

Regional differences in retinoid release from embryonic neural tissue detected by an in vitro reporter assay

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Summary

Retinoic acid and related retinoids have been suggested to contribute to the pattern of cell differentiation during vertebrate embryonic development. To identify cell groups that release morphogenetically active retinoids, we have developed a reporter assay that makes use of a retinoic acid inducible response element (RARE) to drive *lacZ* or *luciferase* reporter genes in stably transfected cell lines. This reporter gene assay allows detection of retinoids released from embryonic tissues over a range equivalent to that induced by femtomole amounts of retinoic acid. We have used this assay first to determine whether the floor plate, a cell group that has polarizing properties in neural tube and limb bud differentiation, is a local source of retinoids within the spinal cord. We have also examined whether the effects of exogenously administered retinoic acid on anteroposterior patterning of cells in the developing central nervous system correlate with differences in retinoid release from anterior and posterior neural tissue. We find that the release of morphogenetically active retinoids from

the floor plate is only about 1.5-fold that of the dorsal spinal cord, which does not have neural tube or limb polarizing activity. These results suggest that the spatial distribution of retinoid release from spinal cord tissues differs from that of the neural and limb polarizing activity. This assay has also shown that retinoids are released from the embryonic spinal cord at much greater levels than from the forebrain. This result, together with previous observations that the development of forebrain structures is suppressed by low concentrations of retinoic acid, suggest that the normal development of forebrain structures is dependent on the maintenance of low concentrations of retinoids in anterior regions of the embryonic axis. This assay has also provided initial evidence that other embryonic tissues with polarizing properties in vivo release retinoids in vitro.

Key words: retinoids, neural patterning, floor plate, notochord, zone of polarizing activity.

Introduction

The induction and patterning of many vertebrate tissues depends on signals that derive from restricted groups of cells in the early embryo. The induction and anteroposterior (A-P) patterning of neural ectoderm in amphibia is controlled by signals from cells in the dorsal lip of the blastopore, the organizer region (Spemann, 1938; Hamburger, 1988; Dixon and Kintner, 1989; Ruiz i Altaba, 1990). The patterning of neural cell types along the dorso-ventral (D-V) axis of the neural tube appears to depend on signals from axial mesodermal cells of the notochord and from floor plate cells at the ventral midline of the neural tube (van Stratten et al., 1988; Placzek et al., 1990a, 1991; Yamada et al., 1991; Hatta et al., 1991). Grafts of the notochord or floor plate to dorsal regions of the neural tube induce an ectopic floor plate and the dorsal differentiation of motor neurons and other classes of neurons normally located in the ventral spinal cord (Yamada et al., 1991; Placzek et al., 1991). Inversely, elimination of the notochord and floor plate results in the formation of a spinal cord that is devoid of ventral neurons (Placzek et al., 1991; Yamada et al., 1991).

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The molecules responsible for the patterning properties of the organizer, the notochord and floor plate have not been identified. These three cell groups may, however, have conserved signalling properties. One line of evidence for this derives from studies of chick limb development. The A-P polarity of the chick limb appears to be regulated by signals that derive from a group of mesenchymal cells located in the posterior margin of the limb, the zone of polarizing activity (ZPA). Grafts of ZPA cells into the anterior region of the limb bud result in mirror-image duplication in the pattern of digits (Saunders and Gasseling, 1968; Tickle et al., 1975). Similar mirror-image digit duplications result when Hensen's node (the avian equivalent of the amphibian organizer), the notochord and the floor plate are grafted into the chick limb bud (Hornbruch and Wolpert, 1986; Stoker and Carlson, 1990; Wagner et al., 1990). These studies raise the possibility that the patterning prop-

erties of these three midline cell groups are mediated by similar or identical molecules and that these are related to the polarizing signals present in the ZPA.

Retinoids are the only molecules known to mimic the actions of the ZPA in evoking digit duplication (Eichele, 1989; Tickle, 1991). Grafting beads soaked in retinoic acid (RA) or 3,4 didehydro-RA into the anterior margin of the limb bud leads to a dose-dependent duplication of the digit pattern (Summerbell, 1983; Tickle et al., 1985; Thaller and Eichele, 1990). Moreover, both RA and 3,4 didehydro-RA are found in the limb bud and RA is present at higher concentration in the posterior than in the anterior region (Thaller and Eichele, 1987; Thaller and Eichele, 1990). A graded distribution of retinoids within the limb bud could directly control the pattern of mesenchymal cell differentiation. Alternatively, a local posterior source of RA could induce other signalling molecules within the ZPA region (Thaller and Eichele, 1987; Tickle, 1991; Summerbell and Harvey, 1983; Wanek et al., 1991; Noji et al., 1991).

The application of RA modifies axial patterning in regions of the vertebrate embryo other than the limb bud. In particular, RA modifies the A-P patterning of axial mesoderm and neural ectoderm by suppressing the development of both anterior mesodermal and anterior neural cell types (Durston et al., 1989; Ruiz i Altaba and Jessell, 1991a,b; Sharpe, 1991; Morriss-Kay et al., 1991; Holder and Hill, 1991; Sive et al., 1990; Sive and Cheng, 1991; Sundin and Eichele, 1992). RA also suppresses the differentiation of neural crest cells and has marked effects on the development of the dorsal neural tube (Webster et al., 1986; Yasuda et al., 1987). The widespread changes in the pattern of cell differentiation observed after application of RA to early embryos could therefore reflect a role for retinoids in the patterning of many vertebrate tissues. In particular, it is possible that the patterning properties of the notochord and floor plate reflect the ability of these cell groups to release morphogenetically active retinoids.

The regional distribution of retinoids within vertebrate tissues other than the limb bud has not been determined. This is, in part, because the sensitivity of available biochemical methods precludes measurement of retinoids in small amounts of tissue. Moreover, in identifying local sources of retinoid activity in the early embryo, measurements of the total cellular content of retinoids may not be as relevant as the amount of retinoid released by tissues. To provide more information on the potential role of retinoid signals in embryonic tissue patterning, we have developed retinoid-sensitive reporter cell lines that permit quantitative analysis of retinoid release from small regions of embryonic tissue *in vitro*. In this study, we have used this assay to address two primary issues. First, does the floor plate provide a local source of retinoids within the neural tube which can account for its polarizing activity in the neural tube and in the limb bud? Second, do the effects of RA on anteroposterior patterning of the neural tube coincide with differences in retinoid release from anterior and posterior neural tissue? Our results show that the activity of morphogenetically active retinoids released from the floor plate is only marginally greater than that released from dorsal regions of the spinal cord, suggesting that the release of retinoids is not sufficient to account for the ability of the

floor plate to establish D-V pattern in the neural tube. In addition, we show that retinoids are released from the spinal cord at much greater levels than from the forebrain, providing evidence for marked differences in retinoid release from cells at different axial levels of the embryonic CNS. We also demonstrate that this assay can be used to detect retinoid release from Hensen's node and posterior limb bud mesenchyme, two other embryonic tissues with polarizing properties.

Materials and methods

Construction of plasmids

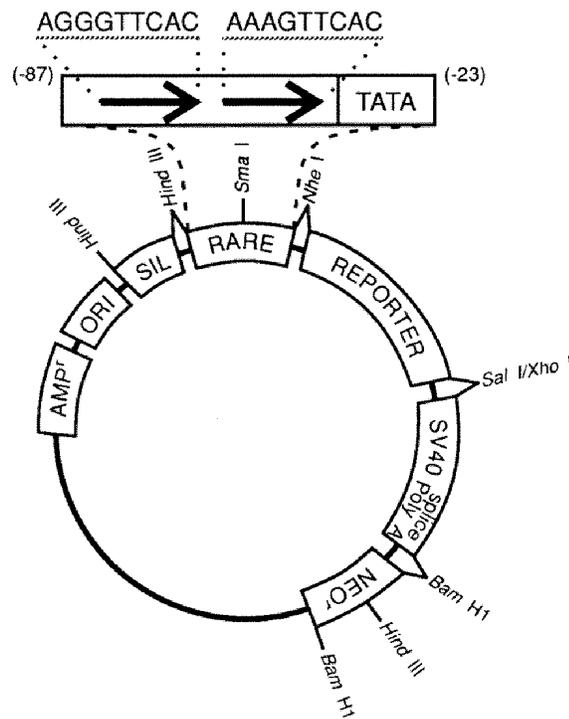
This reporter system uses a retinoic acid-response element (RARE) located within the *cis*-acting regulatory sequences of the human β -retinoic acid receptor gene (de The et al., 1990). This RARE is compact (64 nucleotides) and functions as an inducible enhancer that responds to the α and β retinoic acid receptor (RAR) subtypes (Sucov et al., 1990). A single copy of this RARE was placed immediately upstream of the *E. coli lacZ* or firefly *luciferase* genes, conferring retinoid responsiveness to these genes. The *lacZ* gene is used for histochemical detection of retinoid-responsive cells and the *luciferase* gene is used to provide a quantitative assay of reporter gene activation. The inclusion of an aminoglycoside phosphotransferase (*NEO^r*) gene permits the establishment of transfected cell lines that stably maintain these reporter gene constructs (Fig. 1A,B). The F9 teratocarcinoma cell line was used because it expresses endogenous α and β retinoic acid receptors (Zelent et al., 1989). L cells were used for the luciferase assay to avoid potential problems that might result from the differentiation of F9 cells upon exposure to RA. The presence of retinoic acid receptors in L cells has been inferred from studies in which L cells carrying RA reporter constructs with the same RARE respond specifically to RA (Sucov et al., 1990).

For construction of the Sil-REM/ β -gal-NEO reporter construct (see Fig. 1), the *E. coli lacZ* gene obtained from plasmid pCH110 (Clontech Laboratories, Inc.) by *HindIII/BamHI* digestion, was blunt-ended and cloned into the *SmaI* site of pMSG (Pharmacia). Sequences corresponding to the minimally sized, fully functional RARE and basal promoter associated with the β -RAR gene (de The et al., 1990) were synthesized chemically and subcloned into the β -gal/pMSG vector. *NEO^r* gene sequences were introduced 3' to the reporter gene by use of a *BamHI NEO^r* gene cassette consisting of SV40 early promoter/*NEO^r* gene/SV40 early splice-polyadenylation signal sequences. A *HindIII* "silencer" cassette with trimerized SV40 polyadenylation signal sequences (kindly provided by Dr I. Maxwell, University of Colorado; Maxwell et al., 1989) was placed immediately 5' to the RARE.

For construction of the luciferase reporter construct, Sil-REM/luc-NEO, the firefly luciferase gene including a SV40 splice and polyadenylation signal was obtained from plasmid pSV232AL-A 5' (kindly provided by Dr D. Helinski, University of California, San Diego; de Wet et al., 1986). The *luciferase* gene/SV40 sequences were inserted into a Sil-REM/NEO vector prepared by removing the *lacZ* gene from the Sil-REM/ β -gal-NEO construct.

RARE mutant reporter constructs containing mutated or deleted RARE direct repeat sequences were generated as follows. Oligonucleotides identical to wild-type RARE sequences but with their RARE direct repeat sequences either omitted or completely randomized were chemically synthesized and made double-stranded by PCR amplification. The mutant RAREs were ligated into a β -gal/pMSG vector lacking the *NEO^r* cassette. A *NEO^r* cassette was subsequently inserted into all mutant RARE constructs.

A. Basic reporter construct



B. Reporter genes

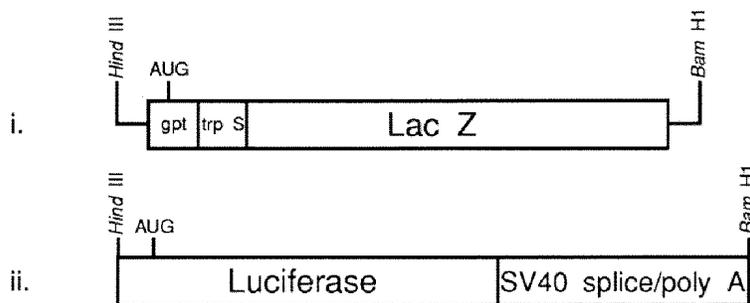


Fig. 1. Plasmid reporter constructs. (A) Basic reporter construct. Essential elements of the reporter construct used in these studies are depicted in this schematic diagram (not drawn to scale). Reporter genes are cloned into a unique *Sma*I site placing them under the transcriptional control of the RARE situated immediately upstream. Transcriptional read-through of the reporter gene is suppressed by the placement of a SV40 poly (A)/splice site cassette ("SIL" = silencer; Maxwell et al., 1989) upstream of the RARE promoter/enhancer cassette. The general alignment of elements within the RARE cassette is depicted in expanded form. Standard SV40 promoter and splice/polyadenylation signals are present, as are *NEO^r* gene sequences for the establishment of stable transformants. (B) Reporter genes. Structure of reporter genes used in these studies. (i) The *lacZ* gene is from the pCH110 plasmid (Clontech). Constructs with this reporter gene are referred to as Sil-REM/ -gal-NEO. (ii) The firefly luciferase gene is from plasmid pSV232A1-5 (de Wet et al., 1986). Constructs with this gene as reporter are referred to as Sil-REM/luc-NEO.

DNA transfections

Between 10 and 20 μ g of plasmid DNA was used per transfection. Transfection into F9 cells was carried out according to published protocols (Espeseth et al., 1989). For transfection of L cells, cells were plated at a density of $0.6-1 \times 10^6$ cells per 10 cm plate, cultured for 1 day, and given fresh media 4 hours prior to transfecting. One day after transfection, cells were given non-selective media. Two days after transfection, cells were split 1:5 and placed in selection media (0.5 mg/ml G418 supplement). Selection was maintained until the appearance of drug-resistant colonies.

To screen for RA-responsive F9 clones carrying Sil-REM/ -gal-NEO plasmid constructs, individual colonies were picked, transferred into triplicate 24-well plates, and grown to near confluency. Stock concentrations of RA in DMSO (5 mM) were diluted to 5×10^{-7} M in serum-free L15-CO2 media containing N3 supplement and antibiotics. Cells were incubated in this media for 16-18 hours; control cultures had media with equivalent dilutions of DMSO. After incubation, the cells were fixed and assayed for -galactosidase production using a standard X-gal in situ assay (see below).

For RA-responsive L cell clones carrying the Sil-REM/luc-NEO plasmid construct, colonies were transferred to 24-well plates and the cells treated in essentially the same way as the F9 transfectants. For assay of luciferase production, cell lysates were prepared and analysed using reagents and protocols provided by the commercially available "Luciferase Assay System" (Promega). An LKB 1250 luminometer was used for light detection (see below).

In addition to these cell lines, NIH-3T3, HeLa and S91-C2 melanoma cells were transfected with the -galactosidase reporter construct.

 β -galactosidase, luciferase and protein assays *β -galactosidase*

Visualization of *lacZ* positive cells in situ was carried out using standard protocols (Lim and Chae, 1989). *LacZ* expression in cells was measured by assay of -galactosidase activity in cell lysates (Reynolds and Lundblad, 1989). Briefly, cultures were washed with PBS and the cells were suspended non-enzymatically and transferred to Eppendorf tubes. Cells were washed again, pelleted, suspended in PBS/1 mM EDTA and lysed by three freeze-thaw

cycles. Cell debris was pelleted and the cleared lysate assayed for β -galactosidase activity with modification for use with ELISA plates. Quantitation was made possible by use of standard curves generated with known amounts of commercially obtained β -galactosidase.

Luciferase

Quantitation of luciferase gene expression was carried out using the reagents and protocols in the Luciferase Assay System (Promega). Light output was detected using a LKB 1219 Rack-beta scintillation counter with the coincidence circuit between photomultiplier detectors turned off (Nguyen et al., 1988). Counting commenced approximately 10 seconds after the cell lysate and luciferase cocktail were mixed and continued for 3 minutes.

The specificity of these reporter cells was determined by testing the ability of various substances to activate reporter gene expression, among them, phorbol 12-myristate 13-acetate (10^{-7} M, 10^{-8} M), forskolin (10^{-5} M, 10^{-6} M) and dexamethasone, estradiol, progesterone, testosterone, L-thyroxine, Vitamin D₃ and d-aldosterone, all at 10^{-6} M. Near-confluent cultures of reporter cells in 35 mm dishes were incubated in culture media containing these compounds at the specified concentration. After incubation, the cells were harvested and expression of reporter gene activity determined as described above.

Protein

Detergent-free cell lysates were assayed for protein content using the Bio-Rad protein assay kit (Biorad). Cell lysates containing Triton X-100 were assayed using the micro-BCA protein assay reagent kit (Pierce).

RA release from ion-exchange beads

AG1-X2 ion exchange beads (Biorad, 100-250 μ m in diameter) were loaded with known concentrations of [³H]RA (53.4 Ci/mmol; 18.72×10^{-6} M stock concentration in DMSO) essentially as described (Eichele et al., 1984). Briefly, single ion-exchange beads of approximately the same size (200 μ m diameter) were placed in 250 μ l of RA solution and shaken for 20 minutes at room temperature. The RA solution was removed and the beads washed three times for 1, 10 and 10 minutes. The final wash is carried out in serum-free culture medium. To monitor the efficiency of bead loading, 100 μ l of the [³H]RA loading solution was taken before and after loading of the AG1-X2 beads and counted by liquid scintillation counting. The beads were then placed in Terasaki wells containing 15 μ l of media and incubated for up to 20 hours. Beads were removed and the culture media assayed directly for released [³H]RA by liquid scintillation counting (efficiency of counting ³H is 42%). Disintegrations/minute were converted to the amount of retinoic acid released into the media. To relate fmol RA released by beads to luciferase activity, similarly loaded AG1-X2 beads were placed on near-confluent L cell luciferase reporter cells grown in Terasaki plates and cultured for the same amount of time in media lacking serum. Beads were removed, the monolayers washed and cells lysed directly in luciferase lysis reagent. Luciferase activity was determined as described above.

Tissue explant studies

Reporter cells were cultured in normal selective media until 80%-90% confluent. Tissues for assay were prepared by dissection in L-15 Liebovitz's media, washed briefly in the appropriate culture media lacking serum and explanted onto monolayers of reporter cells. Care was taken to ensure that tissues adhered to the cell monolayer. Explants were co-cultured on reporter monolayers overnight for 18-20 hours and subsequently processed for analysis of reporter gene expression. We used an 18-20 hour assay time to permit maximal induction of *lacZ* expression in reporter cells

under conditions in which tissue explants remained viable. *LacZ* induction was first detectable in reporter cells about 4 hours after addition of RA in solution or of tissue explants.

F9 cells were seeded in 35 mm culture dishes and grown to near confluency over 48 hours. After co-culturing of tissue explants, both explants and cells were gently washed with PBS, fixed and developed for *lacZ* expression with X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) as substrate.

L cells were cultured in Terasaki wells. After co-culturing with tissue explants, tissue pieces were removed, and the cells washed and lysed directly in luciferase cell lysis reagent (Promega). Light output (in counts/minute) was measured as described. A typical standard curve relating luciferase activity with RA released into the medium is shown in Fig. 4B. To assess the release of RA by tissue, a standard curve of this type is generated in each experiment. Tissue explants that gave luciferase activities outside the standard curve were excluded from further analysis.

The retinoic acid-responsive F9 transformant lines were at times heterogeneous in their response as judged by the number of cells expressing the *lacZ* product, β -galactosidase. To overcome this, cell lines were subcloned to achieve a 95% or greater response to test concentrations of 5×10^{-8} M RA or responsive cells isolated by fluorescence-activated cell sorting using a fluorescent *lacZ* substrate (Nolan et al., 1988). Under these conditions, cell lines were stable with respect to reporter function; F9 transformant lines were used up to passage 10 while L cell lines (which grow less rapidly than F9 cells) showed no evident decline in response up to passage 17.

Results

Characterization of retinoid-responsive reporter cell lines

The actions of retinoic acid (RA) and other morphogenetically active retinoids on vertebrate embryonic tissues are mediated by interactions with nuclear receptors which bind to specific retinoid-responsive elements within the promoters of subordinate genes, activating their transcription (Umesono et al., 1991; Leid et al., 1992; De Luca, 1991). We have made use of this signalling pathway to create stable cell lines capable of responding to retinoids by expression of reporter genes (see Materials and methods).

Of several reporter lines generated, two lines, F9-RARE-*lacZ* and L-RARE-luc, were used in the present experiments (Fig. 2). The response of these cell lines to three major retinoids found in embryonic tissues, i.e. retinol, retinal and RA, was assayed. Both cell lines responded to RA introduced into the culture medium. At RA concentrations between 10^{-13} M and 10^{-7} M, F9-RARE-*lacZ* cells responded with a relatively linear dose-response curve (Fig. 2A), although at concentrations greater than 10^{-7} M there appeared to be a suppression in the maximal response. It was difficult to determine the threshold concentration for activation of reporter lines since responses consistently above background were detected at RA concentrations below 10^{-13} M (Fig. 2A). Responses to these low retinoid levels may result from the concentration of retinoids within cells over the course of the 18-20 hour assay period. Histochemical assays also show that RA applied in solution induced uniform *lacZ* expression in monolayers of reporter cells (Fig. 3A,B) with a threshold response at approximately 10^{-10} M RA (not shown).

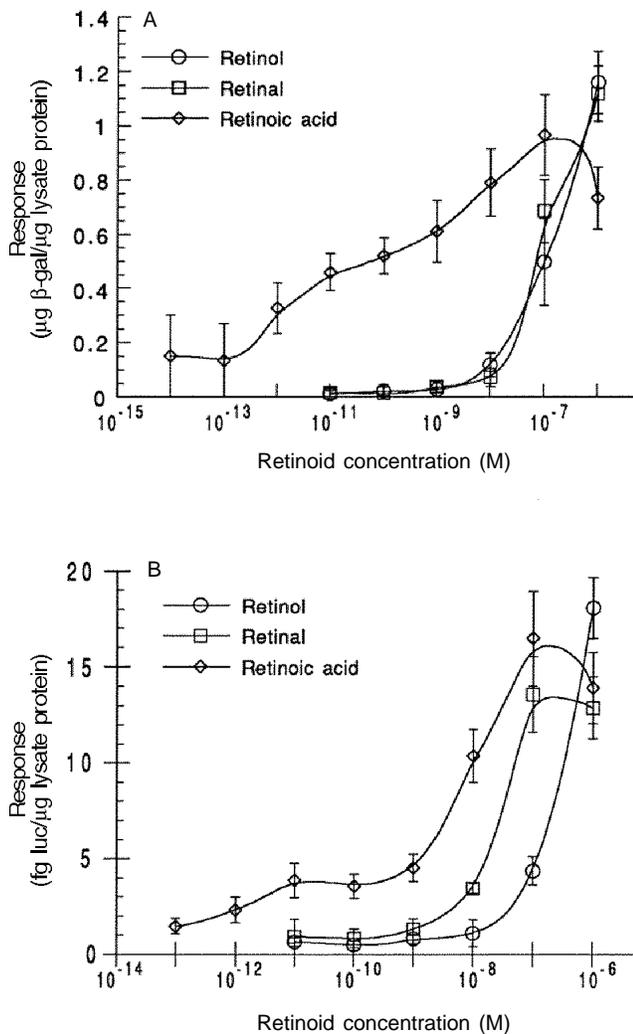


Fig. 2. Response of reporter cell lines to retinoids. (A) Dose-response curve for the F9-RARE-*lacZ* reporter cell line. Individual 35 mm cultures were treated with a given concentration of retinoid in media lacking serum, harvested and assayed for β -galactosidase activity (see Materials and methods). To avoid variation between cultures and experiments, each data point represents the specific activity of the reporter cells defined as $\mu\text{g } \beta\text{-galactosidase}/\mu\text{g cell lysate protein}$. Each point is the mean \pm s.e.m. of 4-6 separate experiments done during early passages of the reporter line. (B) Dose-response curve for the L-RARE-*luc* reporter cell line. Reporter cells were cultured in 24-well plates and treated for 18-20 hours with different concentrations of retinoids in serum-free media. Cells were harvested and assayed for luciferase activity as described in Materials and methods. Luciferase activity was converted to fg luciferase by comparison with standard curves relating light output (counts/minute) to known amounts of luciferase. Each point represents the response of the reporter cells at a given retinoid concentration with response defined as fg luciferase/ $\mu\text{g lysate protein}$. Each point is the mean \pm s.e.m. of four separate measurements.

Exposure of L-RARE-*luc* cells to RA resulted in a dose-response curve similar to that of F9-RARE-*lacZ* cells, but with a plateau in response between 10^{-11} M and 10^{-9} M RA (Fig. 2B). The basis of this plateau is not clear but it could result from concentration-dependent differences in

the rate of RA transport into cells, or from the presence of cellular RA-binding proteins with limited buffering capacity (Yang et al., 1991). An alternative possibility is that the plateau results from changes in the cellular content of RA receptors. Low concentrations of RA may activate the reporter construct without significantly elevating the levels of endogenous β -RARs. Higher RA concentrations may result in a marked elevation in cellular β -RAR levels and consequently in an increase in reporter gene activation (Hu and Gudas, 1990).

The response of these reporter cells, which reflects the activation of endogenous RARs, is similar to that obtained for cells co-transfected with both reporter constructs and cloned RAR cDNAs (Giguere et al., 1987). In addition, the enhancer properties of the RARE cassette used in these reporter constructs appears to have been maintained since it is activated over a concentration range similar to that of the RARE associated with the β -RAR gene (Nervi et al., 1990).

Both reporter lines also responded to retinol and retinal although at concentrations much higher than required for RA (Fig. 2). This may result from a weak affinity of RARs for these retinoids (see also Giguere et al., 1987; Petkovich et al., 1987). A more likely possibility is that retinol and retinal are converted into RA, 3,4-didehydro-RA or other active RA analogs that activate RARs or RXRs (Thaller and Eichele, 1990; Williams and Napoli, 1985; Heyman et al., 1991; Levin et al., 1992). Differences in the ability of L cells and F9 cells to convert retinol and retinal to RA may underlie the difference in the dose-response profiles observed for the three retinoids in these two cell lines (Fig. 2A,B).

To establish that the response to retinoids was dependent on the core enhancer elements of the RARE (de The et al., 1990), constructs containing *lacZ* driven by RAREs with both direct repeat sequences deleted or randomized were introduced into F9 cells and stable transformants tested with 10^{-6} M retinol, retinal and RA. No response over background was observed (not shown). The specificity of the reporter cell lines for retinoids was tested by exposure of cells to non-retinoid ligands including steroid hormones at a concentration of 10^{-6} M, and phorbol 12-myristate 13-acetate (10^{-8} M) and forskolin (10^{-6} M), two agents that activate second messenger pathways leading to the activation of gene expression via retinoid-independent pathways (see Materials and methods). There was no significant increase in β -galactosidase activity after treatment of F9-RARE-*lacZ* cells with these compounds (not shown) or with any embryonic tissue (see below). These results provide evidence that the reporter cell lines used in this study respond selectively to retinoids through activation of the RARE.

Reporter assay for local tissue sources of retinoids

The ability to detect retinoids released from small numbers of cells requires the generation of a sensitive and quantitative reporter assay. To establish the spatial extent of activation of reporter cells by local RA sources, we first used the β -galactosidase histochemical assay. AG1-X2 ion-exchange beads were used to generate a local source of RA release (Eichele et al., 1984; Tickle et al., 1985). The

amount of RA released from these beads was linear with the loading concentration of RA (see Fig. 4A, also Eichele et al., 1984). Beads loaded with different concentrations of RA were placed on F9-RARE-*lacZ* reporter cell monolayers plated in 35 mm dishes. The majority of cells expressing β -galactosidase reaction product were restricted to the immediate vicinity of the bead and there was a clear increase in the number of responding cells with increasing amounts of RA released (Fig. 3D,E,F). To increase the proportion of reporter cells that respond to a local source of RA, reporter cell monolayers were grown in Terasaki wells (1.4 mm diameter). Histochemical analysis of the distribution of β -galactosidase-positive cells indicated that RA released from beads loaded at 5×10^{-9} M RA (see Fig. 4A) resulted in an induction of *lacZ* expression in cells at a distance of about 400 μ m from the bead (Fig. 3H), or approximately 25% of reporter cells. At a RA loading concentration of 5×10^{-8} M, essentially 100% of reporter cells expressed detectable *lacZ* levels (Fig. 3I).

To quantify this assay, L-RARE-luc cells were plated in Terasaki wells and a standard curve relating luciferase activity to the concentration of RA released from beads was determined (Fig. 4B). A linear relationship between the amount of RA released and luciferase activity was obtained over the range of RA concentrations tested (0-10 fmol RA released by beads, Fig. 4). A standard curve of this type was generated for each quantitative assay performed. Since the identity of the retinoids released by tissues is not revealed by this assay, the amount of retinoid released is referred to as "fmol RA equivalents".

Analysis of retinoid release from embryonic spinal cord tissue

To determine whether retinoids are released from neural tissue and whether the amount released falls within the linear response range of the reporter cell assay, embryonic day 13 (E13) rat floor plate tissue was used as a potential source of released retinoids. Co-culture of floor plate tissue on F9-RARE-*lacZ* cells plated in Terasaki wells resulted in a local induction of β -galactosidase activity (Fig. 5A). The extent of *lacZ* induction was similar to that obtained by beads soaked in 5×10^{-9} M RA and releasing approximately 0.8 fmol RA per 20 hour incubation period (compare Fig. 5A with Fig. 3H). To examine further whether neural tissue releases retinoids over the linear range of the dose-response curve, different lengths of floor plate explants were cultured on L-RARE-luc cells. The increase in luciferase activity in response to floor plate was roughly proportional to the increase in length of the explant (Fig. 5B). The level of retinoid released from a 1 mm segment of floor plate was about 0.6 fmol RA equivalents, close to the value estimated from the histochemical assay. These results show that the L-RARE-luc reporter assay can detect the release of retinoids from neural tissue over a range in which the dose-response curve generated by RA released from ion-exchange beads is linear.

Grafts of the floor plate into the anterior region of the chick limb bud induce mirror-image digit duplications whereas dorsal spinal cord grafts do not (Wagner et al., 1990). These observations raise the possibility that the polarizing properties of the floor plate reflect the release of

retinoids. However, floor plate tissue and more dorsal regions of the spinal cord are able to convert retinol to RA at approximately similar levels in vitro (Wagner et al.,

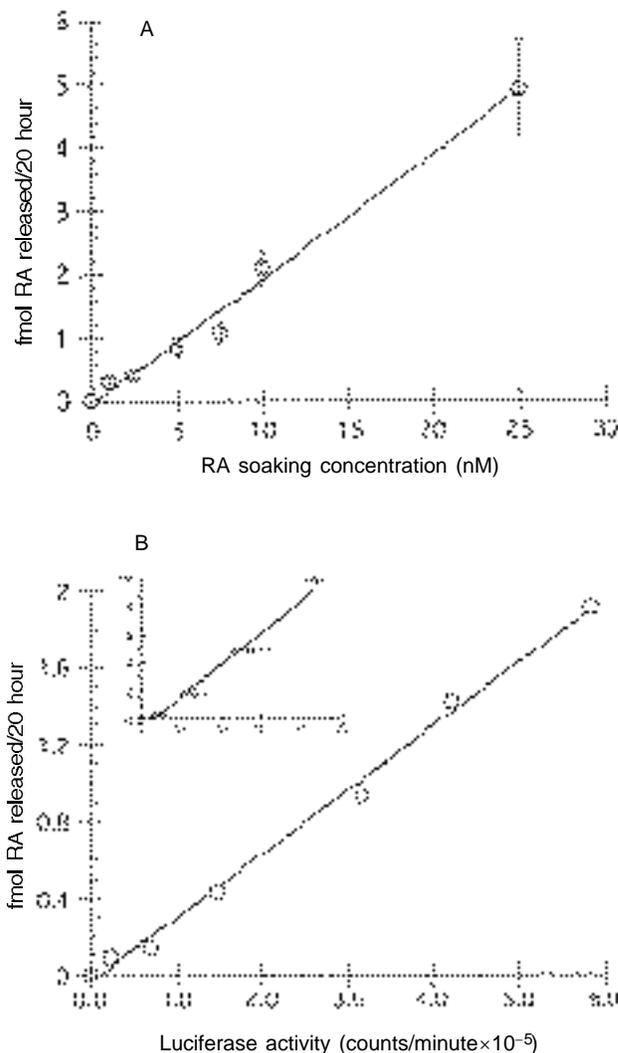


Fig. 4. Detection of luciferase activity in reporter cells as a function of RA concentration. (A) Determination of the amount of RA released as a function of loading concentration of retinoic acid. Ion-exchange beads of uniform size (approximately 200 μ m in diameter) were soaked in [3 H]RA solutions ranging in concentration from 1-25 nM. Beads were placed in Terasaki wells (volume = 15 μ l) with serum-free media and incubated at 37°C for 18-20 hours. The amount of RA released from beads was determined as described in Materials and methods and plotted against loading concentration. Data points are the mean \pm s.e.m. obtained from triplicate samples from each of five different experiments. The amount of RA released from beads is linear with the loading concentration of RA. (B) Detection of luciferase activity as a function of RA release. A typical dose-response curve is shown relating luciferase activity of L reporter cells with the amounts of RA released from ion-exchange beads. Beads loaded with RA concentrations between 7.5×10^{-10} M and 10×10^{-8} M stimulated luciferase activity above background levels. Inset: Abscissa and ordinate axes are as in Fig. 4B. Four different dose-response data sets were averaged to give the dose-response curve shown in the inset. Response is linear up to 10 fmol of RA released from beads.

1990). To resolve whether the floor plate releases greater amounts of retinoid activity than other regions of the spinal cord, segments of floor plate tissue approximately 0.1×1.0 mm in dimension and similarly sized regions of the dorsal spinal cord of E11 rat embryos were cultured as explants on L-RARE-luc reporter cells. At this stage of development, the rat floor plate has polarizing activity when assayed for changes in neural cell pattern and in the chick limb bud assay (Yamada et al., 1991; Wagner et al., 1990). The floor plate was found to release retinoids at levels about 1.4-fold greater than that of dorsal spinal cord explants (Table 1 and Fig. 6). The results of this analysis are consistent with *in vitro* measurements of retinoid synthesis in stage 16-17 chick spinal cord in which floor plate was found to convert retinol to RA at levels about 1.5-fold greater than that of dorsal spinal cord tissue (Wagner et al., 1990).

The polarizing activity of the floor plate is maintained well beyond the stage at which the floor plate is likely to influence D-V neural cell pattern (Wagner et al., 1990; Yamada et al., 1991). We therefore examined whether the distribution of retinoid release in the spinal cord was maintained at later stages of development. Retinoid release from E13 floor plate was about 1.7-fold greater than that from the dorsal spinal cord (Table 1 and Fig. 6). E11 floor plate explants released about 2.5 fmol of RA equivalent (eq.) per μg of protein and E13 floor plate explants released about 0.5 fmol of RA eq. per μg of protein. Thus, there is a five-fold decline in retinoid activity released from spinal cord tissues between E11 and E13 (Fig. 6).

The ability of both chick and rat floor plate and dorsal spinal cord tissue to release retinoids was also determined using F9-RARE-*lacZ* cells. Floor plate and dorsal spinal cord explants from rat (Fig. 7A,B,C) and chick (Fig. 7E,F) induced the expression of *lacZ* in reporter cells within a region of about 350-400 μm from the tissue explant. Although we did not quantify the response in F9-RARE-*lacZ* cells, similar numbers of reporter cells were induced to express *lacZ* by floor plate and dorsal spinal cord explants from both rat and chick.

Collectively, these results show that the all-or-none difference in polarizing activity of the floor plate and dorsal spinal cord when assayed in the neural tube or in the limb bud is not associated with marked differences in retinoid release from these two regions.

Retinoid release at different A-P levels of the embryonic CNS

The establishment of the A-P pattern of cells in the developing nervous system is markedly affected by RA (Durstun et al., 1989; Sive et al., 1990; Ruiz i Altaba and Jessell, 1991b; Morriss-Kay et al., 1991; Holder and Hill, 1991; Sundin and Eichele, 1992). Application of RA to *Xenopus* embryos immediately after the formation of the neural plate causes a truncation of anterior neural structures and leads to the expression of posterior neural cell types in anterior regions (Ruiz i Altaba and Jessell, 1991b). These observations suggest that the normal development of anterior neural structures occurs in the absence of exposure to retinoids.

We therefore examined whether there are differences in retinoid release by E11 and E13 rat neural tissues obtained

Table 1. Comparison of floor plate and dorsal spinal cord as sources of released retinoids

Expt. no.	E11		E13	
	<i>n</i>	Mean F.P.:Dorsal Ratio	<i>n</i>	Mean F.P.:Dorsal Ratio
1	9	1.44 \pm 0.34	11	2.09 \pm 0.31
2	11	1.76 \pm 0.34	5	1.40 \pm 0.29
3	8	1.04 \pm 0.49	14	1.54 \pm 0.20
Average	<i>n</i> =28	1.45 \pm 0.22	<i>n</i> =30	1.72 \pm 0.16

Approximately 0.1×1.0 mm segments of E11 and E13 rat floor plate and dorsal rat spinal cord were assayed for retinoid activity by co-culturing with L-RARE-luc reporter cells. In all cases, floor plate tissue was matched with dorsal tissue from the same A-P level of the spinal cord (the brachial/thoracic region). The floor plate (F.P.):dorsal ratio reflects the pairwise comparison of retinoid activity from these tissues, in contrast to results depicted in Fig. 6 in which values for all tissue pieces in a single experiment were pooled. To control for differences due to tissue size, the amount of RA produced was normalized to μg protein from a lysate of equivalently sized tissue pieces.

from different A-P levels of the neuraxis. Anterior neural tissue obtained from E11 dorsal diencephalon or telencephalon and posterior tissue from the dorsal spinal cord were cultured on L-RARE-luc reporter monolayers (Fig. 8A). Dorsal diencephalic explants released very low levels of retinoid activity (0.21 ± 0.10 fmol RA eq./20 hours/ μg protein) whereas the dorsal spinal cord released 2.9 ± 0.36 fmol RA eq./20 hours/ μg protein. The majority (greater than 50%) of explants obtained from the telencephalic region and all explants from the diencephalic region did not induce histochemically detectable *lacZ* product when placed on F9-RARE-*lacZ* cells (Fig. 7G,H). These results provide evi-

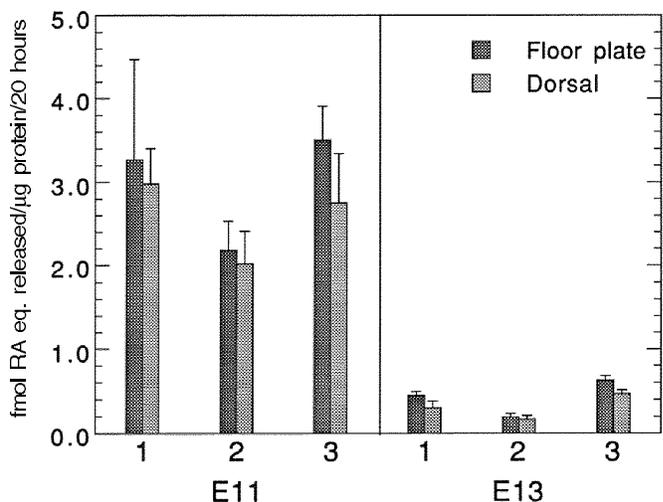


Fig. 6. Quantitative analysis of retinoid release from floor plate and dorsal spinal cord tissue. Retinoid activity released by E11 and E13 floor plate and dorsal neural tube tissues was determined in three separate experiments. The mean \pm s.e.m. for retinoid activity expressed in RA equivalents is presented for combined tissue samples from each individual experiment. The number of samples for each assay are as follows: E11: 1, *n*=9; 2, *n*=11; 3, *n*=8; E13: 1, *n*=11; 2, *n*=5; 3, *n*=14.

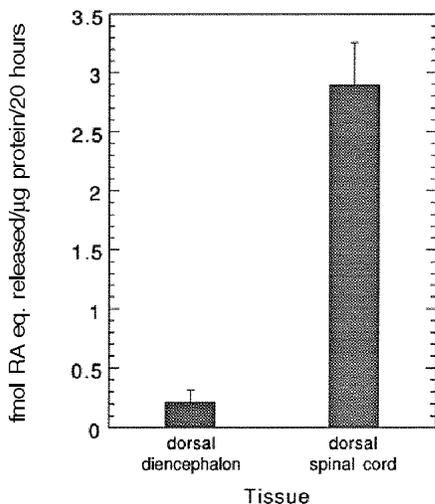


Fig. 8. Quantitative analysis of retinoid release from diencephalic and spinal cord tissue. 500 mm² tissue pieces from the dorsal region of the diencephalon of E11 rat embryos were co-cultured on L-RARE-luc reporter monolayers plated in Terasaki wells ($n=12$). Similarly sized pieces of dorsal spinal cord tissue from the same embryo were explanted onto reporter monolayers in parallel cultures ($n=10$). Luciferase activity was determined as described in Materials and methods.

dence that forebrain regions of the embryonic CNS release much lower levels of retinoid activity than the spinal cord.

Reporter lines can detect retinoid release from other tissues with polarizing properties

To examine whether these reporter lines can detect retinoid release from other tissues that have been suggested to be a source of polarizing signals, we used the histochemical assay on F9-RARE-*lacZ* cells to compare retinoid release from limb bud mesoderm with that of limb ectoderm which has no polarizing activity. Posterior limb mesoderm consistently induced *lacZ* expression (28/28 explants) whereas limb bud ectoderm did not induce detectable *lacZ* expression (0/24 explants; Fig. 9A,B). These studies provide evidence that posterior limb mesoderm which includes the ZPA region can release retinoids in vitro. In preliminary studies (not shown), anterior limb bud mesoderm also induced *lacZ* expression (M. W. and G. E., unpublished observations). Studies to quantify differences in retinoid release from anterior and posterior limb bud mesenchyme are in progress.

We also compared the release of retinoid activity from Hensen's node which has limb polarizing activity (Hornbruch and Wolpert, 1986) with that of epiblast tissue lateral to the node which does not (Wagner et al., 1990). Tissues were taken from stage-4 chick embryos and cultured on F9-RARE-*lacZ* reporter cells. Hensen's node induced significant *lacZ* expression in adjacent cells ($n=13$), whereas the lateral epiblast did not ($n=20$; Fig. 9C,D). Primitive streak tissue isolated from stage-4 embryos also induced *lacZ* expression in a manner similar to that of Hensen's node ($n=6$; not shown). One complicating factor in these experiments is that Hensen's node can differentiate into notochord and floor plate tissue in vitro as detected

by cell-specific markers (M. Placzek, personal communication). Thus, in both these in vitro assays and in the in vivo grafts of Hensen's node into the limb bud (Hornbruch and Wolpert, 1986; Stoker and Carlson, 1990; Wagner et al., 1990), it is not clear whether retinoid release and polarizing activity result from signals derived from Hensen's node itself or from its derivatives, the notochord and floor plate.

These reporter lines have also revealed differences in retinoid synthesis from dorsal and ventral regions of the embryonic neural retina (McCaffery et al., 1992).

Discussion

Evaluating the contribution of endogenous retinoids to the patterning of cell types in vertebrate embryos requires the ability to identify local tissue sources of morphogenetically active retinoids. The in vitro reporter assay described here has been used to detect the release of retinoids from different regions of the embryonic nervous system. Our results provide evidence that morphogenetically active retinoids are released at high but similar levels from cells of the floor plate and the dorsal region of the embryonic spinal cord, whereas cells in the embryonic forebrain release retinoids at much lower levels.

Selectivity and sensitivity of in vitro reporter assays

The reporter lines used in the present assay appear to provide a selective measure of the release of morphogenetically active retinoids. The RARE used to generate the reporter lines contains a direct repeat derived from the regulatory elements of the β -RAR gene which has been shown to be a target for the ligand-bound form of RARs in vitro (de The et al., 1990; Sucof et al., 1990). Other RA-responsive elements have also been described, in particular, a palindromic sequence generated as an inverted repeat of a thyroid-response element (Umesono et al., 1991). The direct repeat element, in contrast to the palindromic sequence, appears highly specific for RARs and is not activated at detectable levels by other members of the steroid/thyroid hormone receptor family. Moreover, mutations in this repeat element abolish the response of reporter lines to retinoids, making it unlikely that alternative regulatory elements for non-retinoid signals were inadvertently generated in the construction of these reporter plasmids. At present, however, we cannot exclude that the induction of reporter genes involves as yet uncharacterized receptor systems with the capacity to bind to the RARE element.

Although the reporter system provides a sensitive assay for retinoids with the ability to activate RARs, the assay does not distinguish between different retinoids. Both RA and 3,4-didehydro RA have approximately equal affinities for RARs, as determined by their potency in evoking digit duplications when introduced into the chick wing bud (Thaller and Eichele, 1990) and for RXRs in transactivation assays (Mangelsdorf et al., 1992). Thus, the identity of the retinoids released from different tissues is more directly addressed by biochemical analysis. The use of an endogenous RARE found within the β -RAR gene promoter nev-

ertheless suggests that the response of the reporter assay is physiologically relevant, independent of the identity of the retinoid. In addition, since the reporter cells detect retinoids released from tissue sources, this *in vitro* assay provides a more accurate indication of retinoid activity than biochemical analysis of the total retinoid content of embryonic tissue which is likely to include retinoids present in compartments not available for release.

We cannot completely rule out that the activation of reporter genes in F9 and L reporter cell monolayers reflects the release of non-retinoid factors that induce the synthesis of retinoids by the reporter cells themselves. The F9 and L cells used in this study, as well as other cell lines such as NIH-3T3, HeLa and S91-C2 melanoma cells which carry RA reporter constructs (data not shown) have widely divergent phenotypes. It seems unlikely that many different cell types would express the capacity both to detect and to respond to this putative retinoid synthesis-inducing factor. Indeed, if this is the case, then the tissues responsible for secreting this factor should themselves respond with enhanced retinoid synthesis. Moreover, the co-culture reporter assay is performed in the absence of the synthetic precursors of RA, retinol and retinal, which decreases the likelihood that reporter activity and induction reflects *de novo* retinoid synthesis by the reporter lines. Another possible way in which reporter genes could be activated by non-retinoid ligands is by agents that modify RA receptor activity, for example, by phosphorylation. Although we cannot exclude this, activation of the protein kinase A and C pathways by forskolin and phorbol esters did not induce reporter gene activity. Thus, the most likely explanation for the activation of reporter genes in F9 and L cells is the release of retinoids from test tissues.

RARE reporter constructs similar to that described here (Rossant et al., 1991; Balkan et al., 1992) and constructs with *lacZ* driven by promoter sequences upstream of the β -RAR gene (Mendelsohn et al., 1991; Reynolds et al., 1991) have been used to generate transgenic mice in which retinoid activity was detected by a β -galactosidase histochemical assay. These studies showed high levels of *lacZ* activity in the embryonic hindbrain, spinal cord and underlying mesodermal tissues. Much lower levels of *lacZ* were detected in anterior regions of the CNS. Our *in vitro* reporter assay complements these *in vivo* analyses and provides certain advantages. First, a single RARE was used in the *in vitro* assay as opposed to the tandem array of three RAREs used in the *in vivo* assay. Most RA-sensitive genes have only a single RARE in their regulatory regions (De Luca, 1991). Thus, the use of a single RARE may provide a more accurate reflection of the response of cells to physiologically relevant concentrations of retinoids. Second, the use of the luciferase assay provides a quantitative analysis of retinoid release in contrast to the histochemical detection of β -galactosidase. Third, the *in vitro* reporter assay permits the selective detection of retinoids released from cells, whereas the *in vivo* assay does not distinguish between reporter gene activation by retinoid synthesis in the cells that express the reporter gene or by retinoid release from nearby cells. Fourth, the use of a reporter line eliminates variability in the properties of different cell types that express the reporter construct *in vivo*, for example, the pres-

ence of different levels of RA-binding proteins (Maden et al., 1989) or of nuclear factors that modify RAR function (Glass et al., 1990). Finally, the histochemical analysis of β -galactosidase expression using reporter cell lines *in vitro* provides an assay that permits the range of action of released retinoids to be estimated.

β -galactosidase expression was detected for approximately 300-400 μm from a neural tissue source. These estimates of the distance over which retinoids can act do not, however, take into account the possibility of the division or migration of reporter cells during the assay which could increase the apparent range of action of retinoids. Nevertheless, estimates of distances obtained in this assay are similar to those reported in studies of the diffusion of labelled retinoids released from a point source in the chick limb bud *in vivo* (Eichele and Thaller, 1987). The local induction of β -galactosidase in response to a point source of retinoids could reflect the release and rapid degradation of retinoids resulting in a steady state gradient in the concentration of retinoid in solution. Alternatively, retinoids may be transmitted within the plane of the reporter cell monolayer. Retinoids are lipophilic and could be transferred between cells by passive diffusion, or by a facilitated transport process involving extracellular and intracellular retinoid-binding proteins (Blomhoff et al. 1990; Eichele and Thaller, 1987).

Retinoid release from the floor plate and dorsal spinal cord

When grafted into the anterior margin of the chick limb bud, the floor plate, but not other regions of the spinal cord, changes the pattern of digits that form along the A-P axis (Wagner et al., 1990). Similarly, grafts of the floor plate adjacent to the neural tube change the pattern of cell types that appears along the D-V axis of the spinal cord (Yamada et al., 1991; Placzek et al., 1991). These observations raise three questions. First, is the floor plate a local source of retinoids within the neural tube? Second, is the ability of the floor plate to change the pattern of digits along the A-P axis of the limb mediated by retinoids? Third, is the ability of the floor plate to change the pattern of neural differentiation along the D-V axis of the spinal cord mediated by retinoids?

In chick embryos, the ability of the floor plate to synthesize RA and 3,4-didehydro-RA from precursor retinol is only slightly greater (1.5-fold for RA, 3-fold for 3,4-didehydro-RA) than that of dorsal spinal cord tissue, which does not exhibit limb or neural tube polarizing activity (Wagner et al., 1990). The present *in vitro* reporter assay provides evidence that the E11 floor plate releases morphogenetically active retinoids at only about 1.4-fold greater levels than the dorsal spinal cord. These two sets of results indicate that limb polarizing ability is restricted to the floor plate whereas retinoids are synthesized and released at similar levels throughout the embryonic spinal cord. One possible explanation for this difference is that the slightly greater ability of the floor plate to synthesize and release retinoids is translated into an all-or-none difference in limb polarizing activity *in vivo*. This seems unlikely since quantitative studies of the digit patterns obtained in response to different numbers of ZPA cells or to different concentrations of RA suggest that 1.5-fold differences in RA levels

do not result in marked differences in polarizing activity, even at the threshold level for changes in digit pattern (Tickle, 1981; Tickle et al., 1985). A second possibility, which we cannot exclude, is that the ability of the floor plate and dorsal spinal cord to release retinoids in vitro does not provide an accurate measure of the release in vivo under conditions in which precursor retinoids are present. One line of evidence against this is that the results of this in vitro assay are generally consistent with the analysis of β -galactosidase expression in the nervous system of transgenic mice expressing RARE reporter constructs (Rossant et al., 1991).

A third possibility is that limb polarizing activity is not mediated by retinoids but by the release of a non-retinoid signal which is restricted to the floor plate. Recent studies on digit pattern in the chick limb bud have suggested that RA induces a non-retinoid signal in posterior mesenchymal cells of the ZPA (Noji et al., 1991; Wanek et al., 1991). It is possible that the floor plate provides a local source of this or a related non-retinoid signal that has polarizing activity in the limb bud. As has been suggested from studies in the limb bud (Noji et al., 1991; Wanek et al., 1991), RA may act on floor plate cells to induce the non-retinoid polarizing signal.

In contrast to the striking effect of RA on A-P patterning in the chick limb bud, local applications of RA do not evoke changes in neural cell pattern in the spinal cord similar to those produced by the floor plate (T. Yamada and T. Jessell, unpublished observations). Thus, at present, there is no evidence linking the release of retinoids by the floor plate with the signals that control the D-V pattern of cells in the spinal cord. It remains possible that retinoids regulate the pattern of cell differentiation in the neural tube by acting in conjunction with other signalling molecules that are restricted to the floor plate.

Alternatively, RA released by the floor plate and by other regions of the neural tube may have a role in the later differentiation of spinal cord neurons, including effects on axon outgrowth and cell survival (Hunter et al., 1991; Wuarin and Sidell, 1991). However, the present results do not support the proposal (Maden and Holder, 1991) that a local source of RA released from the floor plate may account for the chemoattractant properties of the floor plate for commissural axons (Tessier-Lavigne et al., 1988; Placzek et al., 1990b). RA released by neural tissue could also control the differentiation of cells outside the neural tube. Sclerotomal cells migrate from the somites into the vicinity of the floor plate and notochord where they begin to condense into cartilage. The ventral spinal cord and notochord induce cartilage differentiation in mesenchymal cells (Watterson et al., 1954) and this effect is mimicked by RA (Ide and Aono, 1988) raising the possibility that retinoids released from the notochord and floor plate function in the control of sclerotomal cell differentiation. In addition, neural crest cells have been shown to be sensitive to retinoids and to express members of the RXR receptor family (Pratt et al., 1987; Rowe et al., 1991; Heyman et al., 1991). Many neural crest cells migrate ventrally past the ventral neural tube and notochord and appear to require signals from these cell groups for their subsequent differentiation along the sympathoadrenal lineage pathway (Stern et al., 1991). Retinoids released

from the floor plate and notochord could therefore influence the differentiation of neural crest cells.

Differences in retinoid release along the A-P axis of the CNS

Application of RA has marked effects on the patterning of cell types along the A-P axis of the developing nervous system. In particular, RA suppresses the differentiation of forebrain structures and causes neuronal cell types of posterior character to appear in more anterior regions of the CNS (Ruiz i Altaba and Jessell, 1991b). The marked suppression of anterior neural development by low concentrations of RA would suggest that anterior regions of the embryo are not exposed to significant concentrations of RA during development. The ten-fold lower levels of retinoid released from anterior regions of the rat embryonic CNS when compared with the spinal cord are consistent with this suggestion. Moreover, transgenic mouse embryos expressing a (RARE)₃-lacZ reporter construct show low levels of β -galactosidase reaction product in the forebrain of the embryonic mouse CNS (Rossant et al., 1991). Systemic administration of RA to vertebrate embryos may induce or elevate the expression of RARs in anterior mesoderm and CNS, thereby initiating the expression of RA-responsive genes that are incompatible with anterior neural development.

The role of retinoids in establishing the A-P pattern of the developing CNS remains unclear. A local posterior source of retinoids may operate at the time of gastrulation. The release of retinoids from primitive streak, Hensen's node or from the notochord, could expose adjacent ectoderm to retinoids, specifying the development of posterior neural tissue. The forebrain, which derives from regions of the epiblast anterior to Hensen's node, may not be exposed to equivalent concentrations of retinoids, permitting anterior neural differentiation.

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References

- Balkan, W., Colbert, M., Bock, C. and Linney, E. (1992). Transgenic indicator mice for studying activated retinoic acid receptors during development. *Proc. Natl. Acad. Sci. USA* **89**, 3347-3351.
- Blomhoff, R., Green, M. H., Berg, T. and Norum, K. R. (1990). Transport storage of vitamin A. *Science* **250**, 399-404.
- De Luca, L. (1991). Retinoids and their receptors in differentiation, embryogenesis, and neoplasia. *FASEB J.* **5**, 2924-2933.
- de The, H., del Mar Vivanco-Ruiz, M., Tiollais, P., Stunnenberg, H. and Dejean, A. (1990). Identification of a retinoic acid responsive element in the retinoic acid receptor gene. *Nature* **343**, 177-180.
- de Wet, J., Wood, K. V., DeLuca, M., Helinski, D. R. and Subramani, S.

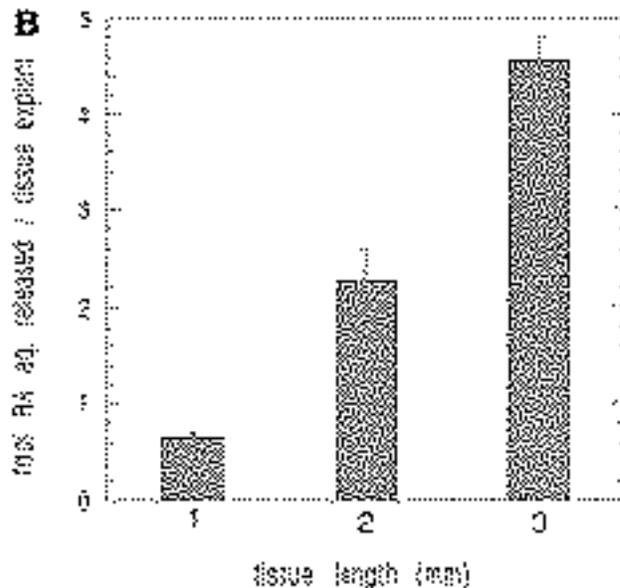
- (1986). Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.* **7**, 725-737.
- Dixon, J. E. and Kintner, C. E.** (1989). Cellular contacts required for neural induction in *Xenopus* embryos: evidence for two signals. *Development* **106**, 749-757.
- Durston, A. 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.
- Eichele, G., Tickle, C. and Alberts, B. M.** (1984). Microcontrolled release of biologically active compounds in chick embryos: Beads of 200- μ m diameter for the local release of retinoids. *Anal. Biochem.* **142**, 542-555.
- Eichele, G. and Thaller, C.** (1987). Characterization of concentration gradients of a morphogenetically active retinoid in the chick limb bud. *J. Cell Biol.* **105**, 1917-1923.
- Eichele, G.** (1989). Retinoids and vertebrate limb pattern formation. *Trends in Genetics* **5**, 246-251.
- Espeseth, A. S., Murphy, S. P. and Linney, E.** (1989). Retinoic acid receptor expression vector inhibits differentiation of F9 embryonal carcinoma cells. *Genes Dev.* **3**, 1647-1656.
- Giguere, V., Ong, E. S., Segui, P. and Evans R. M.** (1987). Identification of a receptor for the morphogen retinoic acid. *Nature* **330**, 624-629.
- Glass, C. K., Devary, O. V. and Rosenfeld, M. G.** (1990). Multiple cell type-specific proteins differentially regulate target sequence recognition by the retinoic acid receptor. *Cell* **729**-738.
- Hamburger, V.** (1988). *The Heritage of Experimental Embryology*. New York: Oxford University Press.
- Hatta, K., Kimmel, C. B., Ho, R. K. and Walker, C.** (1991). The cyclops mutation blocks specification of the floor plate of the zebrafish central nervous system. *Nature* **350**, 339-341.
- Heyman, R. A., Mangelsdorf, D. J., Dyck, J. A., Stein, R. B., Eichele, G., Evans, R. M. and Thaller, C.** (1991). 9-Cis retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* **68**, 397-406.
- Holder, N. and Hill, J.** (1991). Retinoic acid modifies development of the midbrain-hindbrain border and affects cranial ganglion formation in zebrafish embryos. *Development* **113**, 1159-1170.
- Hornbruch, A. and Wolpert, A.** (1986). Positional signalling by Hensen's node when grafted to the chick limb bud. *J. Embryol. Exp. Morph.* **94**, 257-265.
- Hu, L. and Gudas, L. J.** (1990). Cyclic AMP analogs and retinoic acid influence the expression of retinoic acid receptor , , and mRNAs in F9 teratocarcinoma cells. *Mol. Cell. Biol.* **10**, 391-396.
- Hunter, K., Maden, M., Summerbell, D., Eriksson, U. and Holder, N.** (1991). Retinoic acid stimulates neurite outgrowth in the amphibian spinal cord. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3666-3670.
- Ide, H. and Aono, H.** (1988). Retinoic acid promotes proliferation and chondrogenesis in the distal mesodermal cells of chick limb bud. *Dev. Biol.* **130**, 767-773.
- Leid, M., Kastner, P., Lyons, R., Nakshatri, H., Saunders, M., Zacharewski, T., Chen, J.-Y., Staub, A., Garnier, J.-M., Mader, S. and Chambon, P.** (1992). Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. *Cell* **68**, 377-395.
- Levin, A. A., Sturzenbecker, L. J., Kazmer, S., Bosakowski, T., Huselton, C., Allenby, G., Speck, J., Kratzeisen, C., Rosenberger, M., Lovey, A. and Grippo, J. F.** (1992). 9-Cis retinoic acid stereoisomer binds and activates the nuclear receptor RXRa. *Nature* **355**, 359-361.
- Lim, K. and Chae, C. B.** (1989). A simple assay for DNA transfection by incubation of the cells in culture dishes with substrates for beta-galactosidase. *Biotechniques* **7**, 576-579.
- Maden, M., Ong, D. E., Summerbell, D. and Chytil, F.** (1989). The role of retinoid-binding proteins in the generation of pattern in the developing limb, the regenerating limb and the nervous system. *Development* Supplement, 109-119.
- Maden, M. and Holder, N.** (1991). The involvement of retinoic acid in the development of the vertebrate central nervous system. *Development* Supplement, 87-94.
- Mangelsdorf, D. J., Borgmeyer, U., Heyman, R. A., Zhou, J. Y., Ong, E. S., Oro, A. E., Kakizuka, A. and Evans, R. M.** (1992). Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. *Genes Dev.* **6**, 329-344.
- Maxwell, I. H., Harrison, G. S., Wood, W. M. and Maxwell, F.** (1989). A DNA cassette containing a trimerized SV40 polyadenylation signal which efficiently blocks spurious plasmid-initiated transcription. *Biotechniques* **7**, 276-280.
- McCaffery, P., Lee, M., Wagner, M. A., Sladek, N. E. and Drager, U. C.** (1992). Asymmetrical retinoic acid synthesis in the dorso-ventral axis of the retina. *Development*, in press.
- Mendelsohn, C., Ruberte, E., LeMeur, M., Morriss-Kay, G. and Chambon, P.** (1991). Developmental analysis of the retinoic acid-inducible RAR-b2 promoter in transgenic animals. *Development* **113**, 723-734.
- Morriss-Kay, G., Murphy, P., Hill, R. E. and Davidson, D. R.** (1991). Effects of retinoic acid excess on expression of Hox 2.9 and Krox-20 and on morphological segmentation in the hindbrain of mouse embryos. *EMBO J.* **10**, 2985-2995.
- Nervi, C., Vollberg, T. M., Grippo, J. F., Lucas, D. A., George, M. D., Sherman, M. I., Shudo, K. and Jetten, A. M.** (1990). Expression of nuclear retinoic acid receptors in wild-type and mutant embryonal carcinoma PCC4.aza 1R cells. *Cell Growth Devel.* **1**, 535-542.
- Nguyen, V. T., Morange, M. and Bensaude, O.** (1988). Firefly luciferase luminescence assays using scintillation counters for quantitation in transfected mammalian cells. *Anal. Biochem.* **178**, 404-408.
- Noji, S., Nohno, T., Koyama, E., Muto, K., Ohshima, 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.
- Nolan, G. P., Fiering, S., Nicolas, J.-F. and Herzenberg, L. A.** (1988). Fluorescence-activated cell analysis and sorting of viable mammalian cells based on -D-galactosidase activity after transduction of *Escherichia coli LacZ*. *Proc. Natl. Acad. Sci.* **85**, 2603-2607.
- Petkovich, M., Brand, N. J., Krust, A. and Chambon, P.** (1987). A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature* **330**, 444-450.
- Placzek, M., Tessier-Lavigne, M., Yamada, T., Jessell, T. M. and Dodd, J.** (1990a). Mesodermal control of neural cell identity: floor plate induction by the notochord. *Science* **250**, 985-988.
- Placzek, M., Tessier-Lavigne, M., Jessell, T. and Dodd, J.** (1990b). Orientation of commissural axons in vitro in response to a floor plate-derived chemoattractant. *Development* **110**, 19-30.
- Placzek, M., Yamada, T., Tessier-Lavigne, M., Jessell, T. and Dodd, J.** (1991). Control of dorsoventral pattern in vertebrate neural development: induction and polarizing properties of the floor plate. *Development*. Supplement 105-122.
- Pratt, R. M., Goulding, E. H. and Abbott, B. D.** (1987). Retinoic acid inhibits migration of cranial neural crest cells in the cultured mouse embryo. *J. Cran. Genet. Dev. Biol.* **7**, 205-217.
- Reynolds, A. and Lundblad, V.** (1989). Yeast vectors and assays for expression of cloned genes. In *Current Protocols in Molecular Biology* (ed. F.A. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl) pp 13.6.1-13.6.4. New York, Greene Publishing and Wiley-Interscience.
- Reynolds, K., Mezey, E. and Zimmer, A.** (1991). Activity of the -retinoic acid receptor promoter in transgenic mice. *Mech. Develop.* **36**, 15-29.
- Rossant, J., Zirngibl, R., Cado, D., Shago, M. and Giguere, V.** (1991). Expression of a retinoic acid response element-hsplacZ transgene defines specific domains of transcriptional activity during mouse embryogenesis. *Genes Dev.* **5**, 1333-1344.
- Rowe, A., Eager, N. S. C. and Brickell, P. M.** (1991). A member of the RXR nuclear receptor family is expressed in neural crest derived cells of the developing chick peripheral nervous system. *Development* **111**, 771-778.
- Ruiz i Altaba, A.** (1990). Neural expression of the *Xenopus* homeobox gene Xhox3: Evidence for a patterning neural signal that spreads through the ectoderm. *Development* **108**, 595-604.
- Ruiz i Altaba, A. and Jessell, T. M.** (1991a). Retinoic acid modifies mesodermal patterning in early *Xenopus* embryos. *Genes Dev.* **5**, 175-187.
- Ruiz i Altaba, A. and Jessell, T. M.** (1991b). Retinoic acid modifies the pattern of cell differentiation in the central nervous system of neurula stage *Xenopus* embryos. *Development* **112**, 945-958.
- Saunders, J. W. and Gasseling, M. T.** (1968). In *Epithelial-Mesenchymal Interactions* (ed. Fleischmajer, R. and Billingham, R.E.) pp. 78-97. Baltimore: Williams & Wilkins.
- Sharpe, C. R.** (1991). Retinoic acid can mimic endogenous signals involved in transformation of the *Xenopus* nervous system. *Neuron* **7**, 239-247.
- 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.
- Sive, H. L. and Cheng, P. F.** (1991). Retinoic acid perturbs the expression of *Xhoxlab* genes and alters mesodermal determination in *Xenopus laevis*. *Genes Dev.* **5**, 1321-1332.
- Spemann, H.** (1938). *Embryonic Development and Induction*. (New Haven: Yale University Press).
- Stern, C. D., Artinger, D. B. and Bronner-Fraser, M.** (1991). Tissue interactions affecting the migration and differentiation of neural crest cells in the chick embryo. *Development* **113**, 207-216.
- Stoker, K. M. and Carlson, B. M.** (1990). Hensen's node, but not other biological signalers, can induce supernumerary digits in the developing chick limb bud. *Roux's Arch. Devl Biol.* **198**, 371-381.
- Sucov, H. M., Murakami, K. K. and Evans, R. M.** (1990). Characterization of an autoregulated response element in the mouse retinoic acid receptor type gene. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5392-5396.
- Summerbell, D.** (1983). The effect of local application of retinoic acid to the anterior margin of the developing chick limb. *J. Embryol. Exp. Morph.* **78**, 269-289.
- Summerbell, D. and Harvey, F.** (1983). Vitamin A and the control of pattern in developing limbs. In *Limb Development and Regeneration Part A* (ed. J. F. Fallon and A. I. Caplan), pp. 109-119. New York: Alan R. Liss.
- Sundin, O. and Eichele, G.** (1992). An early marker of axial pattern in the chick embryo and its respecification by retinoic acid. *Development*, in press.
- Tessier-Lavigne, M., Placzek, M., Lumsden, A. G. S., Dodd, J. and Jessell, T. M.** (1988). Chemotropic guidance of developing axons in the mammalian central nervous system. *Nature* **336**, 775-778.
- Thaller, C. and Eichele, G.** (1987). Identification and spatial distribution of retinoids in the developing chick limb bud. *Nature* **327**, 625-628.
- Thaller, C. and Eichele, G.** (1990). Isolation of 3,4-didehydroretinoic acid, a novel morphogenetic signal in the chick wing bud. *Nature* **345**, 815-819.
- Tickle, C., Summerbell, D. and Wolpert, L.** (1975). Positional signalling and specification of digits in chick limb morphogenesis. *Nature* **254**, 199-202.
- Tickle, C.** (1981). The number of polarizing region cells required to specify additional digits in the developing chick wing. *Nature* **289**, 295-298.
- 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.** (1991). Retinoic acid and chick limb bud development. *Dev. Suppl.* **1**, 113-121.
- Umesono, K., Murakami, K. K., Thompson, C. C. and Evans, R. M.** (1991). Direct repeats as selective response elements for the thyroid hormone, retinoic acid and vitamin D₃ receptors. *Cell* **64**, 1255-1266.
- van Stratten, H. W. M., Hekking, J. W. M., Wiertz-Hoessels, E. J. L. M., Thors, F. and Drukker, J.** (1988). Effect of the notochord on the differentiation of a floor plate area in the neural tube of the chick embryo. *Anat. Embryol.* **177**, 317-324.
- Wagner, M., Thaller, C., Jessell, T. M. 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**, 81-82.
- Watterson, R. L., Fowler, I. and Fowler, B. J.** (1954). The role of the neural tube and notochord in development of the axial skeleton of the chick. *Am. J. Anat.* **95**, 337-399.
- Webster, W. S., Johnston, M. C., Lammer, E. J. and Sulik, K. K.** (1986). Isotretinoin embryopathy and the cranial neural crest: An in vivo and in vitro study. *J. Craniofac. Genet. Devl Biol.* **6**, 211-222.
- Williams, J. B. and Napoli, J. L.** (1985). Metabolism of retinoic acid and retinol during differentiation of F9 embryonal carcinoma cells. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4658-4662.
- Wuarin, L. and Sidell, N.** (1991). Differential susceptibilities of spinal cord neurons to retinoic acid-induced survival and differentiation. *Dev. Biol.* **144**, 429-435.
- Yamada, T., Placzek, M., Tanaka, H., Dodd, J. and Jessell, T. M.** (1991). Control of cell pattern in the developing nervous system: polarizing activity of the floor plate and notochord. *Cell* **64**, 635-647.
- Yang, N., Schule, R., Mangelsdorf, D. J. and Evans, R. M.** (1991). Characterization of DNA binding and retinoic acid binding properties of retinoic acid receptor. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3559-3563.
- Yasuda, Y., Konishi, H., Kihara, T. and Tanimura, T.** (1987). Developmental anomalies induced by all-trans-retinoic acid in fetal mice: II. Induction of abnormal neuroepithelium. *Teratology* **55**, 355-366.
- Zelent, A., Krust, A., Petkovich, M., Kastner, P. and Chambon, P.** (1989). Cloning of murine and retinoic acid receptors and a novel receptor predominantly expressed in skin. *Nature* **339**, 714-717.

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Fig. 3. Histochemically detected responses of F9 reporter cells to locally released retinoids. (A, B) F9-RAIRE-*lacZ* reporter cell monolayer untreated (A) and (B) treated with 5×10^{-8} M RA in serum-free media. (C) Reporter cell monolayer in 35 mm dish incubated with one 10^{-6} M retinol-soaked AG1-X2 ion-exchange bead for 18-20 hours. Blue X-gal reaction product appears in cells immediately surrounding the bead. (Bead was in center of field and removed for photography of cells leaving a small area void of cells). Reporter cell monolayers (plated in 35 mm dishes (D-F) or in Terasaki wells (G-I)) incubated with beads soaked at 5×10^{-10} M RA (D, G); 5×10^{-9} M RA (E, H); $5 \times$

10^{-8} M RA (F, I). Increasing numbers of reporter cells respond to increasing amounts of RA released from beads. Note that cells exposed to beads loaded with 10^{-6} M retinol give approximately the same level of induced β -galactosidase expression as the response to beads loaded with 5×10^{-9} M RA. The asymmetric staining evident in panel F was occasionally observed with beads soaked in high concentrations of RA. Beads soaked at these RA concentrations release greater amounts of RA into the media where it may be subjected to the fluid flow of the culture medium or alternatively, the vibration of the incubator may cause small movement of the bead. Scale bar, 0.5 mm.



This is fig 5b, to go on colour tipin with fig 5.

Fig. 5. Use of reporter assay to detect release of retinoid from floor plate tissue. (A) A 1 mm long length of rat E13 floor plate tissue co-cultured on an F9-RARE-*lacZ* monolayer in a Terasaki plate microwell and the co-culture processed for visualization of X-gal-positive cells (see Materials and methods). (B) Different lengths of floor plate tissue were isolated from approximately the same A-P level of E13 rat spinal cord. Tissue samples were co-cultured with L-RARE-luc monolayers for 18-20 hours after which cell monolayers were harvested and assayed for luciferase activity. Note that for purposes of comparison with Fig. 4, retinoid activity released by floor plate tissue segments is expressed as fmol RA equivalents released per explant and not as a function of tissue protein.

Fig. 7. Histochemical detection of retinoid release from different regions of embryonic central nervous system. (A) Low power view of E13 floor plate explant on F9-RARE-*lacZ* cells shows *lacZ*-expressing cells only in vicinity of floor plate tissue. (B) Higher power view of E13 floor plate explant showing expression of *lacZ* in region of reporter cell monolayer immediately adjacent to floor plate. (C) Dorsal spinal cord explants taken from same A-P level of spinal cord as floor plate shown in B. (D) Rat E13 floor plate tissue explant on F9 cell line transfected with mutated RARE reporter constructs (low power). This cell line carries a reporter construct in which the core enhancer elements of the RARE are deleted. The absence of β -galactosidase-expressing cells provides evidence that the response of reporter cells to tissues is dependent on the RARE of the reporter construct. (E) Floor plate tissue taken from stage-16 chick embryo spinal cord. (F) Dorsal spinal cord tissue taken from the same A-P level of chick spinal cord as the floor plate tissue shown in E. Approximately equal numbers of *lacZ*-positive cells are induced by both tissues. (G) Telencephalic tissue from E11 rat embryo cultured on F9 reporter cells fails to induce *lacZ* expression. (H) Dorsal diencephalon from E11 rat embryo cultured on F9 reporter cells does not induce *lacZ* expression. Scale bar, 0.8 mm.

Fig. 9. Use of histochemical reporter assay to detect retinoid release from embryonic tissues with polarizing activity. (A) Limb bud mesoderm cultured on F9-RARE-*lacZ* cells. The induction of *lacZ* expression by limb mesoderm was usually restricted to one side of the tissue explant ($n=40$). Further studies are required to determine whether this region of the explanted limb bud corresponds to the most posterior mesoderm. (B) Limb bud ectoderm isolated free from underlying mesoderm and cultured on reporter cells fails to induce *lacZ* expression ($n=24$). (C) Hensen's node tissue obtained from stage-4 chick embryos induces *lacZ* expression in reporter cells ($n=13$). In the majority of cases, node tissue underwent marked changes in shape to form elongated structures resembling differentiated notochord. (D) Epiblast tissue from regions lateral to Hensen's node fails to induce any detectable *lacZ* expression ($n=13$). Scale bar, 0.5 mm.