Title
Digit Induction by Hensen's Node and Notochord Involves the Expression ofshhbut NotRAR-β2

Journal
Developmental Biology, 173(1)

ISSN
0012-1606

Authors
Selleck, Mark AJ
Hayamizu, Terry F
Ohsugi, Kojune
et al.

Publication Date
1996

DOI
10.1006/dbio.1996.0026

DEVELOPMENTAL BIOLOGY **173,** 318–326 (1996)
Article No. 0026

Digit Induction by Hensen's Node and Notochord Involves the Expression of *shh* but Not *RAR-β2*

Mark A. J. Selleck, Terry F. Hayamizu,[1] Kojune Ohsugi, Marianne Bronner-Fraser, and Susan V. Bryant

Developmental Biology Center, University of California at Irvine, Irvine, California 92717

It is well established that Hensen's nodes can induce the formation of supernumerary digits after grafting into the anterior margin of the developing limb bud. The recent finding that distinct mesodermal cell populations are segregated within the node has made it possible to isolate different prospective cell types in an attempt to correlate digit-inducing ability with cell fate. We find that the prospective notochord cells contained within Hensen's node are able to induce supernumerary digits, whereas presumptive somite cells cannot. This early difference in inducing ability persists into later stages of development: epithelial somites are unable to induce while notochord from all lengths of the neuraxis continues to induce. Using probes to retinoic acid receptor-β2 and sonic hedgehog (*shh*) we find no evidence to support the idea that inducing tissues generate extra digits by releasing retinoic acid into adjacent limb tissue but find that the inducing ability of a tissue correlates with its expression of *shh*.

INTRODUCTION

Hensen's node of amniote embryos is generally considered to be equivalent to the dorsal blastopore lip of the amphibian because of its ''organizer properties.'' For instance, it can induce the formation of a regionalized secondary axis after grafting into extraembryonic regions of a host embryo (Waddington, 1932, 1993; Waddington and Schmidt, 1933; more recently, Dias and Schoenwolf, 1990; Storey *et al.,* 1992) and can induce the formation of supernumerary digits after grafting into the anterior region of developing limb buds (Hornbruch and Wolpert, 1986; Stocker and Carlson, 1990; Wagner *et al.,* 1990; Hogan *et al.,* 1992). In this way, Hensen's node mimics the action of a grafted ZPA, which induces digits when transplanted to the anterior of the limb bud (Saunders and Gasseling, 1968; Tickle *et al.,* 1975).

Many studies have demonstrated that exogenous retinoic acid (RA) induces digit duplication when it is locally applied to the anterior of the limb bud (Tickle *et al.,* 1982, 1985; Summerbell, 1983; Eichele *et al.,* 1984). The evidence suggests that the effect of RA is to convert anterior cells to ZPA cells (Noji *et al.,* 1991; Wanek *et al.,* 1991; Tamura *et al.,* 1993). Digit induction by RA is accompanied by upregulation of *RAR-β2* in the adjacent anterior cells (Noji *et al.,* 1991; Hayamizu and Bryant, 1994), whereas there is no *RAR-β2* response following ZPA grafting (Noji *et al.,* 1991). Since *RAR-β2* is a sensitive reporter of RA levels (de The *et al.,* 1989), this result suggests that digit induction by the ZPA does not require RA release. Instead, the inducing ability of ZPA cells has been attributed to their expression of *sonic hedgehog* (*shh;* Riddle *et al.,* 1993; Laufer *et al.,* 1994; Niswander *et al.,* 1994). Recent experiments have shown that *shh* expression is a downstream consequence of RA treatment and that it is activated several cell diameters away from an implanted source of RA (Helms *et al.,* 1994; Niswander *et al.,* 1994).

In inducing extra digits in limbs, Hensen's node and its derivatives could be acting either by releasing RA, as suggested by Hogan *et al.* (1992) or by signaling to anterior limb cells via the expression of *shh*. There is evidence in support of each possibility. Recent studies have shown that Hensen's node, as well as some of its derivatives, in common with the ZPA, express *shh* (Echelard *et al.,* 1993; Riddle *et al.,* 1993; Roelink *et al.,* 1994). Further, the expression of *shh* by transfected cell lines, or by virally infected limb cells, is sufficient to replicate the effects of ZPA grafts in limbs (Riddle *et al.,* 1993; Niswander *et al.,* 1994). On the other hand, it has been demonstrated that chick and mouse Hensen's nodes both contain, and have the ability to synthesize, retinoic acid (Chen *et al.,* 1992; Hogan *et al.,* 1992). In this paper, we have assessed the possibility that release of retinoic acid is involved when nodes and node-derived tissues induce digits, by looking for ectopic expression of *RAR-β2* after grafting. In addition, we have attempted to

[1] Present address: Division of Biology and Medicine, Brown University, Providence RI 02912.

0012-1606/96 $12.00

correlate the digit-inducing ability of a tissue with its expression of *shh*.

Recent fate mapping and cell lineage experiments have shown that the prospective notochord and somite cells contained within Hensen's node differ in their developmental potential (Selleck and Stern, 1992a) and are segregated populations. For example, prospective notochord cells occupy a V-shaped medial region of the node mesoderm, while prospective somitic mesoderm is located in lateral node and in the rostral portion of the primitive streak (Fig. 1; Selleck and Stern, 1991). Such a subdivision of the node makes it possible to isolate each population of cells and evaluate its organizer properties, an approach recently used to investigate the neural inducing and regionalizing properties of the node by Storey *et al.* (1995). In this study, we have tested the digit-inducing ability of subregions within Hensen's node, corresponding to prospective notochord and prospective somite cells, by grafting them into limb buds. We have compared these results to those of grafts of notochord, neural tissue, and somite from later staged embryos.

By separating regions with different prospective fates within Hensen's node, we were able to show that prospective notochord cells, but not prospective somite cells, are able to induce the formation of extra digits after grafting. These same properties are maintained in later embryos when grafts of notochord but not of somites can induce extra digits. These results support the earlier findings (Hornbruch and Wolpert, 1986; Wagner *et al.*, 1990) that notochord from older embryos can induce, whereas somite and lateral neural tissue cannot. We also report that neither Hensen's node nor its derivative, the notochord, induced ectopic expression of *RAR-β2* in limb bud tissue after 6, 12, or 18 hr, making it unlikely that digit-inducing activity is dependent on RA release. We find that Hensen's nodes (digit-inducing) express *shh* at the time of grafting and continue to do so, at least until 18 hr of incubation: limb cells surrounding the node implant do not express this gene. In contrast to Hensen's nodes, *shh* is not expressed in anterior primitive streak tissue (noninducing), even after grafting. Based on this evidence, we conclude that the digit-inducing activity of Hensen's node and its derivatives is related to their expression of *shh*.

MATERIALS AND METHODS

Preparation of Donor Tissue

Fertile chicken eggs were incubated for 18 to 40 hr to give stage 4–11 embryos (Hamburger and Hamilton, 1951). Embryos were dissected from the egg and explanted into phosphate-buffered saline (PBS). After rinsing, embryos were pinned out in wax-coated dishes containing calcium- and magnesium-free Tyrode's saline and the graft tissues dissected with tungsten needles. In cases in which the graft tissues adhered strongly to surrounding embryonic structures, enzymes were used to help isolate them. Embryos were bathed in either trypsin (0.1% w/v in CMF-Tyrode's) or collagenase (Cooper Biochemical; CLS type II, 1 mg/ml in Ringers saline) during the dissection. Grafts were subsequently transferred to complete culture medium (Eagle's minimum essential medium (MEM; Gibco), with 15% horse serum and 10% chick embryo extract) to recover for 30–90 min prior to grafting.

Hensen's node grafts. Hensen's nodes were obtained from definitive streak stage (stage 4) embryos. In some instances, whole nodes were grafted, while in other experiments, subregions of the node were used (Fig. 1A). Hensen's nodes were dissected into medial and lateral triangle-shaped sectors. In such cases, we were careful to exclude the intermediate region which is thought to contain multipotent stem cells (Selleck and Stern, 1992b). In a subset of experiments, we further divided Hensen's node sectors into their ectodermal and mesendodermal components after enzymatic treatment. Rostral primitive streak, which contains presumptive lateral somite cells, was isolated from stage 4 embryos and used in some experiments.

Notochord, somite, and neural tissue grafts. Embryos ranging from stages 8 to 11 were used for notochord, somite, or neural tissue grafts. Embryos were explanted ventral-side up and pinned out in the dish. In the case of older embryos, the foregut was opened by tearing rostrad from the anterior intestinal portal with a needle. The midline endoderm was reflected to access the underlying mesodermal tissues. In some cases, no enzymatic treatment was used and small lengths of chordamesoderm (about 200 μm), taken from different levels of the neuraxis, were isolated along with adjacent floor plate. In the remaining experiments, notochord and whole somites were isolated following enzymatic treatment (Fig. 1B). For neural tissues grafts, embryos were explanted dorsal-side uppermost in CMF-Tyrode's containing trypsin, and lateral wall of the neural tube was isolated from different points along the neuraxis.

Preparation of Host Embryos and Grafting

Fertilized White Leghorn chicken eggs were incubated at 38°C to generate embryos at stages 20–21 (Hamburger and Hamilton, 1951). The eggs were prepared by withdrawing some of the albumen and creating a window in the shell overlying the embryo, as described previously (Tickle, 1993). The graft tissues were implanted into right wing buds at their anterior margin, immediately subjacent to the apical ectodermal ridge (AER), and the eggs resealed and incubated at 38°C.

Embryo Fixation and Data Analysis

At varying time points following implants, embryos were fixed in alcoholic Bouin's solution. Left wings were used as controls for assessing the effects on the treated right wings. The pattern of cartilage structures in the limbs were determined in whole-mount preparations of limbs stained with Victoria blue (Bryant and Iten, 1974) or Alcian blue (Wanek *et al.*, 1989) and cleared in methyl salicylate. Tissue samples were photographed under either incident or transmitted

light using a Wild M8 Stereomicroscope with a Wild Photoautomat MPS50 and Kodak Ektachrome 160 Tungsten film.

Whole-Mount in Situ Hybridization

Digoxigenin-labeled antisense RNA probes specific for chick *RAR-β2* were transcribed as previously described (Hayamizu *et al.*, 1994) from cloned DNA provided by B. Blumberg and K. Umesono. Detection of *RAR-β2* transcripts in whole-mount chick limb preparations was performed as previously described (Hayamizu *et al.*, 1994). For *shh* probes (provided by J.-C. Izpisua-Belmonte), the hybridization temperature was 70°C, with a wash temperature of 65°C. Following the alkaline phosphatase-mediated color reaction, limbs were analyzed either after dehydration in methanol or after clearing in methyl salicylate. Tissue analysis and photography were performed as described above.

RESULTS

Hensen's Node Grafts

Whole Hensen's nodes, as well as subregions of the node and anterior primitive streak, were isolated from stage 4 embryos (Fig. 1A) and grafted into the anterior margin of chick wing buds at stages 20/21. The results of 52 surviving experiments are summarized in Table 1.

Whole Hensen's node grafts induce supernumerary digits in 67% of cases. Medial, V-shaped portions of the node induce with a similar frequency to whole nodes (63%). Lateral portions of Hensen's node also induce extra digits, but with a somewhat lower frequency (40%). In some experiments, medial and lateral sectors were separated into their ectodermal and mesendodermal components. The ectodermal portions of both medial and lateral node regions induce supernumerary digits with a low frequency (25% for medial ectoderm, Fig. 2B; 20% for lateral ectoderm). The greatest difference in inducing ability was seen between the mesendodermal portions of medial and lateral Hensen's node. While the medial mesendoderm induced additional digits with a frequency of 75% (Fig. 2C), lateral node mesendoderm rarely induced extra digits after grafting to the anterior limb bud (10%). Similarly, anterior portions of the primitive streak induced in only a small number of cases (1/9; 11%).

In most of the inductions (15 of 19 cases), a single extra digit 2 was produced. In one other case, a single digit 3 was induced after grafting a whole Hensen's node. In the remaining 3 cases, in which medial node, medial mesendoderm, or rostral primitive streak were grafted, extra digits 2 and 3 were generated.

Our previous fate mapping experiments performed on Hensen's nodes at the definitive streak stage reveal that the medial and lateral mesodermal portions of the node and the anterior primitive streak differ in their content of prospective notochord and somite cells. Presumptive notochord cells are present in medial mesoderm, while lateral mesoderm and anterior primitive streak contain cells destined for the medial parts of somites (Selleck and Stern, 1991). Future notochord cells are present in both medial and lateral portions of the node ectoderm. Therefore, the digit-inducing ability of subregions of Hensen's node correlates well with their content of prospective notochord cells.

Notochord, Somite, and Neural Tissue Grafts

To investigate whether the differences in inducing ability exhibited by prospective notochord and somite cells in Hensen's node persist to later stages of development, we grafted notochord and somites from older embryos.

Small pieces of notochord from different axial levels of embryos ranging from stages 8 to 11 were grafted to the anterior margin of chick limb buds. In some cases notochord was isolated along with adherent floor plate, while in other experiments, trypsin and collagenase were used to ensure that only notochord tissue was grafted.

From the 50 surviving experiments, 40 grafts induced supernumerary digits in the chick limb (Figs. 2A and 2D). In cases in which notochord was grafted with adherent floor plate, extra digits were seen in 100% of cases, with the exception of notochord/floor plate isolated from forebrain and midbrain levels, where supernumerary digits were produced in 75% of cases. Enzymatically isolated notochords induced with a similar frequency (90%) when taken from spinal cord levels, and at 70% when the notochord was removed from forebrain or midbrain levels. In most instances of supernumerary digit induction, one extra digit 2 was generated. In two cases, notochord lying adjacent to somites 7 and 8 was grafted to the limb and, in both instances, two supernumerary digits (2 and 3) were induced. One graft of mesencephalic notochord resulted in an extra digit 3, and in one other case the supernumerary digits were both digit 2 (Fig. 2D).

In contrast to notochord, somites that had been isolated from stage 8–10 embryos, at different rostrocaudal levels, never induced supernumerary digits (Table 2). Similarly, neural tissue grafts isolated from the lateral wall of neural tube at various levels of the neuraxis never induced.

RAR-β2 in Situ Hybridization Studies

To address the question of whether Hensen's node induces extra digits by acting as a source of retinoic acid, we used *in situ* hybridization to detect expression of the retinoic acid-sensitive gene *RAR-β2* following grafts to anterior limb bud. *RAR-β2* contains a retinoic acid response element (RARE) that initiates transcription following an elevation in exogenous retinoic acid levels (de The *et al.*, 1989). Therefore, limb bud cells that have been exposed to a source of retinoic acid will upregulate expression of this gene (Noji *et al.*, 1991). Host embryos ranged from stages 19 to 21. Limbs receiving Hensen's node grafts were fixed after 6 ($n = 4$), 12 ($n = 2$), or 18 ($n = 2$) hr of incubation.

In control limbs (Fig. 3A), *RAR-β2* transcripts were restricted to proximal parts of the limb bud adjacent to the embryonic axis, a finding in agreement with previous stud-

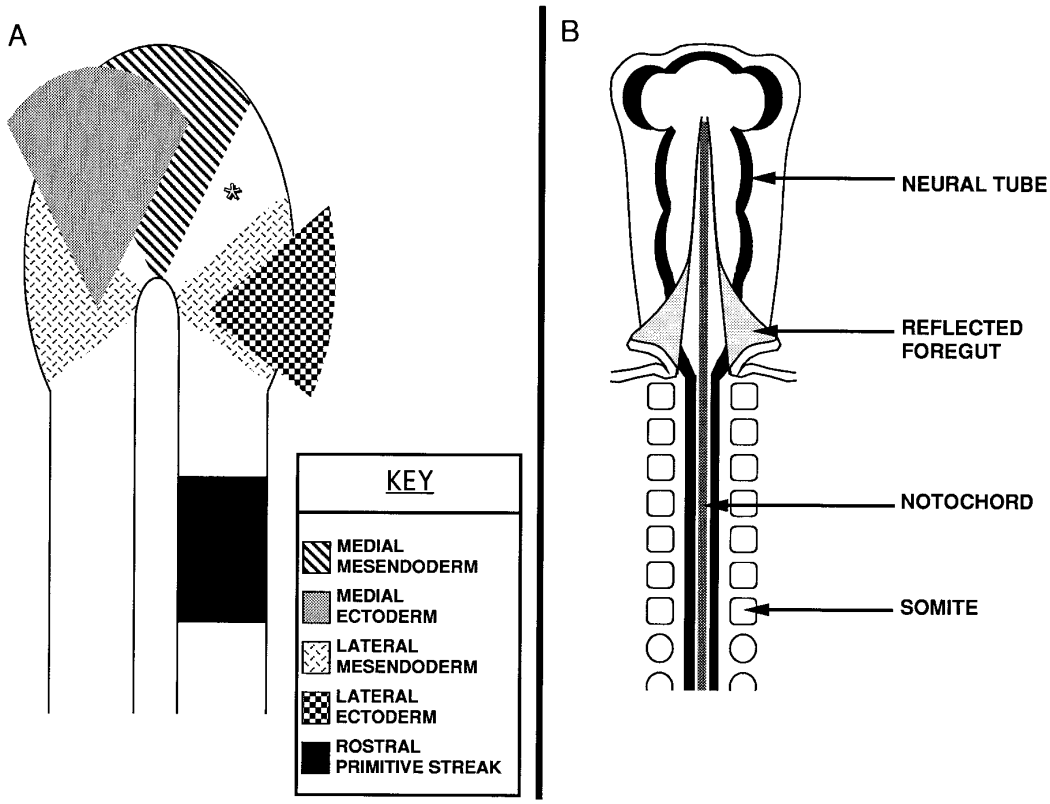


FIG. 1. (A) Diagrammatic representation of Hensen's node and the anterior primitive streak, illustrating the regions (shaded blocks) that were used for grafting experiments. In some experiments, Hensen's nodes were dissected into medial and lateral wedges, both of which could be further subdivided into ectodermal and mesendodermal portions. In this figure, the ectodermal portions of the medial and lateral node sectors are shown displaced to the left and right, respectively, revealing the location of the underlying mesendodermal wedges. A region of cells lying between the medial and lateral node sectors (asterisk), thought to contain stem cells (Selleck and Stern, 1992b), was excluded from the isolates. (B) A schematic view of the ventral aspect of an embryo from which notochord and somites were isolated. Access to the cephalic notochord was gained after cutting the anterior intestinal portal and reflecting the ventral wall of the foregut.

ies (Noji *et al.*, 1991; Smith and Eichele, 1991; Schofield *et al.*, 1992; Hayamizu and Bryant, 1994). When retinoic acid-containing beads are grafted into the limb bud, *RAR-β2* is upregulated in cells adjacent to the implant (Fig. 3D; Hayamizu and Bryant, 1994). Following grafts of Hensen's node (Fig. 3B), no ectopic expression of *RAR-β2* could be detected in the limb bud, and no staining could be detected distal to the graft site at any of the three time points. Similarly, no ectopic expression of *RAR-β2* was detected after grafting fragments of notochord to developing limb buds. Furthermore, RAR-β2 transcripts were not detected within the grafted Hensen's nodes or notochord.

TABLE 1

Grafted tissue	Number of grafts	Limbs with extra digits	%
Hensen's node[a]	3	2	67
Medial node[a]	8	5	63
Medial-meso[a]	8	6	75
Medial-ecto[a]	4	1	25
Lateral node[a]	5	2	40
Lateral-ecto[a]	5	1	20
Lateral-meso	10	1	10
Primitive streak	9	1	11

[a] Contains presumptive notochord cells.

Shh in Situ Hybridization Studies

In an attempt to correlate digit-inducing ability with the expression of *shh* in grafted tissues, we performed a series of experiments in which Hensen's nodes or fragments of primitive streak were grafted into limb buds and subsequently processed for *in situ* hybridization with a probe to *shh* (Fig. 4).

Of 8 Hensen's node grafts, 5 were fixed and processed after 2 hr of development and 3 were processed after 18 hr.

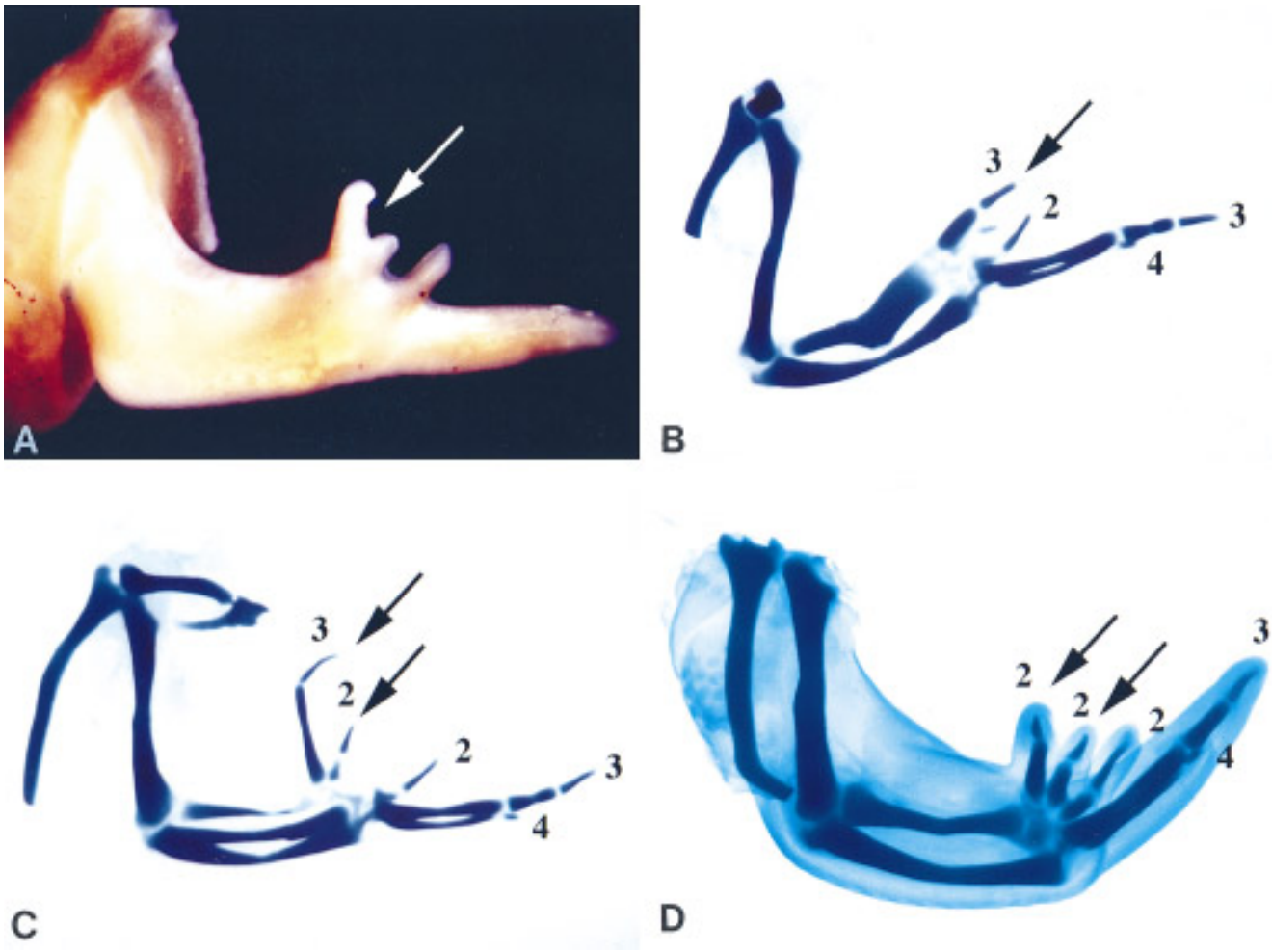


FIG. 2. Skeletal analysis of limb buds following grafts of subregions of Hensen's node and notochord. (A) Following a graft of notochord, a supernumerary outgrowth (arrow) can be seen on the anterior side of this whole mount, unstained limb. Limbs in B, C, and D were stained for visualization of the skeleton as described in the text. Grafts of medial sector ectoderm (B) and medial sector mesoderm (C) of Hensen's nodes both induce supernumerary digits (arrows). Frequently, a single extra digit 2 is produced, but other combinations of supernumerary digits were seen occasionally, such as an extra 3 and 2 (C) and two extra digit 2s seen after one experiment in which notochord was grafted (D).

TABLE 2

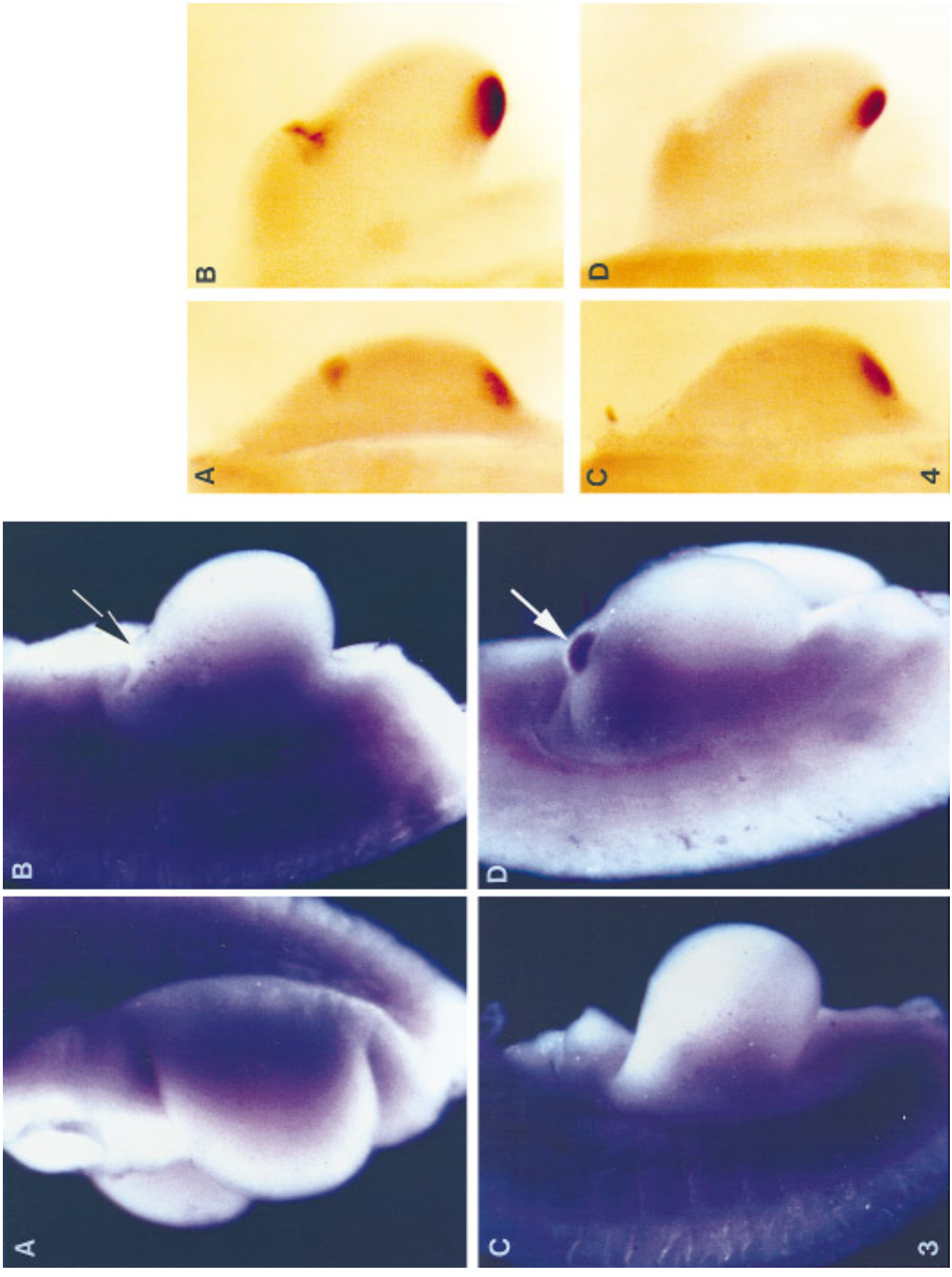
Grafted tissue	Number of grafts	Limbs with extra digits	%
Notochord + floor plate			
Hindbrain/spinal cord level	9	9	100
Midbrain/forebrain level	4	3	75
Notochord alone			
Spinal cord level	10	9	90
Brain level	27	19	70
Somites	18	0	0
Neural tube	8	0	0

In all cases, *shh* was expressed within the grafted nodes, although no *shh* transcripts could be found within adjacent limb bud cells (Figs. 4A and 4B).

Fragments of anterior primitive streak were grafted in 11 experiments, 6 of which were fixed after 2 hr incubation (Fig. 4C). Labeling was found in only 1 case, and the transcripts were restricted to the grafted tissue. The remaining 5 specimens were processed after 18 hr incubation and in no cases were transcripts detected (Fig. 4D).

DISCUSSION

In the present study, we have analyzed the regions of Hensen's node that are capable of inducing supernumerary


B
D
A
C
4
B
D
A
C
3

digits. By grafting subregions of Hensen's node, we find that the digit-inducing ability of grafted tissue correlates with its content of prospective notochord cells. In addition, our results confirm earlier reports that notochord isolated from older embryos can induce, whereas somite and neural tissue which does not include the floorplate cannot (Hornbruch and Wolpert, 1986; Wagner *et al.*, 1990). The frequency and complexity of the digits induced in this study are comparable to those reported by other authors. We have attempted to determine whether the ability of Hensen's node and notochord to induce supernumerary digits can be accounted for by the previously documented expression of *shh* (Echelard *et al.*, 1993; Riddle *et al.*, 1993; Roelink *et al.*, 1994) or whether induction involves the release of diffusible RA, as previously suggested (Hogan *et al.*, 1992). We have used whole-mount *in situ* hybridization with a probe to *RAR-β2* as an assay for diffusible retinoic acid. Additionally, we have used a probe to *shh* to investigate whether inducing tissues express this gene. We find no evidence to suggest that retinoic acid is released in amounts capable of affecting the expression of this sensitive reporter gene of RA levels from either Hensen's node or notochord into neighboring limb bud tissue after transplantation. This result, combined with our finding that *shh* is expressed in inducing (Hensen's node) grafts but not in noninducing (primitive streak) grafts, suggest that expression of *shh* alone is sufficient to account for the inducing activity.

We have used *RAR-β2* in this study because it is known to be a very sensitive indicator of changes in RA levels. Cell lines show an immediate and marked response in *RAR-β2* mRNA levels after the addition of as little 1 n*M* RA to the culture medium (de The *et al.*, 1989). Although a detailed examination of the *in vivo* changes in *RAR-β2* expression in response to beads soaked in different concentrations of RA has not been reported, Hayamizu and Bryant (1994) showed that RA bead soaking solutions that led to digit 2 duplications in about half the cases evoked transient, albeit weak, induction of *RAR-β2*. Most of the results in the present study gave a somewhat higher frequency, and either an equivalent or occasionally more complex response, leading us to conclude that if RA release were involved, *RAR-β2* gene expression is sensitive enough to have reported it. We infer that Hensen's node does not induce by acting as a source of diffusible RA.

Our experiments have shown that grafts which contain notochord or prospective notochord cells are able to induce supernumerary digits, while those lacking chordamesoderm are generally not able to signal. In common with other tissues having polarizing ability, such as ZPA and floor plate, Hensen's node and notochord contain cells which express *shh* (Echelard *et al.*, 1993; Riddle *et al.*, 1993; Roelink *et al.*, 1994; Levin *et al.*, 1995). Transfected tissue culture cells which express *shh* can induce digit duplications after grafting to anterior limb bud (Riddle *et al.*, 1993; Niswander *et al.*, 1994), thereby establishing a causal link between SHH signaling and digit induction. If chordamesoderm cells induce because they express *shh*, one might predict that *shh* expression is limited to Hensen's node ectoderm and medial mesendoderm at definitive streak stages.

While the retinoic acid in grafted Hensen's nodes may never leave the node and enter limb bud tissue, it may still play an important role in the ontogeny of the node's inducing properties. In limbs, it has been shown that *shh* is expressed in response to RA (Helms *et al.*, 1994; Niswander *et al.*, 1994), raising the possibility that endogeneous retinoids in Hensen's node could be important for inducing *shh* expression and polarizing activity in the chordamesoderm. Helms *et al.* (1994) have shown that *shh* expression can be induced in limb cells near to beads that have been loaded with as little as 10 μg/ml of RA. This treatment generates an average concentration of RA in the limb bud that is similar to that measured in untreated limb buds, 30 n*M* (Thaller and Eichele, 1987), and it also leads to induction of a localized ectopic domain of *shh* expression close but not immediately adjacent to the bead (Helms *et al.*, 1994; Niswander *et al.*, 1994). It is likely that the calculated average concentration masks differences in concentration at different distances from the RA bead.

Very similar average concentrations of RA have been calculated for stage 4 Hensen's nodes (33 n*M;* Chen *et al.*, 1992), lending strong support to the idea that in the node, the levels of endogenous RA are sufficient to induce *shh* expression, and by inference, polarizing activity. It is likely that in the node, RA may be restricted to a subpopulation of cells, rather than being evenly distributed. Since it has been shown in the limb that *shh* is expressed at a distance of several cell diameters away from the RA source, it is possible that RA will not be found in the prospective notochord cells, since these are most likely the populations that express *shh* and show polarizing activity. An intriguing possibility is that RA will be concentrated in the stem cells that have been proposed to exist in the intermediate regions of the node (Selleck and Stern, 1992b).

One interesting finding is that the chordamesoderm un-

FIG. 3. The results of *RAR-β2 in situ* hybridizations in limbs. In control limbs which have received no grafts (A), *RAR-β2* staining is restricted to proximal areas of the limb. After grafting a Hensen's node (arrow; B) or notochord (C) a similar expression pattern is seen, and no ectopic expression of *RAR-β2* can be found along the anterior margin of the limb. In contrast, grafts of RA-containing beads (0.1 mg/ml, fixed after 6 hr) induce ectopic expression of *RAR-β2* in adjacent limb bud cells (D). In this case, staining can be seen extending distal to the implant site (arrow). This specimen is taken from a previous study (Hayamizu and Bryant, 1994).

FIG. 4. *shh in situ* hybridizations after grafting Hensen's nodes (A, B) or anterior primitive streak (C, D) to limb buds. (Hensen's node grafts) Two hours after grafting nodes to anterior limb bud (A), *shh* transcripts can be found within the graft tissues. Grafted Hensen's nodes continue to express *shh* after 18 hr incubation (B), but no transcripts are found in adjacent host limb bud cells. (Primitive streak grafts) In contrast to Hensen's nodes, primitive streak fragments do not express *shh* around the time of grafting (C) nor 18 hr later (D).

derlying anterior regions of the neuraxis (i.e., forebrain and midbrain) induces with lower frequency than notochord taken from more posterior, spinal cord regions. This difference may reflect some regional variations in the notochord. Consistent with this, Chen *et al.* (1992) reported that the amount of RA in stage 6 Hensen's nodes is about three times higher than that in stage 4 nodes. A role for retinoic acid in the anteroposterior regionalization of the CNS has been proposed on the basis of its ability to posteriorize or delete anterior neural tissue (Durston *et al.*, 1989; Sive *et al.*, 1990; Chen and Solursh, 1992). It is conceivable that retinoic acid conveys anteroposterior identity to the developing nervous system via effects on the notochord. Posterior notochord emerging from older nodes would have been exposed to higher concentrations of RA for a longer time. RA is known to affect the expression not only of *shh*, but of numerous other genes, including Hox complex genes (Kessel and Gruss, 1991; Mavilio, 1993) and differences in exposure to RA during axis formation might be expected to translate into different patterns of gene expression at different levels along the notochord (see also Hogan *et al.*, 1992). These regional differences could in turn lead to patterning of the overlying nervous system.

ACKNOWLEDGMENTS

We are grateful to Drs. David Gardiner and Mary Dickinson and the anonymous reviewers for helpful comments on the manuscript. We thank Bruce Blumberg and Kaz Umesono for the RAR-β2 probe and Juan Carlos Izpisua-Belmonte for the *shh* probe. M.A.J.S. was supported by a Muscular Dystrophy Association research fellowship. Research was supported by HD25138 and a grant from the March of Dimes Birth Defects Foundation to M.B.-F. and HD25620 to S.V.B.

REFERENCES

Bryant, S. V., and Iten, L. (1974). The regulative ability of the limb regeneration blastema of *Notophthalmus viridescens:* Experiments *in situ*. *Wilhelm Roux' Arch. Dev. Biol.* **174,** 90–101.

Chen, Y., Huang, L., Russo, A. F., and Solursh, M. (1992). Retinoic acid is enriched in Hensen's node and is developmentally regulated in the early chicken embryo. *Proc. Natl. Acad. Sci. USA* **89,** 10056–10059.

Chen, Y., and Solursh, M. (1992). Comparison of Hensen's node and retinoic acid in secondary axis induction in the early chick embryo. *Dev. Dyn.* **195,** 142–151.

de The, H., Marchio, A., Tiollais, P., and Dejean, A. (1989). Differential expression and ligand regulation of the retinoic acid receptor α and β genes. *EMBO J.* **8,** 429–433.

Dias, M. S., and Schoenwolf, G. C. (1990). Formation of ectopic neurepithelium in chick blastoderms: age-related capacities for induction and self-differentiation following transplantation of quail Hensen's nodes. *Anat. Rec.* **228,** 437–448.

Durston, A. J., Timmermans, J. P. M., Hage, W. J., Hendriks, H. F. J., de Vries, N. J., Heideveld, M., and Nieuwkoop, P. D. (1989). Retinoic acid causes an anteroposterior transformation in the developing central nervous system. *Nature* **340,** 140–144.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A., and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75,** 1417–1430.

Eichele, G., Tickle, C., and Alberts, B. M. (1984). Microcontrolled release of biologically active compounds in chick embryos: Beads of 200-μm diameter for the local release of retinoids. *Anal. Biochem.* **142,** 542–555.

Hamburger, V., and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88,** 49–92.

Hayamizu, T. F., and Bryant, S. V. (1994). Reciprocal changes in *Hox D13* and *RAR-β2* expression in response to retinoic acid in chick limb buds. *Dev. Biol.* **166,** 123–132.

Hayamizu, T. F., Wanek, N., Taylor, G., Trevino, C., Shi, C., Anderson, R., Gardiner, D. M., Muneoka, K., and Bryant, S. V. (1994). Regeneration of *HoxD* expression domains during pattern regulation in chick wing buds. *Dev. Biol.* **161,** 504–512.

Helms, J., Thaller, C., and Eichele, G. (1994). Relationship between retinoic acid and sonic hedgehog, two polarizing signals in the chick wing bud. *Development* **120,** 3267–3274.

Hogan, B. L. M., Thaller, C., and Eichele, G. (1992). Evidence that Hensen's node is a site of retinoic acid synthesis. *Nature* **359,** 237–241.

Hornbruch, A., and Wolpert, L. (1986). Positional signalling by Hensen's node when grafted to the chick limb bud. *J. Embryol. Exp. Morphol.* **94,** 257–265.

Kessel, M., and Gruss, P. (1991). Homeotic transformations of murine vertebrae and concomitant alteration of Hox codes induced by retinoic acid. *Cell* **67,** 89–104.

Laufer, E., Nelson, C. E., Johnson, R. L., Morgan, B. A., and Tabin, C. (1994). Sonic hedgehog and Fgf-4 act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell* **79,** 993–1003.

Levin, M., Johnson, R. L., Stern, C. D., Kuehn, M., and Tabin, C. (1995). A molecular pathway determining left-right asymmetry in chick embryogenesis. *Cell* **82,** 803–814.

Mavilio, F. (1993). Regulation of vertebrate homeobox-containing genes by morphogens. *Eur. J. Biochem.* **212,** 273–288.

Niswander, L., Jeffrey, S., Martin, G. R., and Tickle, C. (1994). A positive feedback loop coordinates growth and patterning in the vertebrate limb. *Nature* **371,** 609–612.

Noji, S., Nohno, T., Koyama, E., Muto, K., Ohyama, K., Aoki, Y., Tamura, K., Ohsugi, K., Ide, H., Taniguchi, S., and Saito, T. (1991). Retinoic acid induces polarizing activity but is unlikely to be a morphogen in the chick limb bud. *Nature* **350,** 83–86.

Riddle, R. D., Johnson, R. L., Laufer, E., and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **75,** 1401–1416.

Roelink, H., Augsburger, A., Heemskerk, J., Korzh, V., Norlin, S., Ruiz i Altaba, A., Tanabe, Y., Placzek, M., Edlund, T., Jessell, T. M., and Dodd, J. (1994). Floor plate and motor neuron induction by *vhh-1,* a vertebrate homolog of hedgehog expressed by the notochord. *Cell* **76,** 761–775.

Saunders, J. W., Jr., and Gasseling, M. T. (1968). Ectodermal-mesenchymal interactions in the origin of limb symmetry. *In* "Epithelial–Mesenchymal Interactions" (R. Fleishmajer and R. E. Billingham, Eds.), pp. 78–97. Williams & Wilkins, Baltimore.

Schofield, J. N., Rowe, A., and Brickell, P. M. (1992). Position-dependence of retinoic acid receptor-beta gene expression in the chick limb bud. *Dev. Biol.* **152,** 344–353.

Selleck, M. A. J., and Stern, C. D. (1991). Fate mapping and cell lineage analysis of Hensen's node in the chick embryo. *Development* **112,** 615–626.

Selleck, M. A. J., and Stern, C. D. (1992a). Commitment of mesoderm cells in Hensen's node of the chick embryo to notochord and somite. *Development* **114,** 403–415.

Selleck, M. A. J., and Stern, C. D. (1992b). Evidence for stem cells in the mesoderm of Hensen's node and their role in embryonic pattern formation. *In* ''Formation and Differentiation of Early Embryonic Mesoderm'' (R. Bellairs, E. J. Sanders, and J. W. Lash, Eds.), pp. 23–31. Plenum, New York.

Sive, H. L., Draper, B. W., Harland, R. M., and Weintraub, H. (1990). Identification of a retinoic acid-sensitive period during primary axis formation in *Xenopus laevis*. *Genes Dev*. **4,** 932–942.

Smith, S. M., and Eichele, G. (1991). Temporal and regional differences in the expression pattern of distinct retinoic acid receptor-β transcripts in the chick embryo. *Development* **111,** 245–252.

Stocker, K. M., and Carlson, B. M. (1990). Hensen's node, but not other biological signallers, can induce supernumerary digits in the developing chick limb bud. *Roux's Arch. Dev. Biol.* **198,** 371–381.

Storey, K. G., Crossley, J. M., De Robertis, E. M., Norris, W. E., and Stern, C. D. (1992). Neural induction and regionalisation in the chick embryo. *Development* **114,** 729–741.

Storey, K., Selleck, M. A. J., and Stern, C. D. (1995). Neural induction and regionalisation by different subpopulations of cells in Hensen's node. *Development* **121,** 417–428.

Summerbell, D. (1983). The effect of local application of retinoic acid to the anterior margin of the developing chick limb. *J. Embryol. Exp. Morphol.* **78,** 269–289.

Tamura, K., Aoki, Y., and Ide, H. (1993). Induction of polarizing activity by retinoic acid occurs independently of duplicate formation in developing chick limb buds. *Dev. Biol.* **158,** 341–349.

Thaller, C., and Eichele, G. (1987). Identification and spatial distribution of retinoids in the developing chick limb bud. *Nature* **327,** 625–628.

Tickle, C. (1993). Chick limb buds. *In* ''Essential Developmental Biology: A Practical Approach'' (C. Stern and P. Holland, Eds.), pp. 119–125. IRL Press, Oxford.

Tickle, C., Alberts, B., Wolpert, L., and Lee, J. (1982). Local application of retinoic acid to the limb bond [sic] mimics the action of the polarizing region. *Nature* **296,** 564–566.

Tickle, C., Lee, J., and Eichele, G. (1985). A quantitative analysis of the effect of all-*trans*-retinoic acid on the pattern of chick wing development. *Dev. Biol.* **109,** 82–95.

Tickle, C., Summerbell, D., and Wolpert, L. (1975). Positional signalling and specification of digits in chick limb morphogenesis. *Nature* **254,** 199–202.

Waddington, C. H. (1932). Experiments on the development of chick and duck embryos, cultivated in vitro. *Philos. Trans. R. Soc. London* **221,** 179–230.

Waddington, C. H. (1933). Induction by the primitive streak and its derivatives in the chick. *J. Exp. Biol.* **10.**

Waddington, C. H., and Schmidt, G. A. (1933). Induction by heteroplastic grafts of the primitive streak in birds. *Wilhelm Roux's Arch. Dev. Biol.* **128,** 522–563.

Wagner, M., Thaller, C., Jessell, T., and Eichele, G. (1990). Polarizing activity and retinoid synthesis in the floor plate of the neural tube. *Nature* **345,** 819–822.

Wanek, N., Gardiner, D. M., Muneoka, K., and Bryant, S. V. (1991). Conversion by retinoic acid of anterior cells into ZPA cells in the chick wing bud. *Nature* **350,** 81–83.

Wanek, N., Muneoka, K., Holler-Dinsmore, G., Burton, R., and Bryant, S. V. (1989). A staging system for mouse limb development. *J. Exp. Zool.* **249,** 41–49.

Received for publication June 14, 1995
Accepted October 31, 1995