Hensen's Node from Vitamin A-Deficient Quail Embryo Induces Chick Limb Bud Duplication and Retains Its Normal Asymmetric Expression of Sonic hedgehog (Shh)

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Both Hensen's node, the organizer center in chick embryo, and exogenous retinoic acid are known to induce limb duplication when grafted or applied to the host chick limb bud. Retinoic acid is known to be present in the node and has been proposed as the putative morphogen for chick limb development. Here, we report that Hensen's node from vitamin A-deficient quail embryo induces limb duplication in the host chick embryo similar to that induced by the node from vitamin A-sufficient control embryos. We also demonstrate that the expression of Sonic hedgehog (Shh), recently shown to be the mediator of polarizing activity in the chick limb bud, is not affected by the endogenous vitamin A status of the embryo. Furthermore, whole-mount in situ hybridization revealed asymmetry of Shh expression in the Hensen's node of both vitamin A-sufficient and -deficient quail embryos. Retinoids were not detectable in the eggs from which vitamin A-deficient embryos were obtained. Extracts from normal embryos induced a level of expression of reporter gene equivalent to the presence of 3.4 pg of active retinoids per embryo, while those from vitamin A-deficient embryos induced a baseline level of reporter gene expression similar to that of the controls. Our studies suggest that endogenous retinoic acid is not involved in Shh expression nor in regulating its asymmetry during normal early avian embryogenesis and support the current view that endogenous retinoic acid may not be a direct morphogen for limb bud duplication.

INTRODUCTION

Studies with exogenous retinoic acid (RA) have led to the speculation that this vitamin A-active molecule is the diffusible morphogen specifying positional information along the anteroposterior axis of the embryo (Durston et al., 1989; Mitrani and Himoni, 1989; Sive et al., 1990; Summerbell and Maden, 1990; Ruiz I Altaba and Jessell, 1991; Eichele, 1993; Hofmann and Eichele, 1994; Gudas, 1994; Linney and Mantia, 1994), best exemplified by the numerous studies on induction of limb duplication when RA is applied to the anterior domain of the developing chick limb bud (reviewed in Hofmann and Eichele, 1994; Scadding and Maden, 1994; Helms et al., 1994). The polarizing region, or zone of polarizing activity (ZPA), is a tissue located on the posterior edge of the chick limb bud, discovered by Saunders and Gas- seling (1968). When this tissue is grafted to a more anterior region of a host limb bud, it causes additional digits to develop in a typical mirror image pattern as the result of a diffusible morphogen that forms a gradient across the anteroposterior axis of the wing bud (Tickle et al., 1975; Saunders and Gasseling, 1983). Polarizing activity has been demonstrated in various embryonic tissues, including the Hensen's node (Hornbruch and Wolpert, 1986; Stocker and Carlson, 1990), a cluster of organizer cells regulating the early primary axial patterning of the three germ layers. The ability of Hensen's
node to induce secondary axis formation in the early chick embryo has been confirmed (Chen and Solursh, 1992) and has not been disputed. However, the hypothesis that RA is the putative morphogen for chick limb development has been seriously challenged (Wanek et al., 1991; Nöji et al., 1991; Colbert et al., 1993) and recently disproved by the demonstration that the product of Sonic hedgehog (Shh) expression is the long-hypothesized morphogen and that RA may function indirectly via induction of Shh (Riddle et al., 1993; Smith, 1994; Niswander et al., 1994).

Shh, the vertebrate homolog of the Drosophila segment polarity gene hedgehog (Mohler and Vaní, 1992), is expressed in ZPA and other regions of the developing chick embryo, including Hensen’s node, notochord, and floor plate (Riddle et al., 1993; Johnson et al., 1994). Implantation of Shh-expressing cell mass into the anterior domain of chick limb bud results in the formation of supernumerary digits by a mechanism that excludes the role of RA as a proximal morphogen (Riddle et al., 1993). It has been suggested that exogenous RA causes chick limb bud duplication by initially converting anterior cells into ZPA cells (Wanek et al., 1991; Nöji et al., 1991). Subsequently, ZPA cells either secrete a morphogen (Nöji et al., 1991) or interact with adjacent anterior cells to bring about digit duplication (Wanek et al., 1991). Exogenous RA can induce ZPA activity and the expression of Shh in the limb bud, suggesting that the actions of exogenous RA in limb bud duplication may be mediated through the induction of Shh, which is the direct signal for anterior–posterior patterning (Riddle et al., 1993; Smith, 1994; Niswander et al., 1994; Helms et al., 1994). In accord with this hypothesis is the localization of Shh in the ZPA of the chick limb bud (Riddle et al., 1993; Helms et al., 1994) and the observation that other Shh-expressing tissues such as Hensen’s node, notochord, and floor plate are also able to induce duplication. Since Hensen’s node is enriched in RA (Chen et al., 1992) and also has the capability to biosynthesize RA (Hogan et al., 1992), the role of RA as a putative morphogen remains under investigation. As the result of these new developments, the role of retinoic acid-regulated gene transcription in limb bud outgrowth has recently received renewed attention (Paulsen, 1994; Madden, 1994a,b; Scadding and Madden, 1994; Helms et al., 1994; Tabin, 1995). The recent findings that fibroblast growth factor is capable of inducing limb development (Cohn et al., 1995) and that Hoxb-8 expression may also be involved (Charite et al., 1994) clearly indicate that Shh is only one of several signaling molecules that regulate the polarizing activity of ZPA.

We have developed an avian embryo model that allows the examination of the role of retinoids in the expression of early developmental genes such as Shh. This model is based on the discovery by Thompson (1969) and the subsequent confirmation by others (Heine et al., 1985; Dersch and Zile, 1993; Twal et al., 1995) that vitamin A is required for early avian embryogenesis. Vitamin A deficiency results in numerous abnormalities in the avian embryo leading to 100% lethality by 72 hr of development, unless vitamin A-active molecules are provided during the first 24 hr of development (Dersch and Zile, 1993). The major gross anatomical defect in the vitamin A-deficient avian embryo is an abnormal heart characterized by situs inversus in 60–70% of cases (Twal et al., 1995). Furthermore, the vascular connections between the heart and the extraembryonal blood pools are absent (Thompson, 1969; Heine et al., 1985; Dersch and Zile, 1993; Twal et al., 1995). A retinoic acid-specific monoclonal antibody (Zhou et al., 1991), when administered to normal quail embryo during early development, causes abnormalities and death of the embryo similar to those resulting from vitamin A deficiency (Twal et al., 1995).

In the studies described here, we further examine the role of retinoic acid and Shh in limb bud duplication using retinoid-deficient quail embryos from eggs laid by quail that had been raised on a vitamin A-deficient diet. Our results demonstrate that there is a temporal asymmetric expression of Shh but that the expression of Shh is not altered by vitamin A deficiency of the embryo during early stages of avian development. We also show that Hensen’s nodes from stage 6–7 vitamin A-deficient quail embryos grafted into the anterior domain of stage 20–21 developing host chick limb buds induce limb duplication similar to that obtained when grafting Hensen’s nodes from normal control quail embryos.

**MATERIALS AND METHODS**

**Quail and Chicken Embryos**

Eggs from Japanese quail (Coturnix coturnix japonica) were obtained from the Poultry Research Farm at Michigan State University. Birds were fed either a normal or a vitamin A-deficient diet (Teklad) supplemented with 10 mg of retinoic acid/kg of diet as the only source of vitamin A, as described previously (Dersch and Zile, 1993; Twal et al., 1995). The embryos from eggs of hens fed the normal diet were termed “normal” or “+A” because they developed normally; the embryos from eggs of hens fed the vitamin A-deficient diet supplemented with retinoic acid were termed “vitamin A-deficient” or “−A” because they developed a severe vitamin A deficiency which is lethal in 100% of the embryos after 72 hr of incubation, but can be prevented by a prior in ovo administration of retinoic acid (Dersch and Zile, 1993). Eggs were collected daily, stored at 13°C for less than 7 days, and incubated at 38°C at 98% relative humidity in an incubator with automatic rotation. Whole quail embryos were dissected from eggs at various stages of development, staged according to Hamburger and Hamilton (1951), and frozen on dry ice and stored at −80°C until analysis or prepared for whole-mount in situ hybridization, or after staging their Hensen’s node was grafted into host chicken embryos. Fertilized normal White Leghorn eggs were obtained from the Poultry Research Farm at Michigan State University and incubated as needed for the generation of host embryos.
Hensen’s Node Grafting

Whole quail embryos of stages 6–7 were removed from eggs and transferred into Ringer’s solution and their Hensen’s nodes were dissected out under a microscope. The egg shell of the chick host was windowed, the host embryo was stained with neutral red, and a small cut was made with a fine glass needle in the anterior margin of the stage 20–21 host wing bud, into which a Hensen’s node from either a normal or a vitamin A-deficient donor quail embryo was implanted. The graft was placed onto the host incision site in a drop of Ringer’s solution. The host egg was then sealed with tape and returned to the incubator for additional 6 days. Control chick hosts were manipulated as above but received no implants.

Analysis of Retinoids by High-Pressure Liquid Chromatography (HPLC)

Yolks and albumen from fertilized –A and normal (+A) eggs were analyzed for retinoids by modification of HPLC methods used routinely in our laboratory (Cullum and Zile, 1985; Salyers et al., 1993). Retinoids were extracted from 0.1-g samples of lyophilized yolks first with methanol (MeOH) and then with hexane in the presence of internal standard (retinyl acetate), 0.001% butylated hydroxytoluene (BHT), and nitrogen. Supernatants were concentrated and redissolved in MeOH and aliquots were analyzed for retinoids by HPLC using a Partisil ODS-3 10-μm reverse-phase column (Whatman) and a Waters Guard-Pak precolumn with a C18 cartridge. Retinoids were separated by a stepwise elution with MeOH:0.01 M ammonium acetate (7:3), 20 min; MeOH:water (88:12), 15 min; and MeOH:CHCl3 (85:15), 10 min (Figs. 1A and 1B). All-trans-retinol and retinal were separated on an Excalibar Spherisorb ODS 5-μm column (Milton Roy Co) by a stepwise elution with MeOH:0.01 M ammonium acetate (65:35), 15 min; MeOH:water (88:12), 17 min; and MeOH:CHCl3 (85:15), 8 min (Fig. 1A, inset). All procedures were performed under amber lighting. Recovery of 5 ng of RA by this analytical procedure was >70%. The detection limit was 2 ng.

Analysis of Retinoids in Embryos by Reporter Assay

For the determination of retinoid activity in the early stage quail embryos, a reporter F9 cell line was used, as described previously (Chen et al., 1992). Briefly, a plasmid containing a luciferase reporter gene, thymidine kinase promoter, and two copies of an RA response element from the RA receptor gene was transiently cotransfected with a β-galactosidase expression vector into F9 cells by electroporation. To obtain a standard RA response curve, transfected cells were cultured in 1 ml of DMEM medium in 35-mm culture dishes containing different concentrations of all-trans-RA.

Fifty-five +A and 55 –A quail embryos from stage 6–7 were pooled and dispersed in 1 ml of phosphate-buffered saline, pH 7.2, containing 28 mM ascorbate and 13 mM ethylenediaminetetraacetic acid; after the addition of 1 ml of MeOH containing 0.001% BHT, the samples were sonicated for 15 min and centrifuged, supernatants were collected, and residues were extracted with hexane containing BHT. The extracts were stored at –80°C until used in the assay. Throughout all procedures the samples were kept under N2 and cold. The pooled supernatants from each set of embryos were concentrated and aliquots were used for assays of retinoid activity in the above reporter system. Tissue extracts from 11 embryos dissolved in 2 ml of dimethyl sulfoxide (DMSO) was added to transfected cells in different man-ipulated as above but received no implants. Dishes. For control, 2 ml of DMSO was added to the medium. After 24 hr of culture, cells were extracted and luciferase activity was measured and plotted against the standard RA response curve after normalization for protein content in cell extracts. All experiments involving retinoids were conducted under amber lights.

In Situ Hybridization

DNA fragment, approximately 550 bp from the 3′ end of chick Sonic hedgehog cDNA (a gift from Dr. Izpisua-Belmonte), was used for generating riboprobes. Whole-mount in situ hybridization was performed with some modifications (Kostetskii et al., 1995) of the method of Riddle et al. (1993). Embryos were fixed overnight at 4°C with 4% paraformaldehyde in PBS, washed twice in PBS/0.1% Tween 20, dehydrated through an ascending methanol/PBS gradient, and postfixed with 4% paraformaldehyde/0.2% glutaraldehyde in PBS. Following prehybridization and hybridization with digoxigenin-labeled riboprobe, embryos were washed twice with 2× SSC/1% SDS at 60°C and twice with 0.5% SSC/1% SDS at 65 and 70°C. After overnight incubation at 4°C with the preadsorbed antibody, embryos were washed extensively and the antibody was detected with nitroblue tetrazolium in presence of X phosphate. After color development, embryos were washed with PBS and photographed under transmitted light using a Nikon stereo microscope. For negative controls hybridization was performed with a sense probe. Sections were made at 7 μm.

Analysis of Wing Patterns

Chick embryos were fixed in 3% trichloroacetic acid, stained in Alcian green, treated with acidic ethanol, cleared with histosol, then examined for limb patterns, and photographed.

RESULTS

Retinoid Content of Eggs

The HPLC analysis of retinoids in yolks from quail eggs is shown in Fig. 1. Normal (+A) quail yolk (Fig. 1A) contained...
Hensen's Node, Shh Asymmetry, and Retinoids

FIG. 1. HPLC analysis of retinoids in quail eggs. (A) Representative HPLC profile of yolk extract from normal (+A) quail eggs. Arrows indicate elution positions of authentic retinoids: 4-oxo-RA (1), 3,4-didehydroretinoic acid (2), 13-cis-RA (3), 9-cis-RA (4), all-trans-RA (5), 3,4-didehydroretinol (6), all-trans-retinol (7), all-trans-retinal (8), retinyl acetate (9), and retinyl palmitate (10); solvent front (SF). Peaks without designation do not contain retinoids. Data represent three pooled samples of 13 yolks each and six determinations for each set of pooled samples. Albumen does not contain any retinoids. Inset shows chromatography of combined peaks 7 and 8 in an HPLC system that resolves retinol and retinal. Retinal, retinoic acids, and polar retinoids were not detected in normal quail egg yolk. Detection limit, 2 ng. Details of quantitation are described under Materials and Methods.

20.6–51.0 μg of retinol, 1.8–2.7 μg of didehydroretinol, and 11.0–32.2 μg of retinyl esters. The data represent three pooled samples of 13 yolks each and six determinations for each set of pooled samples. Albumen did not contain any retinoids. Figure 1A (inset) shows chromatography of the combined peaks 7 and 8 from Fig. 1A using an HPLC system that resolves retinol and retinal. Retinal, retinoic acids, and polar retinoids were not detected in normal quail egg yolk. Figure 1B represents the HPLC profile of pooled yolk extracts from vitamin A-deficient quail eggs. The data represent the HPLC analysis of 11 individual yolk extracts. No retinoids were detected in the yolks of the –A quail eggs.

TABLE 1
Induction of Host Chick Limb Duplication by Hensen’s Node Grafts from Quail Embryos

<table>
<thead>
<tr>
<th>Source of Hensen's node</th>
<th>Total number of grafts</th>
<th>Normal limbs in host embryo</th>
<th>Limbs with supernumerary digits in host embryo</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Digital pattern</td>
<td>Digital pattern</td>
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<tr>
<td></td>
<td></td>
<td>2 3 4</td>
<td>2 2 3 4 or 3 3 4 or 2 3 4</td>
</tr>
<tr>
<td>Normal quail embryo</td>
<td>23</td>
<td>11 (48%)</td>
<td>9 (39%)</td>
</tr>
<tr>
<td>Vitamin A-deficient quail embryo</td>
<td>24</td>
<td>10 (42%)</td>
<td>9 (38%)</td>
</tr>
</tbody>
</table>

Retinoid Content of Embryos

Figure 2 shows the results of luciferase activity in RARE-TK plasmid-transfected F9 cells, incubated with pooled extracts from either +A or –A stage 6–7 quail embryos. Cells showed a graded response of luciferase activity to increasing concentrations of retinoic acid. Luciferase activity induced by tissue extracts from +A and –A embryos was plotted against the RA standard response curve. The luciferase activity induced by the pooled tissue extracts from 11 normal embryos that resolves retinol and retinal. Retinal, retinoic acids, and polar retinoids were not detected in normal quail egg yolk.
embryos corresponded to $1.25 \times 10^{-10}$ M retinoic acid equivalent and thus represents 3.4 pg of active retinoids per embryo, which is in the range of the amount of retinoids reported in chick embryo (Chen et al., 1992). The luciferase activity in cells cultured with medium containing pooled tissue extracts from 11 vitamin A-deficient embryos induced a baseline level of reporter gene expression similar to that of controls, demonstrating an absence of biologically active retinoids. These findings are in agreement with our earlier demonstration of the absence of immunoreactivity of a monoclonal antibody against RA in −A embryos, while normal embryos were immunostained (Twal et al., 1995).

Effect of Grafted Hensen’s Nodes on Digit Patterns in Host Chick Limbs

Hensen’s nodes from stage 6–7 normal quail embryos, when grafted onto the anterior domain of stage 20–21 wing buds of developing host chick embryos, induced supernumerary digits 2 and 3 with 52% frequency (Table 1; Fig. 3a), in agreement with the induction frequency and duplication pattern reported previously (Hornbruch and Wolpert, 1986; Stocker and Carlson, 1990). Notably, Hensen’s nodes from vitamin A-deficient embryos were equally potent in the induction of digit duplication in the host chick limb bud (Table 1; Fig. 3b), with an induction frequency of 58% and a duplication in the digit pattern similar to that induced by nodes from normal embryos.

Expression of Sonic Hedgehog

Whole-mount in situ hybridization was performed on stage 5–7 normal and −A quail embryos using riboprobes generated from chick Shh cDNA (Fig. 4). The expression pattern of Shh in −A quail embryos was the same as that in normal embryos and corresponds to that reported for normal chick embryos (Riddle et al., 1993). Shh was expressed in Hensen’s node and in the midline of the axial structures above it. No Shh mRNA was detected in normal quail embryos hybridized with sense Shh probe (Fig. 4G). A cross section of the whole mount of stage 7 + vitamin A-deficient quail embryo localizes Shh transcripts in the notochord and floor plate (Fig. 4H). Interestingly, Shh is expressed asymmetrically in the Hensen’s node, with localization to the left side of the node. This asymmetric expression of Shh is evident in both normal and vitamin A-deficient stage 5 and 6 quail embryos (Figs. 4A, 4B, 4D, and 4E), but diminishes at stage 7 (Figs. 4C and 4F).

DISCUSSION

The proposed function of retinoic acid as an endogenous morphogen (Tickle et al., 1975) has resulted in a critical

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**FIG. 4.** Whole-mount in situ hybridization showing the expression of Shh in stage 5, 6, and 7 normal quail embryos (A, B, and C) and vitamin A-deficient quail embryos (D, E, and F). A control stage 7 normal quail embryo hybridized with sense Shh probe is shown in G. Shh mRNA is detected in the midline of the axial structures (arrowheads) and in Hensen’s node of all normal and vitamin A-deficient embryos examined. The localization of Shh transcripts in the notochord (NC) and floor plate (FP) is shown in the cross section of a stage 7 + vitamin A-deficient embryo (H). Note that the transcripts of Shh are asymmetrically localized to the left edge of Hensen’s node in stage 5 (A) and 6 (B) normal embryos. Similar localization of Shh transcripts is also observed in stage 5 (D) and 6 (E) vitamin A-deficient embryos. This asymmetric expression of Shh in the node disappears at stage 7 in both normal (C) and vitamin A-deficient (F) embryos. All embryos are shown in ventral view. Hensen’s node pit is indicated by arrows. Diagram shows approximate level of sectioning (dotted line across anterior part of the embryo) for H. Headfold, HF; somites, S; Hensen’s node, HN; primitive streak, PS. Bars, 250 μm.
examination of the role of vitamin A-active molecules in early development in various embryonic model systems (Summerbell and M aden, 1990; Savard, 1992; Eichele, 1993; Paulsen, 1994; Hofmann and Eichele, 1994). While the significance of retinoids, particularly retinoic acid, as important signaling molecules in early development becomes increasingly more evident (Summerbell and M aden, 1990; M aden and Holder, 1992; Savard, 1992; Linney and LaMantia, 1994), recent studies do not support the morphogen role of endogenous retinoic acid in limb patterning (Wanek et al., 1991; Bryant and Gardiner, 1992; Colbert et al., 1993; Riddle et al., 1993; Smith, 1994; Johnson et al., 1994; Hayamizu and Bryant, 1994). We used a vitamin A-deficient quail embryo model to further clarify the role of retinoic acid in avian limb duplication. We grafted Hensen’s nodes from vitamin A-deficient quail embryos into the anterior margin of host chick limb buds and discovered that these nodes, similar to those obtained from vitamin A-sufficient quail embryos, were capable of inducing digit duplication in the host, with a similar digit duplication pattern and induction frequency. This observation suggests that the presence of retinoic acid in the donor Hensen’s node is not required to induce digit duplication in the host limb bud and thus indirectly supports the accumulating evidence invalidating the theory of the morphogen function of retinoic acid (Johnson et al., 1994). Questions can be raised about the retinoid-deficient status of Hensen’s nodes from the vitamin A-deficient quail embryos. While the reporter assay failed to detect any retinoid activity above the baseline in the vitamin A-deficient quail embryos, our methodology cannot exclude the small absolute amounts of retinoids that might be present at early stages of development and that might be derived from maternal materials. However, the small absolute amount of retinoids that might be present in the early stage vitamin A-deficient embryo is not sufficient to induce limb bud duplication, since the lowest concentration of exogenously applied RA that is able to induce limb duplication is 3 µg/ml (Helms et al., 1994). Support for the vitamin A-deficient status of the embryos used in this work comes from our routine observations that all vitamin A-deficient quail embryos develop the vitamin A-deficient phenotype at the same stage in development (Dersch and Zile, 1993; Twal et al., 1995) and that in these embryos the expression of RARβ1 is significantly decreased and the mRNA of RARβ2, known to be regulated by vitamin A status, is not detectable (Kostetskii and Zile, 1993, 1995; Kostetskii et al., 1995). We have also demonstrated that the expression of Msx-1 is altered in the vitamin A-deficient early quail embryos (Chen et al., 1995). These interpretations are corroborated by our demonstration of the absence of immuno-reactivity of a monoclonal antibody against retinoic acid in the vitamin A-deficient embryos, while normal embryos, including their Hensen’s nodes, were immunostained (Twal et al., 1995).

However, the absence of bioactive retinoids from the Hensen’s node does not preclude the potential for the Hensen’s node, when grafted into the normal chick limb bud, to secrete retinoic acid precursor(s) from the host and to generate retinoic acid. In order to further explore the role of endogenous retinoids in early development, we asked the question whether Shh can be expressed in the vitamin A-deficient embryo, since Shh is a required component of the limb bud induction pathway (Tabin, 1995) and since the function of retinoic acid in limb patterning may be linked to the expression of Shh. We were particularly interested in those tissues known to be important embryonic organizers and to have polarizing activity, i.e., the Hensen’s node, the notochord, and the floor plate (Hornbruch and Wolpert, 1986; Stocker and Carlson, 1990; Wagner et al., 1990; Izpisua-Belmonte et al., 1992), where Shh expression has already been demonstrated (Riddle et al., 1993; Johnson et al., 1994). If Shh expression is required for the induction of chick limb bud and if Shh expression is dependent on retinoic acid, then one would expect Shh expression also to be regulated by endogenous retinoic acid in other polarizing tissues such as the Hensen’s node, the notochord, and the floor plate. However, the studies reported here unequivocally demonstrate that the expression of Shh in these important signaling tissues was not affected by the absence of retinoids. It is therefore very unlikely that retinoids directly participated in the limb bud duplication that was initiated by grafting the Hensen’s nodes from the vitamin A-deficient quail embryos into the anterior margin of the limb bud of the host. Direct proof of the role of endogenous retinoids in limb patterning is yet to be obtained. It was recently shown that Shh expression can be induced by the fibroblast growth factors, FGFs (Cohn et al., 1995; Yang and Niswander, 1995), which are present in the early avian embryo (Mitrani et al., 1990). It is thus possible that FGFs are responsible for the induction and expression of Shh in the early quail embryo.

The salient finding from our studies reported here is that Shh expression in the early quail embryo is not affected by the absence of vitamin A-active molecules. Not only was Shh expression in the vitamin A-deficient quail embryo co-localized with all the important organizer tissues (Johnson et al., 1994), but also the asymmetric expression of Shh in the Hensen’s node that we observed in the vitamin A-sufficient quail embryo was unaltered by the lack of retinoids. A similar asymmetric expression pattern of Shh was also observed in the chick Hensen’s node (M. Levin and C. Tabin, personal communication). The transcripts of Shh in normal as well as in vitamin A-deficient stage 5 and 6 quail embryos were asymmetrically localized to the left edge of the Hensen’s node. It is interesting to note that Wetzels in 1929 reported an asymmetry in the chick Hensen’s node, which at the primitive streak stage was observed to consist of two parts, with the right-hand part being bigger than the left (Wetzel, 1929). We observed that the asymmetric expression of Shh in the Hensen’s node disappeared at stage 7 in both normal and vitamin A-deficient quail embryos. During avian embryogenesis, the first visible morphological asymmetry between the left and right sides is the looping of the early heart, which begins at stage 11. Thus it is likely
that the temporal asymmetric expression of Shh that we observed coincides with the determination of the looping direction of the heart tube in the precardiac mesoderm at stage 5 and 6, as proposed by Hoyle et al. (1992). Shh is the first molecule reported to be expressed asymmetrically in the Hensen’s node in the early chicken (M. Levin and C. Tabin, personal communication) and quail embryo, indicating that left–right handedness is regulated at the molecular level during the stages of gastrulation/neuralization. The significance of this asymmetric expression of Shh in the Hensen’s node remains to be elucidated.

The principal findings of our studies are (a) the expression of Sonic hedgehog in the important signaling tissues of the early quail embryo is not regulated by endogenous retinoids; (b) there is a temporal asymmetric expression of Sonic hedgehog in the Hensen’s node and it is not affected by the absence of endogenous retinoids; and (c) the lack of endogenous regulation of Shh by retinoids in tissues with polarizing activity strongly suggests that retinoids are not directly involved in the duplication of digits induced by the vitamin A-deficient Hensen’s node grafted in the host limb bud. Further experiments are needed to provide direct evidence for the role of endogenous vitamin A–active molecules in limb patterning.

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REFERENCES


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