

Continuing organizer function during chick tail development

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SUMMARY

Development of the posterior body (lumbosacral region and tail) in vertebrates is delayed relative to gastrulation. In amniotes, it proceeds with the replacement of the regressed node and primitive streak by a caudal blastema-like mass of mesenchyme known as the tail bud. Despite apparent morphological dissimilarities, recent results suggest that tail development in amniotes is in essence a continuation of gastrulation, as is the case in *Xenopus*. However, this has been inferred primarily from the outcome of fate mapping studies demonstrating discrete, regionalized cell populations in the tail bud, like those present at gastrulation. Our analysis of the tail bud distribution of several molecular markers that are expressed in specific spatial domains during chick gastrulation confirms these results. Furthermore, we present evidence that gastrulation-like ingression movements from the surface continue in the early chick tail

bud and that the established tail bud retains organizer activity. This 'tail organizer' has the expected properties of being able to recruit uncommitted host cells into a new embryonic axis and induce host neural tissue with posteriorly regionalized gene expression when grafted to competent host cells that are otherwise destined to form only extra-embryonic tissue. Together, these results indicate that chick tail development is mechanistically continuous with gastrulation and that the developing tail in chick may serve as a useful experimental adjunct to investigate the molecular basis of inductive interactions operating during gastrulation, considering that residual tail organizing activity is still present at a surprisingly late stage.

Key words: Gastrulation, Tail organizer, Neural induction, Neural regionalization, *Gnot1*, *Brachyury*, T-box genes, Chick embryo

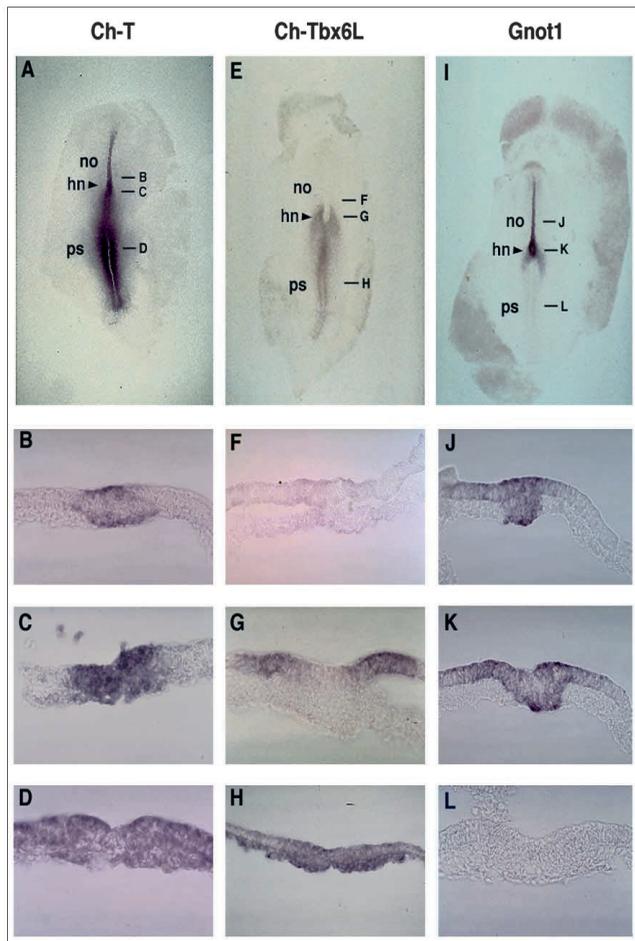
INTRODUCTION

During gastrulation, a uniform population of embryonic cells segregates to form definitive germ layers (definitive ectoderm, mesoderm and endoderm). While the mechanism by which this segregation is achieved may differ slightly from one species to another, the relationship and the fate of definitive germ layers is highly conserved among all vertebrates (Gilbert, 1991; Tam and Quinlan, 1996). An important feature of early gastrulation is the internalization of superficially located mesodermal and endodermal precursors. In amniotes this process occurs via movement of cells through the primitive streak. During this early phase of gastrulation, presumptive neuroectoderm and surface ectoderm precursors remain superficially located. Once formed, the germ layers will undergo a series of inductive interactions to establish and pattern the primary body axis. This induction is mediated by key signalling centers (or 'organizers') which have the defining properties of both contributing to and recruiting other cells into the forming embryonic axis. The properties of these signalling centers are also quite dynamic. The embryonic axis develops in a craniocaudal temporal sequence and there is considerable evidence in amphibians to support the idea first proposed by Mangold (1933), that the organizer contains distinct head, trunk and tail subdomains that function sequentially to 'regionalize' different parts of the CNS with respect to their

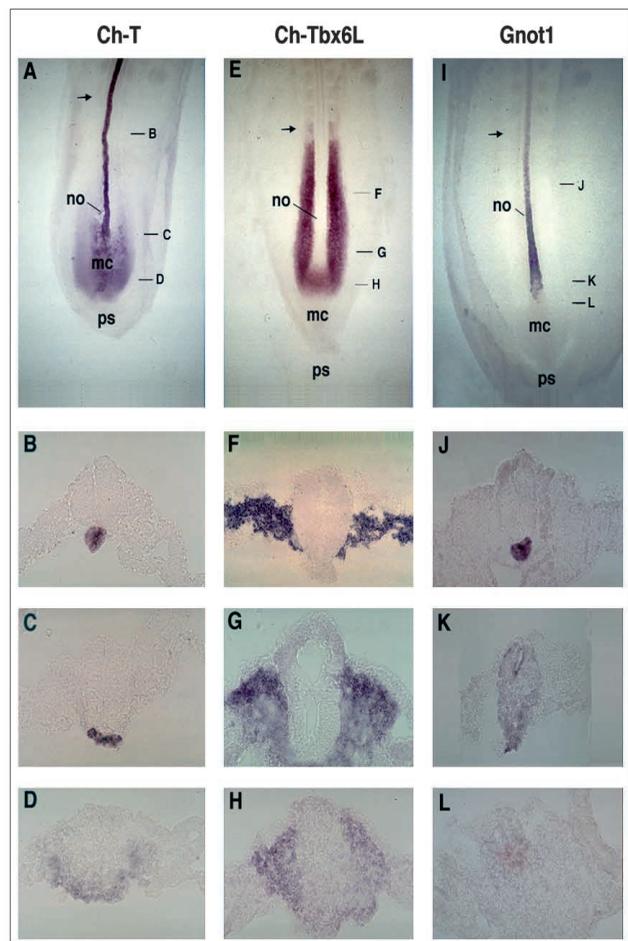
AP (anteroposterior) characteristics (reviewed by Lemaire and Kodjabachian, 1996). Expression patterns of different organizer-related genes in various vertebrate embryos are consistent with structural and functional heterogeneity within the organizer and recent functional studies also point to the presence of separable head and trunk organizer components in mammals, as well as amphibians and fish (reviewed by Tam and Behringer, 1997).

Evidence for the existence and characteristics of a 'tail organizer' is comparatively more nebulous in amniotes and, in fact, entirely different mechanisms have been previously proposed for development of the tail region (the lumbosacral region and caudad in amniotes; Criley, 1969; Holmdahl, 1925a,b, 1939; Lemire et al., 1975; Muller and O'Rahilly, 1987). In the tail bud, the primitive streak and Hensen's node are replaced by a bulb-like structure consisting of a morphologically uniform mass of mesenchyme directly continuous with axial (neural tube, notochord, gut) and paraxial (segmental plate) structures formed during the earlier phases of gastrulation. This appearance has led to the proposal that structures in the tail are formed directly from a 'blastema' without segregation of cells into germ layers (Holmdahl, 1925a,b, 1939; Hughes and Freeman, 1974; reviewed by Griffith et al., 1992). In amphibians, which lack such an apparent mesenchymal condensation, a variety of approaches have been used to demonstrate the continuity of developmental processes during gastrulation and

stage 6



stage 15



tail formation, including analyses of the distribution of molecular markers (Gont et al., 1993); mapping studies with lineage tracers and homotypic grafts (Gont et al., 1993; Tucker and Slack, 1995a,b) and, most notably, the demonstration of functional organizer properties within the tail bud (Gont et al., 1993). However, in amniotes, evidence supporting continuity between gastrulation and tail formation has been more indirect and comes primarily from mapping studies using either lineage tracers in mouse (Wilson and Beddington, 1996) or homotypic grafts in chick (Catala et al., 1995, 1996).

The size and accessibility of the chick embryo and availability of viral expression vectors provides a powerful system to experimentally manipulate gene expression during tail development. If tail development in amniotes is indeed comparable to gastrulation, then such studies would provide an alternate avenue to analyzing the function of genes critical for gastrulation. It was therefore of interest to examine tail development in the chick for various features characteristic of gastrulation. In this paper, we examined the regional distribution of several markers and investigated the movements of cells from the surface during tail bud formation, as well as testing for the presence of a functional tail organizer. Our results show not only that similar cell populations are continuously present through gastrulation and tail elongation, but that mesodermal precursors continue to internalize from the

surface, recapitulating morphogenetic movements during gastrulation. We also demonstrate the presence of a functional organizer with posteriorizing features during tail elongation, strongly supporting the concept that formation of the caudal body axis is conceptually and mechanically a continuation of gastrulation in amniotes.

MATERIALS AND METHODS

Embryos

White Leghorn chick embryos or quail embryos (Truslow Farms) were incubated at 38.5°C and staged as described by Hamburger and Hamilton (1951). Embryos were dissected in phosphate-buffered saline (PBS) and processed for experiments as described below.

Whole-mount in situ hybridization of embryos

Digoxigenin-UTP riboprobes were generated as previously described from either 3' untranslated sequences (*Ch-T* and *Gnot1*) or from coding sequences 3' to the T-box (*Ch-Tbx6L*) (Knezevic et al., 1995, 1997). Other chick riboprobes were generated from plasmids provided by J.C. Izpisua-Belmonte (*Gsc*), A. Simeone (*Otx-2*), A. Bang and M. Goulding (*Pax-3*), R. Lovell-Badge (*Sox-2*) and G. Eichele (*Hoxb-8*). Embryos were prepared for whole-mount in situ hybridization, hybridized with antisense riboprobes, washed, and the hybrids visualized with alkaline phosphatase conjugated anti-digoxigenin as

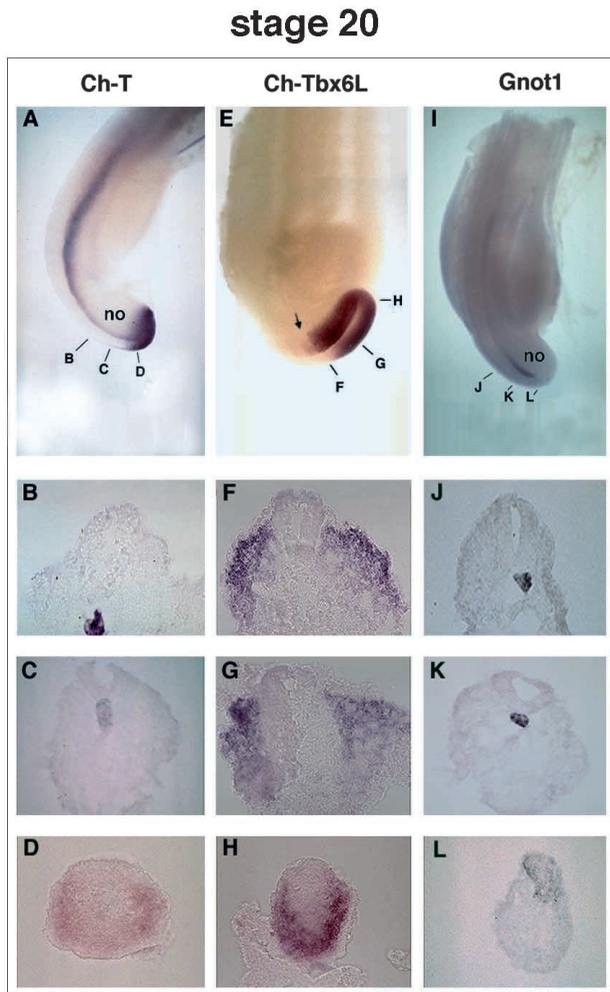


Fig. 1. Expression pattern of primitive streak (*Ch-T*, *Ch-Tbx6L*) and axial mesoderm (*Gnot1*, *Ch-T*) specific molecular markers at stage 6, stage 15 and stage 20. Levels of transverse sections are indicated on the whole-mount panels (A,E,I) for each stage. Arrows in stage 15 A,E and I and stage 20 E indicate the level of the last formed somite. no, notochord; hn, Hensen's node; ps, primitive streak; sp, segmental plate; mc, medullary cord.

described by Conlon and Rossant (1992), except that the length of proteinase K digestion varied from 1-5 minutes depending on the size of the embryos. Upon visualization of the reaction product (usually 0.5 to 6 hours), the reaction was stopped and embryos were stored at 4°C in PBS containing 2.5 mM EDTA and 0.1% Tween-20, and photographed without clearing. Stained embryos were embedded in OCT compound (Tissue-Tek) and 10 µm frozen sections were cut and mounted in Immunon (Thomas Scientific). As a control, sense riboprobes for each gene were also used, and none of these gave a detectable hybridization signal at any of the stages analyzed (data not shown).

Embryos that received quail grafts were first immunostained with QCPN antibody using diaminobenzidine (to reveal quail tissue, described below) followed by hybridization with digoxigenin-UTP riboprobes as above.

Whole-mount immunohistochemistry

Whole-mount immunostaining of embryos with L5 monoclonal antibody (provided by Claudio Stern) was done exactly as described

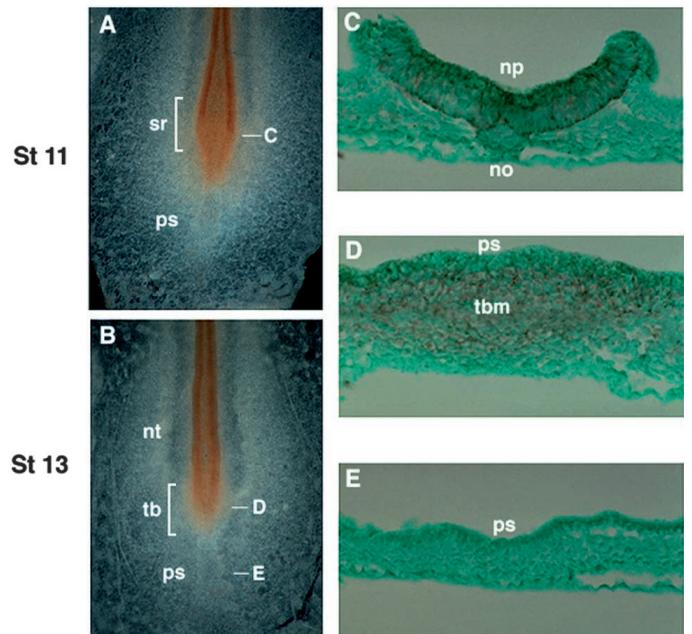


Fig. 2. Expression of the pan-neural marker (L5) during tail bud formation. The L5 epitope is present in the presumptive neuroectoderm of the sinus rhomboidalis at stage 11 (A,C) and most of the central tail bud mesenchyme of stage 13 (B,D). Note that mesodermal precursors located in the ventral rim of tail bud mesenchyme (D) and primitive steak (E) do not express L5. Levels of transverse sections in C,D and E are indicated in A and B. sr, sinus rhomboidalis; ps, primitive streak; nt, neural tube; tb, tail bud; np, neural plate; no, notochord; tbm, tail bud mesenchyme.

by Streit et al. (1997), except that HRP-conjugated goat-anti-rat IgM antibody (Jackson Laboratory) was used and embryos were photographed without counterstaining and clearing. Following immunostaining with L5, embryos that received quail grafts were immunostained with QCPN monoclonal antibody (from Developmental Studies Hybridoma Bank) as described by Streit et al. (1997), except that 1 M LiCl was added to all washes, HRP-conjugated goat-anti-mouse IgG antibody (Jackson Laboratory) was used, and nickel chloride (0.04% final) was added to 0.5 mg/ml of diaminobenzidine (DAB). In some cases, embryos were first stained with QCPN monoclonal antibody using DAB alone, followed by staining with an Engrailed polyclonal antibody (provided by A. Joyner; as described by Davis et al., 1991), using DAB and nickel chloride. Embryos subjected to two rounds of immunostaining were reblocked after the first round. Stained embryos were embedded in paraffin and 10 µm serial sections were cut, counterstained with light green and mounted in *Permount* (Fisher Scientific).

Dil labeling and photoconversion

Dil labeling and photoconversion was done as described by Izpisua-Belmonte et al. (1993). Labeling of the dorsal surface was done in ovo while labeling of the ventral surface was done in a modified New's culture in vitro (New, 1955) in which embryos were removed together with vitelline membrane, placed on a ring of Whatman paper and cultured on a pool of thick albumen (Olszanska et al., 1984). Stained embryos were embedded in paraffin and 10 µm serial sections were cut, counterstained with light green and mounted in *Permount* (Fisher Scientific).

Tail grafting

The tip of the tail (tip mesenchyme only) or region just anterior to it

(caudal end of medullary cord region) were isolated from quail embryos at stage 17-18 (the angle between tail axis and main body axis was $\sim 90^\circ$) and grafted onto the area pellucida or area opaca in stage 4⁻ chick hosts. Each host received grafts on both sides. Host embryos were cultured for 24 hours in modified New's culture (New, 1955), as above. Embryos were fixed in 100% methanol (for L5 immunochemistry) or 4% paraformaldehyde and processed for whole-mount immunohistochemistry and in situ hybridization as described above.

RESULTS

Contiguous expression of a subset of markers during gastrulation and tail bud formation

We and others have observed that several markers specific for axial (*Gnot1* and *Ch-T*) and paraxial mesodermal lineages (*Ch-Tbx6L*) during gastrulation, are also expressed in the tail bud (Kispert et al., 1995; Knezevic et al., 1995, 1997; Stein and Kessel, 1995). Notably, expression of organizer-related markers in developing tail bud was restricted to 'caudal' components (trunk/tail organizer). Not surprisingly, rostral (head) organizer-associated genes that are expressed transiently in the early Hensen's node at gastrulation (Bally-Cuif et al., 1995; Izpisua-Belmonte et al., 1993), were entirely absent from tail bud at all stages (chick *Gsc* and *Otx-2*; data not shown). The expression of representative caudal organizer-associated markers was evaluated during the transition period from streak to tail bud. As previously reported, *Gnot1* and *Tbx6L* were expressed in complementary domains during gastrulation. *Gnot1* was in Hensen's node and notochord (Fig. 1 stage 6, I-K) and *Ch-Tbx6L* in primitive streak and segmental plate (Fig. 1 stage 6, E,G,H), while *Ch-T* was expressed in nascent and axial mesoderm along the entire axis (Fig. 1 stage 6, A-D).

Formation of the tail bud starts in the 13 somite embryo (stage 11) as cells of Hensen's node and primitive streak begin to accumulate caudally in a bulbous mass of uniform mesenchyme. This transformation into tail bud is completed by the 24-28 somite stage (stage 15). Surprisingly, despite its uniform morphological appearance, gene expression in the forming tail bud suggested a segregation of Hensen's node- and primitive streak-derived cells. Expression patterns were comparable throughout this transition period (stage 11-16) and only data for stage 15 is shown in Fig. 1. *Ch-T* and *Ch-Tbx6L* were both selectively expressed in a superficial medial to lateral ventral rim of tail bud mesenchyme (Fig. 1 stage 15, A,D,E,H), in addition to continued expression of *Ch-T* in the notochord (Fig. 1 stage 15, A-C) and of *Ch-Tbx6L* in the segmental plate (Fig. 1 stage 15, E-G). *Gnot1* expression was detected in the formed and nascent notochord (Fig. 1 stage 15, I-K) but did not extend very posteriorly from this region compared to *Ch-T* (Fig. 1 stage 15, A,D,I,L). The caudal limit of *Gnot1* expression corresponded to the chordoneural hinge region (the point where caudal neural tube and notochord unite; Fig. 1 stage 15, K); located between the residual Hensen's node (*Gnot1* and *Ch-T* positive) and primitive streak (*Ch-T* positive).

Cells located within the central region of the condensing tail bud failed to express primitive streak or node-specific markers (Fig. 1 stage 15, D,H,L, and stage 11-13, not shown). Before the tail bud forms, the neuroectoderm at the caudal end of the neural tube/neural plate (sinus rhomboidalis, see

Fig. 2A) is in direct continuity with the underlying mesenchyme where the tail bud will arise (Klika and Jelinek, 1969; Schoenwolf, 1979; Schoenwolf and DeLongo, 1980). In later tailbud stages, the closed neural tube extends beneath the surface as a solid mesenchymal rod (medullary cord) in the tail bud. This medullary cord forms caudal neural tube by central cavitation and mesenchymal-epithelial conversion (Schoenwolf and DeLongo, 1980; Schoenwolf, 1984; Schoenwolf and Smith, 1990). To determine whether the central core of marker-negative cells seen in forming tail bud were early neural progenitors of medullary cord, the pan-neural marker L5 was used (a monoclonal antibody directed against an epitope specific for early neural tissue; Roberts et al., 1991; Streit et al., 1997). The central mesenchymal region that was negative for mesodermal markers stained positively for the L5 neural marker (Fig. 2D), suggesting that caudal neuroectoderm also contributes cells regionally to the early central tail bud mesenchyme prior to formation of a discrete medullary cord.

Once formation of the tail bud is complete, elongation begins. During the ensuing week (stage 16-35), the tail bud elongates posteriorly, leaving behind organized tail structures proximally. Although the tail bud eventually occupies a small region at the tip of the growing tail, distinct regional domains of gene expression were still visible at these later times. *Gnot1* expression continued in the chordoneural hinge region and adjacent caudal notochord (Fig. 1 stage 20, I-L), while *Ch-T* and *Ch-Tbx6L* were expressed in the ventral rim of tail bud mesenchyme (Fig. 1 stage 20, A,D,E,H). *Ch-T* expression also continued in chordoneural hinge and notochord (Fig. 1 stage 20, A-C), and *Ch-Tbx6L* in the tail segmental plate (Fig. 1 stage 20, E-G).

Morphogenetic movements during tail bud formation

Internalization of superficially located cells through the primitive streak and the node is one of the hallmarks of gastrulation. The apparent contiguity of gene expression in discrete cell populations from gastrulation through tail development prompted us to investigate whether morphogenetic movements characteristic of gastrulation were still detectable at tailbud stages. Embryos were labeled either at stage 11 (tail bud condensation just visible; posterior neuropore open) or at stage 13 (distinct tail bud condensation; posterior neuropore closed), and were analyzed at stage 13 or 16 respectively. DiI tracer was applied in the midline to five different sites (diagrammed in Fig. 3A) as follows: (1) dorsal surface just caudal to end of primary neural tube (site I); (2) dorsal surface of tail bud condensation replacing anterior primitive streak (site II); (3) dorsal surface of remaining caudal (flat) primitive streak (site III); (4) ventral surface of tail bud condensation (site IV); (5) ventral surface of the caudal primitive streak (site V).

Contribution of dorsal surface to tail bud

The outcome of dorsal labeling at either site I or site II at stage 11 was the same and the results were therefore combined (see Table 1). Labeling of either the anterior or the posterior sinus rhomboidalis (the latter overlying the tail bud) at stage 11 resulted in labeled cells in tail neural tissue (secondary neural tube and medullary cord; Table 1 and Fig. 4A-C,E-G). Labeled

Table 1. Summary of tailbud surface labeling results

Site and stage labeled	Distribution of labeled cells										
	1°nt	no	sp	en	se	2°nt	mc	vtbm	cnh	tbm	ver
Site I+II stage 11	6/14 43%	0/14 0%	7/14 50%	0/14 0%	12/14 86%	6/12 50%	8/14 57%	7/14 50%	1/12 8%	10/12 83%	4/6 67%
Site I stage 13	1/6 17%	0/6 0%	0/6 0%	0/6 0%	6/6 100%	0/6 0%	0/6 0%	0/6 0%	0/6 0%	0/6 0%	0/6 0%
Site II stage 13	0/6 0%	0/6 0%	4/6 67%	0/6 0%	2/6 33%	0/6 0%	0/6 0%	5/6 83%	0/6 0%	0/6 0%	6/6 100%
Site III stage 11-13	0/11 0%	0/11 0%	0/11 0%	0/11 0%	1/11 † 9%	0/11 0%	0/11 0%	0/11 0%	0/11 0%	0/5 0%	0/5 0%
Site IV stage 11-13	5/19 * 26%	3/19 16%	18/19 95%	18/19 95%	0/19 0%	2/19 11%	2/19 11%	17/19 89%	15/18 83%	11/18 ‡ 61%	0/17 0%
Site V stage 11-13	0/6 0%	0/6 0%	1/6 17%	6/6 100%	0/6 0%	0/16 0%	0/6 0%	4/6 67%	0/6 0%	0/6 0%	0/6 0%

For each site (box), embryos that showed labeling over the total number evaluated are indicated, with the percentage positive given just below. Boxes are shaded for sites with more than 40% positive.

*only ventral side; †, tip of the tail; ‡, localized in the midline; no, notochord; se, surface ectoderm; en, endoderm; cnh, chordoneural hinge; ver, ventral ectodermal ridge; 1°nt, primary neural tube; 2°nt, secondary neural tube; sp, segmental plate; vtbm, ventral tail bud mesenchyme; mc, medullary cord; tbm, tail bud mesenchyme.

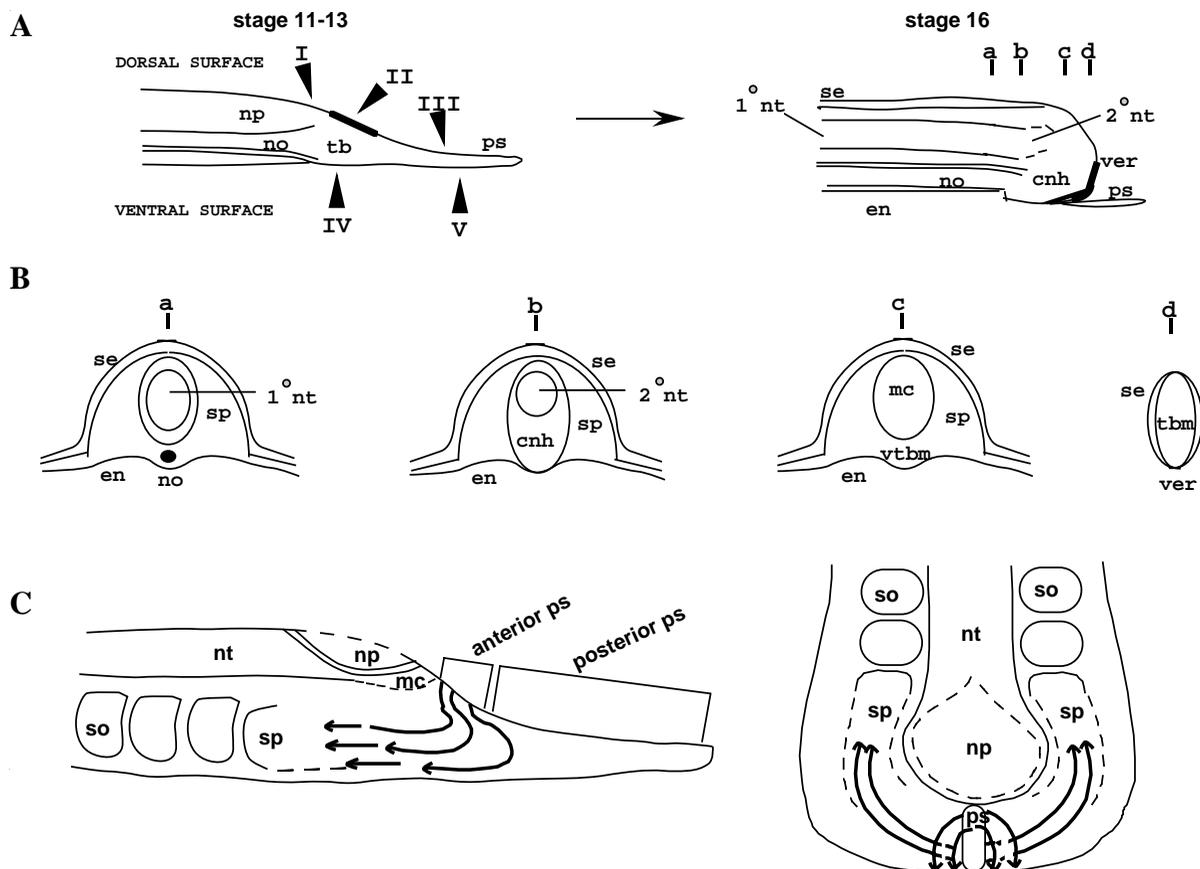


Fig. 3. Diagram of sites labeled and analyzed, and summary of cell movements. (A) Schematic of sites labeled and analyzed. (B) Schematic of transverse sections with sites evaluated indicated (representative examples shown in Fig. 4). (C) Diagram of direction of late ingression movements from dorsal surface. np, neural plate; no, notochord; tb, tail bud; ps, primitive streak; se, surface ectoderm; en, endoderm; cnh, chordoneural hinge; ver, ventral ectodermal ridge; 1°nt, primary neural tube; 2°nt, secondary neural tube; sp, segmental plate; vtbm, ventral tail bud mesenchyme; mc, medullary cord; tbm, tail bud mesenchyme; so, somite.

cells also contributed to the segmental plate of the tail (Table 1 and not shown), the ventral mesenchyme of the tip of the tail (Table 1 and Fig. 4H) and the ventral ectodermal thickening of

the tail (ventral ectodermal ridge or VER, Table 1 and Fig. 4H). These results indicated that in the chick, as in the mouse (Wilson and Beddington, 1996), the anterior end of the late

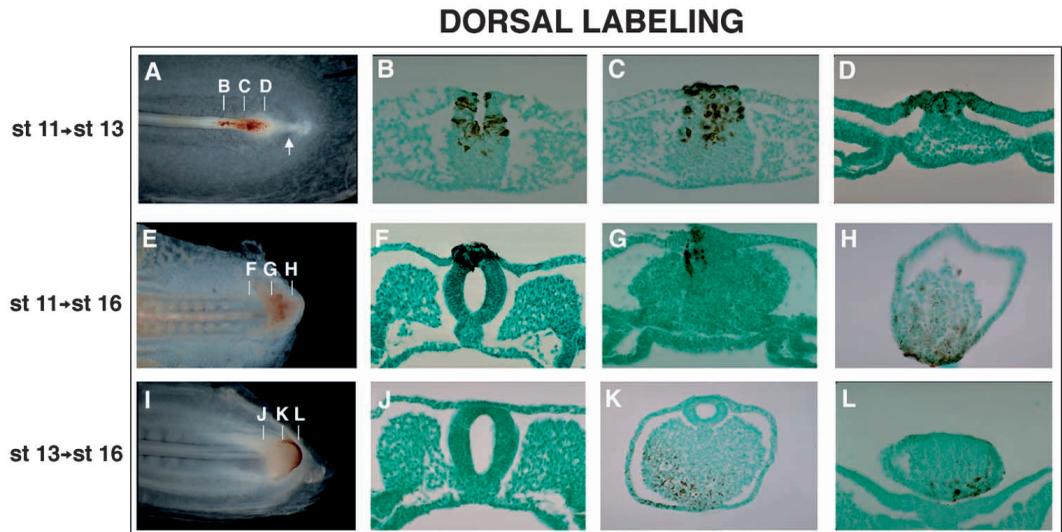
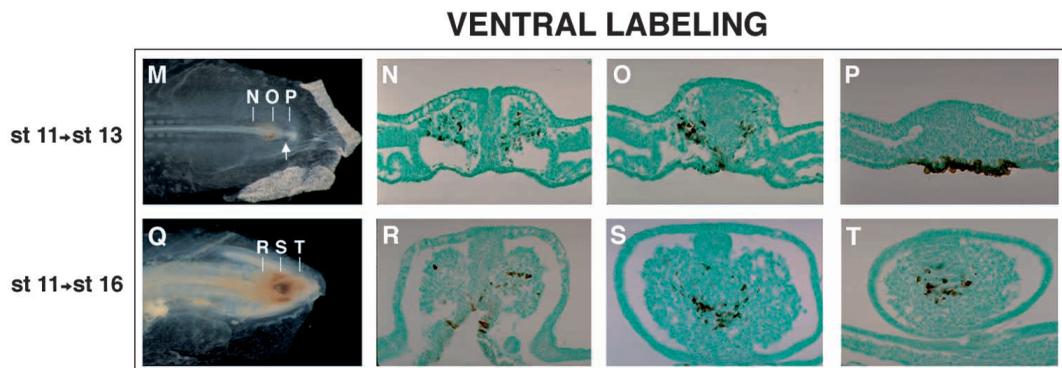


Fig. 4. Distribution of labeled cells after DiI labeling of dorsal or ventral surface. Embryos were labeled: (A-H) site I, II, (I-L) site II, (M-T) site IV, and analyzed at the stages indicated on the left side of the figure. Levels of transverse sections in B-D, F-H, J-L, N-P, R-T are indicated on the whole-mount embryo panels (A, E, I, M, Q).



primitive streak was still a site of cell ingress prior to posterior neuropore closure. At stage 13, after closure of the posterior neuropore (and disappearance of the sinus rhomboidalis), labeled cells derived from site I contributed exclusively to the surface ectoderm of the midline (Table 1 and data not shown). However, cells derived from site II at stage 13 still contributed to the segmental plate and the ventral mesenchyme of the tail bud (Table 1 and Fig. 4K,L). This unexpected result indicated that even after posterior neuropore closure, a functioning primitive streak still exists (cell ingress continues from the bulging anterior surface of the primitive streak incorporated into the tail bud condensation). Interestingly, after labelling site II at stage 13, labeled cells were also found in the VER of the elongating tail at later stages (stage 17-19; Table 1 and data not shown). The VER has been proposed to be a derivative of primitive streak (Schoenwolf, 1979). However, when the VER of stage 17-18 was labeled, there was no evidence of subsequent cell ingress from this site (data not shown).

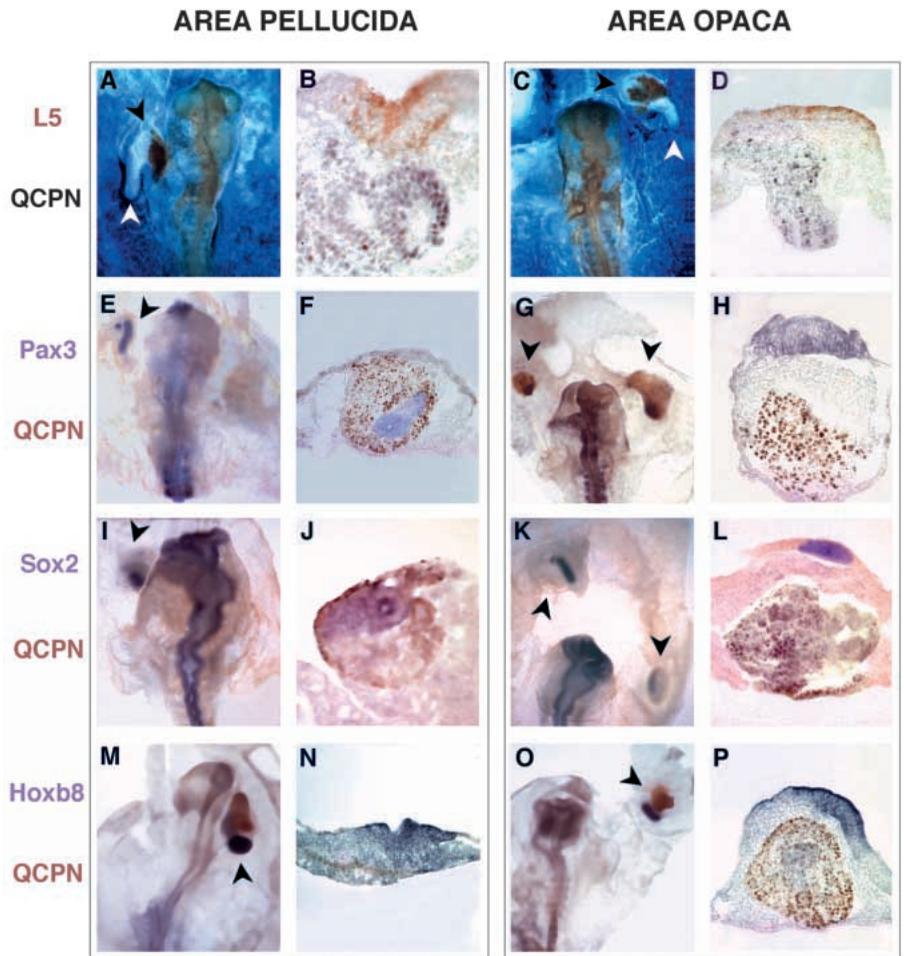
The initial tail bud condensation does not include the posterior part of the primitive streak (see arrow in Fig. 4A,M). When the dorsal surface of the posterior primitive streak (site III in Fig. 3A) was labeled at either stage 11 or stage 13, the majority of labeled cells remained localized at the superficial midline and rare descendants were also found in extraembryonic membranes (Table 1 and data not shown).

Labeled cells derived from site III did not contribute to the tail bud proper. This finding confirms and extends previous results showing that the potential of the posterior end of the streak to contribute cells to the lateral plate and extra-embryonic mesoderm (Psychoyos and Stern, 1996 and ref. therein) is lost by the time of tail bud formation (stage 10-11). Interestingly, by the time of tail bud formation, the posterior end of the streak also stopped expressing primitive streak markers (*Ch-T* and *Ch-Tbx6L*; Fig. 1 stage 15, A,E, and not shown).

Contribution of the ventral surface to the tail bud

Labeling of the ventral surface of the forming tail bud (site IV in Fig. 3A) primarily resulted in labeled cells distributed in hindgut (Fig. 4S), segmental plate of the posterior trunk and tail (Fig. 4N,O,R), chordoneural hinge (not shown), and mesenchyme of the tip of the tail (Fig. 4T, results summarized in Table 1). Labeling at the ventral surface of posterior primitive streak (site V in Fig. 3A) resulted in labeled cells in hindgut endoderm and mesenchyme at the tip of the tail (Table 1 and not shown). The question of ingress of cells arising from the ventral surface, although considered unlikely, could not be definitively answered because of inherent technical difficulties in labeling the ventral surface of the early embryo. Unlike dorsal labeling, in embryos fixed and processed immediately after ventral labeling, there was some labeling of underlying mesenchymal cells as well as of surface endoderm

Fig. 5. Neural inducing and caudalizing activity in late tail bud. Stage 17-18 quail tail tip was grafted to either the area pellucida (left) or area opaca (right) of stage 4⁻ chick recipients and expression of several neural markers was analyzed to demonstrate neural induction and evaluate A-P regionalization. Embryos were also immunostained with QCPN (black A-D; brown E-P) to distinguish quail (graft) from chick (host) cells in induced tissue. Neural markers (indicated to left of figure in staining color; purple for riboprobes, brown or black for antibodies) included early panneural markers L5 epitope (A-D) and *Sox-2* (I-L), and prospective regional markers *Otx-2* (forebrain-midbrain; not shown-see text), *En-1, 2* (midbrain-hindbrain; not shown-see text), *Pax-3* (early hindbrain-spinal cord; E-H), and *Hoxb-8* (early posterior spinal cord; M-P). Whole-mount immunostained and hybridized embryos with area pellucida or area opaca grafts are shown (A,E,I,M and C,G,K,O) with adjacent transverse section of induced axis immediately to the right (B,F,J,N and D,H,L,P). Black arrowheads point to induced secondary axes and white arrowheads to examples of elongated mesodermal projections discussed in text.



(data not shown). However, several conclusions could be drawn from these experiments. The ventral surface and underlying ventral mesenchyme contributed to both endodermal and mesodermal structures, as well as the chordoneural hinge region. Furthermore, mesodermal cells derived from ventral labeling at site IV were always subsequently found in a more anterior location within tail segmental plate than those derived from dorsal labeling at site II (compare Fig. 4F,J with 4N,R). Cells labeled at site II were found predominantly in the caudal ventral rim of tail bud mesenchyme (Fig. 4H,K,L). These labeling results suggest an order of movement of ingressing cells from the dorsal surface first caudally and ventrally and then anteriorly and laterally (shown schematically in Fig. 3C).

The tail bud contains cells with caudal organizer properties

The direct lineage continuity of cells in the chordoneural hinge of the tail bud with Hensen's node (Catala et al., 1995, 1996) and the contiguous expression of certain organizer-associated markers during gastrulation and tail bud formation, as shown above, suggested the possibility that the avian tail bud may still retain some organizer properties. The distinguishing feature of the organizer is its ability to recruit competent surrounding host cells into a new axis (reviewed by Lemaire and Kodjabachian, 1996), in addition to forming a variety of tissue types by self-

differentiation. While the potential of the chick tail bud to differentiate into a variety of tissues has been previously demonstrated, its ability to 'organize' host tissue has not been evaluated.

To evaluate this property, stage 17-18 quail tail tips were grafted to stage 4⁻ chick embryo recipients that are competent to respond to Hensen's node grafts by ectopic axis induction (data not shown; see also Dias and Schoenwolf, 1990; Storey et al., 1992; Streit et al., 1997). In preliminary experiments, grafts to the lateral mid-area pellucida usually contained a morphologically discrete second neural tube or neural plate of chick origin (5/5), as well as loose mesenchyme that sometimes included morphologically distinguishable somites of both chick and quail origin (3/5) and notochord of quail origin (2/5) (data not shown). Grafts derived from regions anterior to tail tip formed only structures that were entirely of graft origin (data not shown).

Grafts to area pellucida are situated on tissue that will contribute to the host embryonic axis and so are likely to recruit host epiblast cells already committed to form neural tissue into an ectopic axis. In one set of experiments, the presence of neural caudalizing activity in tail tip was evaluated by placing grafts adjacent to the forming head region of the host embryo (anterolateral area pellucida). In this case, nearby neural-committed host cells that are recruited into the ectopic axis would normally form anterior neural tissue (forebrain to

hindbrain; discussed in Foley et al., 1997). A total of 28 grafts were analyzed with both early pan-neural (L5 and *Sox-2*; Roberts et al., 1991; Streit et al., 1997) and partly overlapping AP regional neural markers (*Otx-2* for prospective forebrain-midbrain, Bally-Cuif et al., 1995 and Millet et al., 1996; *En-1,2* for midbrain-hindbrain, Davis et al., 1991; *Pax-3* for hindbrain-spinal cord, Bang et al., 1997; and *Hoxb-8* for early posterior spinal cord, Lu et al., 1997). Neural tissue in the ectopic axes was of host origin in all the cases examined (28/28) and pan-neural markers were always expressed (L5, 2/2; *Sox-2*, 1/1; Fig. 5A,B,I,J). However, the induced neural tissue failed to express any anterior regional markers (*Otx-2*, 0/5 and *En-1,2*, 0/11; data not shown), while the consistent expression of *Pax-3* (4/4; Fig. 5E,F) and of *Hoxb-8* (5/5; Fig. 5M,N) was most consistent with caudal (posterior spinal cord) regionalization.

A second set of experiments tested whether stage 17/18 tail tip still had the ability to induce uncommitted, non-neural ectoderm to become neural tissue. Tail tips were grafted to the area opaca, which normally forms only extra-embryonic tissue (Streit et al., 1997 and ref. therein). A total of 55 grafts were analyzed and 47 (85%) formed a morphologically recognizable neural plate which was of host origin. In the majority of cases analyzed, the ectopic neural tissue expressed pan-neural markers (L5, 3/3 and *Sox-2*, 7/10; Fig. 5C,D,K,L) and also selectively expressed caudal (*Pax-3*, 3/6 and *Hoxb-8*, 5/9; Fig. 5G,H,O,P), rather than cranial (*Otx-2*, 0/5 and *En-1,2*, 0/14; data not shown), regional markers. In summary, the tip of the elongating tail at stage 17/18 still retains neural inducing as well as caudalizing activity.

An additional interesting morphological feature of about half of the area pellucida (13/28) and area opaca (29/55) grafts was the outgrowth of elongated mesodermal projections from the ectodermal surface of the host embryo, always in close association with the base of the graft (Fig. 5A,C, white arrowheads). Histologically, these structures contained a central core of mesenchymal cells covered with flat surface ectoderm and, in two thirds of the cases, this mesenchyme was exclusively of host origin (data not shown). In the remaining cases, a small (less than 20%) quail contribution was also present. The mesenchyme was consistently positive for *Hoxb-8*, which is expressed in paraxial mesoderm as well as in neural tissue, but negative for pan-neural markers (Fig. 5A,C and not shown).

DISCUSSION

Based on the observation that primitive streak and Hensen's node become subsumed in a uniform mesenchymal mass directly continuous with caudal neural tube, notochord and gut, Holmdahl first proposed that development of the caudal body in birds and mammals proceeds by direct differentiation of a pluripotent blastema; as distinct from the cell movements and inductive interactions between resulting cell layers that occurs during gastrulation (Holmdahl, 1925a,b; reviewed by Griffith et al., 1992). The potential of ectopically grafted tail bud to self-differentiate into a variety of ectodermal, mesodermal and endodermal derivatives and its phenomenal regenerative properties were cited as evidence in support of the idea that the tail bud mesenchyme is a pluripotent blastema (Fox, 1949;

Schoenwolf, 1978; Seevers, 1932; Tam, 1984; Zwilling, 1942). The mechanism of caudal body formation in amniotes was thus considered to be quite different from gastrulation. Recent studies demonstrating that tail development in amphibians is clearly a continuation of processes initiated during gastrulation (Gont et al., 1993; Tucker and Slack, 1995a,b) have prompted a re-examination of this issue in amniotes. In the chick and mouse, tail development has been previously evaluated primarily by the use of fate mapping techniques (Catala et al., 1995, 1996; Wilson and Beddington, 1996). We have extended this analysis in the avian embryo using three criteria: (1) evidence for continuity of cell lineages; (2) continuation of cell movements; and (3) presence of organizer properties.

The tail bud contains distinct cell populations that are present from the onset of gastrulation

Fate mapping studies in chick and mouse have demonstrated that early Hensen's node, primitive streak and adjacent surface ectoderm always contribute cells to the tail bud (Catala et al., 1996; Ooi et al., 1986; Spratt, 1952; Stein and Kessel, 1995; Wilson and Beddington, 1996). We have taken advantage of several markers that are expressed in discrete cell populations during gastrulation and also expressed in the developing tail, to assess cell behavior and spatial distribution during tail development as compared to gastrulation. Our results support the conclusions of Catala and coworkers in chick (1995, 1996) and Gont and coworkers in frog (1993), as well as a recent study examining multiple gastrulation markers in mouse tail bud (Gofflot et al., 1997), which all concur that the tail bud contains distinct cell populations and therefore is not a blastema. The distribution of these markers in tail bud compared with gastrulation suggests a direct continuity of different cell lineages and conservation of spatial relationships between cells of different origin. The organizer-related genes *Gnot1* and *Ch-T* are both expressed in an anterior ventral domain in the chordoneural hinge region, the descendant of the blastopore dorsal lip in *Xenopus*, and Hensen's node in chick (Catala et al., 1996; Gont et al., 1993). *Ch-T* is additionally expressed in a second distinct posterior ventral domain of mesenchyme at the tip of the elongating tail, along with *Ch-Tbx6L*, which may function as a primitive streak equivalent (see below). It is noteworthy that only a particular subset of genes expressed in axial mesoderm during gastrulation continue to be expressed through tail bud stages. These (*Gnot1* and *Ch-T*) are more 'caudal' (trunk/tail) components, while more 'rostral' (head) components, such as *Gsc* and *Otx-2*, are not expressed.

Morphogenetic movements during early tail bud formation are a continuation of those initiated during gastrulation

During gastrulation, definitive endoderm and mesoderm forms via internalization of cells located superficially in the epiblast. The segregation of notochord and somite precursors is achieved at about the definitive primitive streak stage (Nicolet 1967, 1970, 1971). From then on, in both mouse and chick, these precursors are located within the node and primitive streak, where at least some of them contribute to a stem cell population (Beddington, 1994; Psychoyos and Stern, 1996; Selleck and Stern, 1992; reviewed by Tam and Behringer, 1997). In the present study, cells from the dorsal tail bud

surface did not contribute progeny to notochord; in agreement with previous work indicating that the ingression of notochordal precursors is completed well before tail bud formation (Nicolet, 1970, 1971). However, our experiments indicate that ingression of somitic precursors from the dorsal surface continues during tail bud formation.

Neuroectoderm is not internalized during primary neurulation in which the neural tube forms by elongation and folding up of the prospective neural (or medullary) plate (reviewed by Schoenwolf and Smith, 1990). During secondary neurulation, the terminal neural tube (caudal to lumbar region) forms by cavitation of a solid, internally located medullary cord (Schoenwolf and DeLongo, 1980; Schoenwolf, 1984; Schoenwolf and Smith, 1990). Our results and those of others (Catala et al., 1996; Seichert and Jelinek, 1968) suggest that internalization of surface neuroectoderm occurs during tail bud formation in the transition to secondary neurulation, and stops at the time of posterior neuropore closure. Secondary differs from primary neurulation in the mechanism of tube formation, but it appears to follow the same general blueprint as formation of axial and paraxial structures in the tail; internalization followed by posteriorly directed movement and growth.

The mapping results presented here are in general agreement with those of Catala et al. (1995); the differences being attributable primarily to the evaluation of different tail bud stages using different approaches, because of different aims. We focused on the time period when the tail bud just begins to form (stage 11 to 13), to evaluate whether and how movements of mesodermal precursors and neural cells from the surface contributes to the forming tail bud. Consequently, embryos were evaluated shortly after Dil labeling (6 to 12 hours), which was strictly limited to surface cells dorsally, but also included a few cells just internal to the surface ventrally. In contrast, Catala and coworkers were interested in determining the ultimate fates of cells in different discrete regions of the early tail bud. They evaluated a later definitive tail bud stage (stage 15/16), using quail-chick isotopic grafts that included both internal and surface tissues from particular sites, and analyzed chimeras mainly from 3 to 8 days later to determine contributions to differentiated tissues.

With regard to midline structures, both studies demonstrated that the lateral walls of the neural tube arise from the dorsal anterior tail bud; in the present report this was seen only prior to neuropore closure because only the surface was labeled. Our results also suggest that some secondary neural tube precursors are already present at stage 11, externally in the sinus rhomboidalis region (also seen at stage 9 by Catala et al., 1996). Catala and coworkers (1995, 1996) demonstrated that the floorplate and notochord both arise from the node equivalent, the chordoneural hinge, in the ventral anterior midline of the tail bud. Our ventral tracer analysis showed mainly labeling of the chordoneural hinge with infrequent contribution to notochord and neural tube, presumably reflecting the small numbers of internal cells labelled and short time intervals evaluated.

We have shown that surface ingression of somitic mesodermal precursors continues after posterior neuropore closure, in contrast to results of similar studies in the mouse (Wilson and Beddington, 1996). Apparently these surface movements have ceased by stage 16, in the definitive tailbud, since Catala et al. (1995) found presomitic mesoderm to reside

internally in the ventral tail bud mesenchyme, although they did not directly evaluate surface ingression. Once internalized, both our results and Catala's suggest that these somitic precursors first move posteriorly and turn ventrally in the distal tail bud, and then move laterally and anteriorly to contribute to newly formed segmental plate (see Fig. 3C). Similar movements have also been described in zebrafish tail bud by Kanki and Ho (1997), who termed this 'subduction' as distinct from gastrulation movements, which are primarily in a forward direction. However, taking into account the bulky hemispherical shape of the tail bud compared to the flat early embryo, the movements are not dissimilar.

Evidence for a chick tail organizer

The existence of organizer components with different neural AP regionalizing activities is most evident in amphibians (reviewed by Lemaire and Kodjabachian, 1996). Targeted gene disruption studies in mouse have revealed a head organizer (reviewed by Tam and Behringer, 1997) and recent grafting studies in the chick and mouse indicate anterior neuralizing properties in early head process and prechordal plate emergent from the node (Foley et al., 1997; Pera and Kessel, 1997; reviewed by Tam and Behringer, 1997), as well as the presence of different caudalizing signals along the extent of the AP axis that may, in addition to notochord, reside in primitive streak and paraxial mesoderm as well (Bang et al., 1997; Kintner and Dodd, 1991; Lemaire et al., 1997; Muhr et al., 1997; Storey et al., 1992). The prevailing view in several vertebrates had been that neural induction occurs very early through the action of head organizer genes, producing neural tissue with anterior (forebrain) characteristics, and that the subsequent influence of axial mesoderm and other tissues is confined mainly to caudalizing this prospective neural tissue during elongation of the AP axis (Cox and Hemmati-Brivanlou, 1995; Storey et al., 1992; reviewed by Lemaire and Kodjabachian, 1996 and by Tam and Behringer, 1997). However, the more recent grafting studies suggest that neural inducing, as well as both anterior and posterior regionalizing activities, may segregate within derivatives of the node and may be retained at later times than previously thought.

One of the most intriguing results of this study is the demonstration of residual organizer activity with caudal neural inducing properties in the tip of the tail, which is still capable of recruiting uncommitted, non-neural cells into *de novo* neural tissue in an ectopic axis. The induced neural tissue expressed solely posterior (prospective spinal cord) regional markers and not those specific for more anterior regions (forebrain to hindbrain); consistent with the idea of a heterogeneous organizer, capable of inducing neural tissue with different AP regional characteristics, that segregates into distinct head, trunk and tail organizer components. Neural caudalizing activity was also demonstrated in tail tip by evaluation of regional markers in neural tissue induced by tail tip grafts placed near cells already committed to form anterior neural tissue (host prospective head region). In this case, prospective anterior neural tissue recruited into the second neuraxis induced by tail tip also became caudalized.

The failure of previous studies to identify a tail organizer activity in the chick tail bud may be attributable to both the nature of the host site used and an emphasis on evaluating the self-differentiating capacity of the tail bud (Fox, 1949; Gennaro, 1991; Seevers, 1932). More perplexing is the fact that the neural

inducing properties of Hensen's node rapidly decline during the later stages of gastrulation (Dias and Schoenwolf, 1990; Kintner and Dodd, 1991; Storey et al., 1992), and virtually disappear well before the tail bud stage. Is neuralizing activity discontinuous in the chick? Probably not. One possibility that arises from previous work in the chick (Kintner and Dodd, 1991; Lemaire et al., 1997) and confirmed by our preliminary grafts to area opaca (Knezevic and Mackem, unpublished observations) is that neural inducing properties may reside in primitive streak as well as node. Perhaps at later times such neural inducing activity ultimately becomes localized to the primitive streak equivalent within the tail bud tip. The nature of these signals and whether the neuralizing and caudalizing activities demonstrated in the tail bud are due to the same, or to distinct signals remains to be determined. Work in both *Xenopus* and chick suggests that FGFs, in addition to retinoids, represent good candidates for such activities (Blumberg et al., 1997 and ref. therein; Cox and Hemmati-Brivanlou, 1995; Rodriguez-Gallardo et al., 1997; Storey et al., 1998).

Another interesting feature of the tail organizer was the frequent observation of very elongated mesodermal structures protruding from the ectodermal surface of induced axes in either area pellucida or opaca. Since cells of host origin predominated in these mesodermal 'axes', active recruitment of precursors from host epiblast and/or primitive streak probably occurs. It is thus tempting to speculate that perhaps another function of the early organizer, recruitment and promotion of movements of mesodermal precursors (reviewed by Lemaire and Kodjabachian, 1996), is still present in the tail bud as well.

In conclusion, all of our results suggest that, in birds and probably other amniotes as well, development of the caudal body results from a direct continuation of events initiated during the earlier phase of gastrulation, as it does in amphibians. Furthermore, the persistence of organizer properties in the chick tail reveals evolutionary conservation of a feature found in amphibians and suggests an important role for continuing caudal organizer function in the formation of the posterior body and elongation of the tail in amniotes, as well as amphibians.

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