cardiac mesodermal cells usually formed a heart (MF-20 [+]; arrowhead); the notochord formed in 50% of the cases (Not-1 [+]; arrow). Bar, 100 μm.

Fig. 9. Type-5 (T5) experiments. Cranial transverse blastoderm isolates (top panel) that were capable of reconstituting an organizer usually formed a notochord (Not-1 [+]; arrow), but not a heart (MF-20 [−]), suggesting (as in type-4 experiments) that an insufficient number of ingressed cardiac mesodermal cells were present in the cranial isolates. Caudal transverse blastoderm isolates (bottom panel) that contained both an organizer and a sufficient number of ingressed cardiac mesodermal cells usually formed a heart (MF-20 [+]; arrowheads). However, the notochord failed to form in all cases probably because of mechanical problems that prevented organizer regression (Not-1 [-]). Bar, 100 μm.

ingressed mesodermal cells exists. In support of this possibility, examination of histological sections of stage 3a blastoderms revealed a paucity of ingressed mesodermal cells at distances greater than 250 μm lateral or cranial to the primitive streak (data not shown).

To distinguish between whether the organizer is required for formation of the heart or whether insufficient ingressed precursor cells exist in LBIs, we conducted type-4 through -7 experiments. Collectively, these experiments provide strong evidence that formation and patterning of the heart is under the control of the organizer. Moreover, they show that formation of the heart requires both an inducer (i.e., the organizer or its immediate derivatives) and sufficient ingressed competent mesodermal cells as a responder.

Type-4, -5 and -6 experiments: transverse blastoderm isolates from early mid-gastrula stage embryos (stage 3a) form heart only when both an organizer and ingressing/ingressed prospective cardiac mesodermal cells are present

To establish whether formation and patterning of the heart is under the control of the organizer, 6 kinds of transverse blastoderm isolates were generated (TBIs) at stage 3a: (1) TBIs containing organizer and lacking most ingressed mesoderm (except the ingressed mesoderm immediately adjacent to the organizer) at the time of isolation (Fig. 1D, cranial isolate, type-4.1); (2) TBIs lacking both organizer and most ingressed mesoderm at the time of isolation, but expected (from previous, unpublished experiments on blastoderms at this stage; to be reported elsewhere) to be capable of reconstituting organizer (Fig. 1E, cranial isolate, type-5.1); (3) TBIs lacking organizer and containing copious ingressing/ingressed mesoderm at the time of isolation, but expected to be capable of reconstituting the organizer (Fig. 1D, caudal isolate, type-4.2); (4) TBIs containing both organizer and copious ingressing/ingressed

mesoderm at the time of isolation (Fig. 1E, caudal isolate, type-5.2); (5) TBIs containing organizer and prospective cardiac mesoderm within the primitive streak at the time of isolation (Fig. 1F, cranial isolate, type-6.1); and (6) TBIs lacking organizer and containing copious ingressing/ingressed mesoderm at the time of isolation, but expected to be *incapable* of reconstituting organizer (Fig. 1F, caudal isolate, type-6.2).

Cranial isolates from type-4 experiments (i.e., type-4.1) failed to express MF-20 (i.e., failed to form heart) in all cases, but they expressed Not-1 (i.e., formed organizer and notochord) in all cases (Fig. 8, top panel). Cranial isolates from type-5 experiments (i.e., type-5.1) also failed to express MF-20 in all cases, and they expressed Not-1 (i.e., reconstituted organizer/notochord) in most cases (Fig. 9, top panel). Thus, if insufficient ingressed prospective cardiac mesodermal cells are present (i.e., as in cranial isolates from type-4 and -5 experiments), a heart fails to form regardless of whether an organizer is present (as in cranial isolates from type-4 experiments) or is initially absent and then reconstituted (as in cranial isolates from type-5 experiments).

Caudal isolates from type-4 (i.e., type-4.2) and -5 (i.e., type-5.2) experiments usually (or always in type-5 experiments) expressed MF-20 (Fig. 8, 9, bottom panels), but they expressed Not-1 only 50% of the time in type-4 experiments and never in type-5 experiments (Fig. 8, 9, bottom panels). Based on pilot studies, we expected that not all caudal isolates from type-4 experiments would reconstitute (because of their small size and fragility). Moreover, in other pilot studies Hensen's node failed to undergo normal regression and to form notochord when left attached to the primitive streak and isolated from the immediately lateral epiblast (presumably a mechanical problem), yet maintained its organizer activity (e.g., isolated Hensen's nodes can readily induce ectopic embryos; Dias and Schoenwolf, 1990). Labeling Hensen's node with a fluorescent dye at the time that isolates were constructed revealed that cells
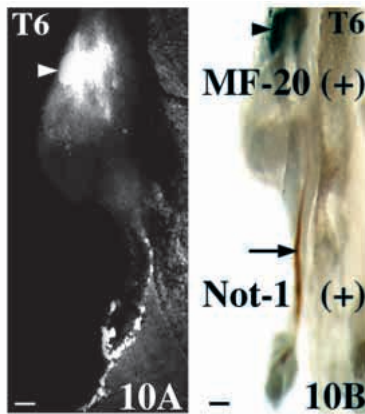
Fig. 10. Type-6 (T6) experiments. (A) Embryo viewed with fluorescence showing that labeled prospective cardiac mesodermal cells ingressed from the primitive streak into the heart-forming area. White arrowhead indicates area to compare with black arrowhead in 10 B. (B) Positive labeling of the same specimen shown in A with MF-20 (+) and Not-1 (+) shows, respectively, that both a heart (arrowhead) and notochord (arrow) formed. Bars, 100 μm.

in the cranial end of the primitive streak eventually moved bilaterally rather than directly caudally (data not shown). Despite the problem with formation of the notochord in type-4 and -5 caudal isolates, these experiments show clearly that in the presence of both sufficient ingressed prospective cardiac mesodermal cells (as in caudal isolates from type-4 and -5 experiments) and an organizer (as in caudal isolates from type-5 experiments) or reconstituted organizer (as in caudal isolates from type-4 experiments), a heart usually (or always in type-5 experiments) forms.

For type-6 experiments, we reasoned that in cranial isolates constructed as shown in Fig. 1F, prospective cardiac mesodermal cells might undergo ingression into the cranial

half of the embryo; however, we were concerned that because cells normally migrate laterally from the primitive streak, ingression might be inhibited in the absence of lateral tissues. Thus, we labeled prospective cardiac mesodermal cells within the primitive streak with a fluorescent dye and followed their movement. In only 5 of 23 cases did these cells ingress and move into the cranial TBIs (Fig. 10A); in the remainder of the cases, labeled cells retained essentially their original positions and further development of the TBIs failed to occur (data not shown). In all 5 of these cases, and in only these 5 cases, the cranial isolate expressed MF-20 and Not-1 (Table 1; Fig. 10B). Although it is not entirely clear why the notochord did not form in the remaining embryos, we speculate that regression of the node is linked to ingression of cells more caudally, and that when the latter failed to occur, so did the former. In summary, in all cases in which the cranial TBIs contained both organizer and a sufficient number of ingressed prospective cardiac mesodermal cells, a heart formed. This provides strong evidence that formation of the heart is under the control of the organizer, and that such control is exerted prior to formation of the fully elongated primitive streak stage.

Type-7 experiments: 'ingressed' prospective cardiac mesodermal cells from the primitive streak of early mid-gastrula stage embryos (stage 3a) cannot self-differentiate heart in the absence of the organizer

In type-4 through -6 experiments, we could not generate one important combination that is necessary to test rigorously the hypothesis that formation and patterning of the heart is under the control of the organizer: isolates containing ingressed cardiac mesodermal cells but lacking organizer. This is because TBIs that might have given this combination (i.e., type-4 caudal TBIs) reconstituted an organizer. Consequently, type-7 experiments were conducted (Fig. 1G) in which prospective cardiac mesodermal cells from the stage 3a primitive streak were grafted into germ cell crescent isolates (GCIs) of stage

Fig. 11. Type-7 (T7) experiments. (A) Embryo viewed with fluorescence showing that labeled prospective cardiac mesodermal cells spread throughout the germ cell crest isolate. Arrowheads, labeled, grafted cells. (B) Negative labeling of the same specimen shown in A with MF-20 [−] and Not-1 [−] shows, respectively, that neither a heart nor a notochord formed. (C) Another specimen in which an ectopic embryo formed. Positive labeling with anti-quail ([+]; carets), MF-20 ([+], arrowheads), and Not-1 ([+], arrow) occurred, indicating that

both a heart and notochord formed. Co-localized labeling of anti-quail and MF-20 occurred, but not of anti-quail and Not-1. (D) Transverse section of the ectopic embryo shown in C at the level indicated by the black dashed line. Co-localized labeling of anti-quail ([+], carets) and MF-20 ([+], arrowheads) is evident. cm, cardiac mesoderm; e, endoderm; ee, epidermal ectoderm. Bars, 200 μm (A,B); 100 μm (C); 50 μm (D).
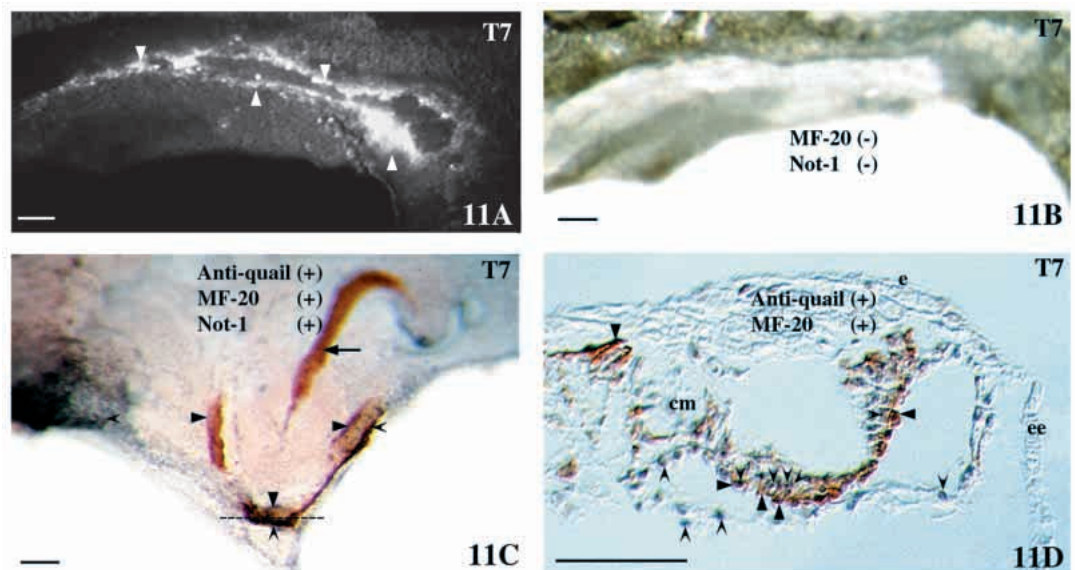
3d/4 hosts. In some cases, grafts were labeled with a fluorescent dye, and in others quail grafts were placed in chick hosts, to track grafted cells. In all cases examined, grafted cells spread throughout the GCI, rather than remaining clumped at their original site (Fig. 11A). In 6 of 10 cases, no evidence of an ectopic embryo was found (i.e., an embryo induced/organized from host tissues by the graft) and a heart failed to form (Fig. 11B). Thus, these 6 cases demonstrated that 'ingressed' prospective cardiac mesodermal cells cannot self-differentiate in the absence of the organizer.

In the remaining 4 cases, an ectopic embryo was evident (Fig. 11C). These 4 cases were all from quail grafts to chick hosts (2 others from quail grafts to chick hosts did not form an ectopic embryo, so this finding was not correlated with the type of donor tissue used). A sample of 2 of these was chosen for further analysis. These were both triple labeled with QCPN (an anti-quail antibody) and with antibodies to Not-1 and MF-20. Both ectopic embryos contained a heart, which labeled with MF-20, and a notochord, which labeled with Not-1. Partial co-localization of QCPN and MF-20 indicated that donor cells contributed to these ectopic hearts (Fig. 11C,D). In contrast, co-localization of QCPN and Not-1 did not occur (Fig. 11C), indicating that the ectopic notochord was induced by the donor graft from host epiblast cells (i.e., an organizer was induced that self-differentiated notochord). Finally, quail cells contributed heavily to the surrounding non-heart mesoderm (i.e., mesoderm that did not express MF-20) and the endoderm of the ectopic embryo. We conclude from these 4 cases, in combination with the results from type-4 through type-6 experiments, that formation of the heart by the 'ingressed' prospective cardiac mesodermal cells is under the control of the organizer.

DISCUSSION

This investigation has resulted in two new major findings. First, it shows that reconstitution of the organizer is both sufficient and required for reconstitution of a fully patterned body plan in LBIs, and that normal craniocaudal, dorsoventral, and mediolateral axes are re-established. Second, it shows that formation and patterning of the heart is under the control of the organizer, and that this control occurs at the early to mid-gastrula stage, prior to formation of a fully elongated primitive streak. By the fully elongated primitive streak stage, differentiation of the heart can occur independently of the organizer.

Reconstitution of the organizer is both sufficient and required for formation of a fully patterned body plan in LBIs

Analysis of the development of supernumerary embryos in avian blastoderms following transplantation of Hensen's node to ectopic sites (typically, the cranial extraembryonic region) has provided considerable insight into body plan formation (Gallera, 1971; Dias and Schoenwolf, 1990; Storey et al., 1992, 1995; and references therein). Most of these experiments focused on the problem of neural induction and regionalization, and the more recent ones, conducted since the advent of molecular markers, have shown clearly that the organizer is sufficient to induce and pattern a normal neuraxis.

Moreover, the reconstituted organizer when transplanted to an ectopic site also is sufficient (Yuan and Schoenwolf, 1998).

Here, we provide further evidence that the reconstituted organizer is sufficient to establish a fully patterned neuraxis as well as a complete body plan. All craniocaudal and dorsoventral markers of the neuroectoderm examined here were expressed in the neuraxis of LBIs undergoing reconstitution (type-1 experiments). These markers were expressed in the appropriate spatial pattern, with the appropriate timing, and confirm results obtained earlier in studies in which the node, or node and part of the primitive streak, were extirpated from whole embryos developing either in ovo or in culture (Waddington, 1932; Waterman, 1936; Grabowski, 1956; Butros, 1967; Gallera and Nicolet, 1974; Smith and Schoenwolf, 1989; Darnell et al., 1992; Schoenwolf and Yuan, 1995; Psychoyos and Stern, 1996). Furthermore, we show that secondary inductions occur within LBIs undergoing reconstitution (type-1 experiments), such that peripherally induced structures – the otocysts – also form normally in reconstituting LBIs. Finally, we show that the reconstituted organizer is sufficient to pattern the mesoderm, as well as the ectoderm. Thus, head and trunk mesoderm form properly in LBIs undergoing reconstitution (type-1 experiments), and normal mediolateral regionalization of the mesoderm occurs. Again, all of the markers we tested for the major mediolateral subdivisions of the mesoderm appeared in the appropriate spatial and temporal order.

We showed previously (Yuan and Schoenwolf, 1998) and have confirmed here that LBIs, regardless of whether they are constructed from the right or left side of the embryo, reconstitute a *left* half-embryo, as indicated by the expression of *nodal* in both right and left reconstituted LBIs (also see Levin and Mercola, 1998). This supports the model proposed by Levin, Tabin and co-workers (Levin et al., 1995, 1997; Logan et al., 1998). The node at the fully elongated primitive streak stage expresses *Sonic hedgehog* (*Shh*) uniformly. By the head process stage, *Shh* expression becomes restricted to the left side of the node. It has been proposed that this restriction results from asymmetric activity of activin to the right of the node, which suppresses *Shh* expression on the right side. Asymmetric expression of *Shh* in the left side of the node in turn leads to left-sided expression of *nodal/Pitx2*. In reconstituting LBIs, the node expresses *Shh* uniformly within 10-12 hours (Yuan and Schoenwolf, 1998), and subsequent asymmetric expression is not obvious. Our results suggest that asymmetric expression of *Shh* within the node fails to occur in LBIs, because LBIs obtained from either side would lack the suppressive signal (i.e., it does not exist on the left side, and the tissue lateral to the node is removed on both the right and left sides, presumably removing the suppressive signal on the right side; alternatively, on the right side, reconstitution may somehow 'erase' the suppressive signal). Because *Shh* expression is present uniformly in the node of LBIs obtained from both the right and left sides, and only one side of the blastoderm is present (i.e., only the right side in LBIs obtained from the right side and only the left side in LBIs obtained from the left side), *nodal* expression is induced in the existing lateral plate mesoderm regardless of its original sidedness.

Although it is clear from the past and present studies that the organizer, or the reconstituted organizer, is sufficient to established a complete body plan in avian embryos, past

studies did not address whether the organizer is *required* to form a complete body plan in these embryos. Previous studies in which the node was ablated from whole embryos could not provide this information, because the organizer reconstitutes (Psychoyos and Stern, 1996). We have shown previously using LBIs that reconstitution of the organizer occurs because of an interaction between an inducer and a responder (Yuan and Schoenwolf, 1998). In type-2 experiments of the present study, we used LBIs at the late gastrula/early neurula stage that *lacked* the inducer. In the absence of the inducer, LBIs fail to reconstitute the organizer, and a body plan also fails to form. This provides strong evidence that the formation of the body plan requires the organizer, and that the body plan is not pre-patterned in LBIs at the late gastrula/early neurula stage.

However, our results do not rule out the possibility that other signaling centers, such as a head organizer (Bouwmeester and Leyns, 1997; Beddington and Robertson, 1998, 1999), are also required. Because LBIs lack cranial tissue, it is unlikely that a head organizer would be present after LBIs are constructed. Nevertheless, we can imagine two scenarios in which a head organizer might still be involved: (1) LBIs might reconstitute a head organizer concomitantly with, but independently of, reconstitution of the organizer; (2) reconstitution of a head organizer might be dependent on prior reconstitution of the organizer; that is, in avian embryos, the organizer may give rise to cells that form or contribute to the head organizer, or the organizer may induce the head organizer. We do not believe that a third possibility is likely: that a head organizer had already functioned and patterned the LBI prior to its construction. Two results do not support this possibility: (1) based on fate mapping (Garcia-Martinez et al., 1993; Yuan et al., 1995b), essentially all cranial neural plate would have been removed in constructing the LBIs; (2) LBIs constructed earlier (i.e., at stage 3a/b) also seem to reconstitute the full length of the neuraxis including its cranial levels. Further studies will be required to address this issue when the appropriate markers that define the head organizer become available in avian embryos.

Reconstitution of the organizer is necessary for upregulation of gene expression during formation of the body plan

In type-1 experiments, LBIs at the time of their construction weakly express *Otx2* and *Sox2*. Within 36 hours in culture, the expression of these genes is dramatically upregulated. In contrast, in type-2 experiments in which reconstitution of the organizer does not occur because LBIs lack inducer, the expression of *Otx2* and *Sox2* is down regulated, becoming undetectable by 36 hours in culture. An interpretation of this result comes from recent studies of the signaling molecules responsible for neural induction: noggin (Smith and Harland, 1992; Lamb et al., 1993), follistatin (Hemmati-Brivanlou et al., 1994; Yamada et al., 1995), and chordin (Sasai et al., 1995), all of which are secreted by the organizer. In type-2 experiments, LBIs lacking the inducer fail to reconstitute the organizer, presumably leading to the loss of expression of these neural-inducing molecules. Consequently, pan-neural and regional-neural markers are lost subsequently. Moreover, the expression of other regional markers that normally occurs after the late gastrula/early neurula stage, such as some neuroectodermal markers (i.e., *fgf8* and *Hoxb9*) and mesodermal markers (i.e., *paraxis*, *Pax2*, and nodal), also fails

to occur (i.e., their expression is not initiated). Collectively, the results from type-2 experiments suggest that regulation of gene expression during embryogenesis requires the formation of an organizer, and that the organizer plays a key role in establishing the body plan through the secretion of instructive molecules.

Differentiation of the prospective cardiac mesoderm at the late gastrula/early neurula stage occurs independently of the organizer

Heterotopic grafting studies of the primitive streak and epiblast have shown that the pattern of cell displacement during gastrulation and the determination of cell fate are still largely labile at the late gastrula/early neurula stage (Schoenwolf and Alvarez, 1991; Garcia-Martinez and Schoenwolf, 1992). Thus, these processes are largely controlled by the local environment in which a cell resides, and plasticity is not limited to fates within a given germ layer (Garcia-Martinez et al., 1997). One population of cells provides a major exception to this finding. The cells of the prospective notochord contained within Hensen's node (i.e., prior to their ingression) are fully committed to a notochordal fate at late gastrula/early neurula stage, although their pattern of migration can be altered experimentally (Garcia-Martinez and Schoenwolf, 1992; Selleck and Stern, 1992; Inagaki et al., 1993).

Type-2 experiments using LBIs lacking the inducer show that at late gastrula/early neurula stage, the ingressed cardiac mesoderm has the potential to differentiate independently of the organizer. In contrast, other populations of ingressed mesodermal cells (i.e., head mesoderm, paraxial mesoderm, and lateral plate mesoderm) have not acquired this ability and fail to form in such isolates (note: the notochord has not yet ingressed at this stage). Whether cardiac mesoderm acquires the ability to self-differentiate shortly after its ingression or whether further interactions (independent of the organizer) occur within such isolates to induce the heart remains to be established (e.g., between the endoderm and ingressed mesoderm; see below). LBIs constructed at the early mid-gastrula stage (i.e., stage 3a) and lacking an inducer (experimental side in type-3 experiments) fail to form a heart. There are two possible explanations of this result: either cardiac mesoderm has not yet acquired the ability to self-differentiate, or an insufficient number of cardiac mesodermal cells has ingressed by this stage. In either case, the control of cardiac differentiation could occur independently of the organizer, or differentiation of the heart could occur under the control of the organizer. Type-4 through -7 experiments were conducted to resolve this issue.

Formation of the heart requires an interaction between the organizer and competent mesodermal cells that is initiated at the early to mid-primitive streak stage

Type-4 to -6 experiments show that a heart forms in TBIs only when two components are present: an organizer or a reconstituted organizer, and sufficient ingressed competent mesodermal cells. Prospective cardiac mesoderm within the primitive streak and epiblast cannot form heart when juxtaposed to an organizer (e.g., type-6 experiments in which ingression failed to occur), unless these cells undergo an epithelial-to-mesenchymal transition and move into the interior of the blastoderm (e.g., type-6 experiments in which ingression

occurred). Thus, prospective cardiac mesodermal cells are not competent to form heart until this transition occurs.

In type-7 experiments, in the absence of the organizer, 'ingressed' prospective cardiac mesodermal cells obtained from the primitive streak of embryos at stage 3a and transplanted to a permissive environment cannot self-differentiate heart; rather, the heart forms only when an organizer is reconstituted in such preparations. Thus, the ingression by itself is not sufficient to cause prospective cardiac mesoderm to form heart. This provides direct evidence that formation and patterning of the heart is under the control of the organizer. This control is initiated during the early to mid-gastrula stage. Also it is the organizer and not the notochord that exerts this control, because type-2 experiments reveal that the heart can form in LBIs at stage 3d/4; that is, prior to formation of the notochord.

We have not been able to determine whether the control exerted by the organizer on ingressed competent mesodermal cells occurs directly or indirectly. Previous studies have shown that formation of the heart from ingressed mesoderm at the early head-process stage (stage 4+/5) requires the presence of cranial endoderm (Antin et al., 1994; Sugi and Lough, 1994). However, removal of endoderm or its rotation craniocaudally (thereby switching cranial and caudal endoderm) in whole embryos fails to inhibit formation of the heart (Inagaki et al., 1993). These studies can be reconciled by proposing that the endoderm provides inductive signals to the ingressed prospective cardiac mesoderm that are necessary for formation of the heart, but that these signals derived from the endoderm are under the control of another region of the embryo, namely, the organizer. Although preliminary, our experiments in which endoderm is removed from LBIs lacking inducer tags (type-2 experiments) support this possibility. Further studies are underway to establish the roles of the endoderm and organizer in induction of the heart.

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