

Evidence that *Shh* cooperates with a retinoic acid inducible co-factor to establish ZPA-like activity

Toshihiko Ogura^{1,*}, Ignacio S. Alvarez^{1,†}, Astrid Vogel¹, Concepción Rodríguez¹, Ronald M. Evans² and Juan Carlos Izpisua Belmonte¹

¹Gene Expression Laboratory and ²Howard Hughes Medical Institute, The Salk Institute for Biological Studies, 10010 North Torrey Pines Rd, La Jolla, California 92037, USA

The two first authors contributed equally to this work

*Present address: Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara, 630-01, Japan

†Present address: Departamento de Biología Celular, Facultad de Ciencias, Universidad de Extremadura, E-06071 Badajoz, Spain

SUMMARY

Patterning across the anteroposterior axis of the vertebrate limb bud involves a signal from the polarizing region, a small group of cells at the posterior margin of the bud. Retinoic acid (RA; Tickle, C., Alberts, B., Wolpert, L. and Lee, J. (1982) *Nature* 296, 554-566) and Sonic hedgehog (Shh; Riddle, R. D. Johnson, R.L., Laufer, E. and Tabin, C.J. (1993) *Cell* 25, 1401-1416; Chang, D. T., Lopez, A., von Kessler, D. P., Chiang, C., Simandl, B. K., Zhao, R., Seldin, M. F., Fallon, J. F. and Beachy, P. A. (1994) *Development* 120, 3339-3353) have been independently postulated as such signals because they can mimic the mirror image digit duplication obtained after grafting polarizing cells to the anterior of limb buds.

Here we show that a embryonal carcinoma cell line, P19, transfected with a *Shh* expression vector shows low polarizing activity, but when cultured with retinoic acid, duplications like those induced by the polarizing region (ZPA) arise. Complete duplications are also obtained by cotransfecting P19 *Shh* cells with a constitutively active human retinoic acid receptor (VP16-hRAR α). These data suggest that Shh and RA cooperate in generating ZPA activity and that Shh, while essential, may not act alone in this process.

Key words: retinoic acid, retinoic acid receptors, *Sonic hedgehog*, P19 cells, *HoxD* genes, polarizing activity, limb development

INTRODUCTION

In the vertebrate embryo, several signaling centers have been identified. One such signaling regions is a group of cells located at the posterior region of the limb bud known as the polarizing region or ZPA. Signaling by the polarizing region is involved in establishing the anteroposterior patterning in the limb, because when the posterior region of the limb bud is grafted at the anterior edge of another limb bud, extra digits are induced in a mirror image fashion (Saunders and Gasseling, 1968). The molecular mechanisms underlying this process, although elusive for long time, are now starting to emerge. RA (Tickle et al., 1982), and *Sonic hedgehog* (Shh; Riddle, 1993; Chang et al., 1994; Lopez Martinez et al., 1995) have been independently postulated as key signals in this process because they can mimic the mirror image digit duplication obtained after grafting polarizing cells to the anterior of limb buds.

In order to explore further the requirement for *Shh* expression for eliciting polarizing activity we made use of a murine embryonal carcinoma cell line, P19. This cell line, when induced to differentiate with retinoic acid, not only expresses markers characteristic of early anterior primitive streak, and Hensen's node, (*Brachyury*, *gooseoid* and *nodal*, etc.), structures that also have a strong polarizing activity in the limb assay, (Hornbruch and Wolpert, 1986) but also expresses activities capable of inducing *Hox* gene expression (Pruitt, 1994; Vidricaire et al.,

1994; and our unpublished data). In the developing vertebrate limb, application of RA beads and overexpression of *Shh* at the anterior margin, like ZPA grafts, induces activation of the more posteriorly expressed *HoxD* genes (Izpisua Belmonte et al., 1991a; Riddle, 1993; Chang et al., 1994 and Lopez Martinez et al., 1995). We have generated several P19 cell lines expressing various levels of *Shh*. These cell lines were unable to elicit complete digit duplications. However, when treated with RA and grafted to the anterior margin of a chick limb bud, ZPA-like duplications arose. Since RA actions are mediated by interactions with nuclear receptors we made use of this signaling pathway and created a cell line expressing *Shh* and a constitutively active human retinoic acid receptor, VP16-hRAR α . This cell line was able to induce the ectopic expression of *HoxD* genes and to generate full digit duplications similar to those resulting from ZPA. We propose that *Shh* and RA interactions are key events in the signaling mechanisms that establish cell proliferation and patterning during vertebrate limb development.

MATERIALS AND METHODS

In situ hybridization

Chicken embryos (MacIntyre Poultry, San Diego) were removed from the egg and fixed in paraformaldehyde overnight. Embedding, sectioning and in situ hybridization were performed as described previ-

ously (Izpisúa Belmonte et al., 1991b). The *hoxd-11* and *hoxd-13* ³⁵S antisense probes used are described by Izpisúa Belmonte et al. (1991a). Whole mount *in situ* hybridization was performed essentially as described by Hemmati-Brivanlou et al. (1990), with some minor modifications (Izpisúa Belmonte et al., 1993). After the chromogenic reaction, embryos were cleared in 80% glycerol in PBS and photographed. The *Shh* antisense probe used, cloned by PCR from ZPA cells, was restricted to the exon 3 of chicken *Shh* (Riddle et al., 1993) in order to avoid possible crosshybridizations with related members of the *hedgehog* family.

RNA isolation and analysis

Total RNA was obtained by the guanidinium thiocyanate method according to Chomczynski and Sacchi (1987). Poly (A)⁺ RNA was selected using a Qiagen oligotex Kit according to manufacturer instructions. For northern blot analysis, 7 µg of total RNA were used. RNA was size fractionated on 1% agarose/2.2 M formaldehyde gel, transferred to Nytran filter (S&S) and hybridized to ³²P-labeled probes. The mouse and chicken *Shh* probes making up part of exon 3 (Riddle et al., 1993 and Echelard et al., 1993) were generated by PCR from mouse and chicken ZPA respectively.

Grafting experiments

To test for polarizing activity and to investigate the regulation of *Shh* expression in the limb tissue, grafts were made under a flap of apical ridge, lifted along the anterior margin of wing buds at stages 20-21. The embryos were then reincubated at 38°C for a further 4-30 hours. After incubation they were removed and fixed overnight in 4% paraformaldehyde and subjected to either hybridization in sections with ³⁵S probes or whole-mount *in situ* hybridization with digoxigenin probes. For assessing the effects of the grafts on limb pattern, embryos were allowed to continue development for up to 6 days after grafting and then the wings were fixed in 5% trichloroacetic acid and stained with alcian green to show the skeletal pattern.

Cells of embryonal carcinoma cell line P19 were transferred from standard tissue culture dishes to bacterial Petri dishes to allow the formation of cell aggregates. These aggregates were then transplanted to the anterior side of the chicken limb buds as described above for the tissue fragments.

Cell culture and transfections

P19 murine embryonal carcinoma and CV-1 (monkey kidney epithelial) cells were maintained in D-MEM-10%FBS (Gemini). For transfection, cells were dissociated with trypsin-EDTA and resuspended in DMEM-10%CBS (Hyclone) and seeded at 20-30% confluency 24 hours before transfection. 1 hour before transfection, cells were resuspended again by pipetting and transfected with the calcium phosphate method essentially as described by Kliewer et al. (1992). 24 hours after transfection, cells were replenished with DMEM-10%FBS. Stable transformants were selected in the presence of 1 mg/ml of G418 and single colonies were picked after 10-14 days of selection. The stable transformants were routinely cultured under the same conditions as the parental cell line. Cells were exposed to 1 µM RA for 2 days in bacterial Petri dishes to allow the formation of cell aggregates. In order to avoid RA carryover they were cultured for another day in the absence of RA. Transient transfections were performed as described above except that cells were transferred to bacterial Petri dishes 24 hours after transfection and cultured for 2 days before transplantation assays. The vector used for stable transfection was in all cases pRC-CMV (Invitrogen). The VP16-hRARα clone was inserted into the pCMX expression vector (Kliewer et al., 1992).

Western blotting

Cell extracts from P19, P*Shh*27 and CV*Shh*14 cells were prepared according to Bumcrot et al. (1995) and separated by SDS-PAGE. Proteins were transferred to a nitrocellulose filter (S&S) and then

blocked in 5% non-fat dry milk in PBS-T (PBS-0.1% Tween20). The filter was then incubated in a 1:500 dilution of affinity-purified rabbit antiserum (Ab180, a gift from A. P. MacMahon) raised against an N-terminal fragment of the mouse *Shh* protein. After washing in PBS-T, filters were incubated in a 1:10000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin (Amersham). Bands were visualized with the Enhanced Chemiluminescence Kit (Amersham) according to the manufacturer's instructions.

RESULTS

Cultured cells with inducible ZPA activity

We initially generated various cell lines stably transfected with chick *Shh* under the control of the cytomegalovirus (CMV) promoter, expressing different *Shh* levels. We chose three lines for further work (P*Shh*3, P*Shh*27 and P*Shh*28) with *Shh* levels either comparable or approximately ten times higher than that found in the posterior region of the limb bud (Fig. 1A). In all three lines selected, the endogenous *Shh* mouse transcripts were absent both before and after 2 days of RA treatment (data not shown). Transcript levels of the transfected chick *Shh* did not significantly change after culturing the cells for 2 days in 1 µM RA (Fig. 1B, lines 5-8). Small aggregates of cells of comparable size to the ZPA grafts were grafted into a slit made at the anterior margin of stage 19-20 host limbs. The embryos with the grafts were either incubated for several hours (4, 16 and 30 hours) and then subjected to whole-mount *in situ* hybridization with various probes (*Shh* and *HoxD* genes) or incubated for 7 days to examine the digit patterns.

The results described in Figs 2 and 4 and Table 1 are a compilation of data from the three cell lines. The digit pattern obtained after grafting was either normal (Fig. 2B) or showed the presence of an extra digit 2 (Fig. 2C). Morphological and histo-

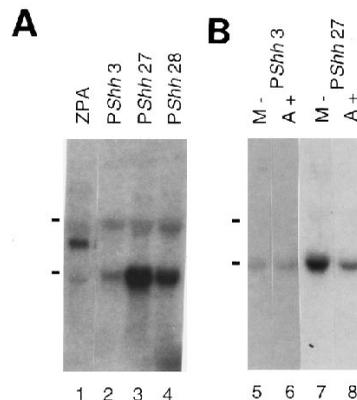


Fig. 1. Northern analysis. (A) Autoradiograph of northern blot of RNA isolated from chicken ZPA cells (lane 1) and from different P19 cell lines transfected with chick *Shh*, P*Shh*3, 27 and 28 (lanes 2, 3 and 4 respectively) and hybridized against a chick *Shh* probe. ZPA cells express the endogenous *Shh* message which migrates between the 18S and 28S rRNAs. The position of 18S and 28S rRNAs is indicated by small bars. The three P19*Shh* cell lines express the entire open reading frame of *Shh* and the untranslated region derived from the expression vector and comigrate with the 18S rRNA. The *Shh* probe used seems to weakly hybridize with the 18S and 28S rRNAs. (B) The levels of chick *Shh* transcripts in P19 transfected cells, both in monolayer and aggregates, seems to remain relatively constant after RA treatment (lanes 5, 6, 7 and 8). M, monolayer; A, aggregates. +, 1 µM RA treatment for 2 days. -, absence of RA. The quantity of total RNA loaded in both northern blots was 7 µg per line.

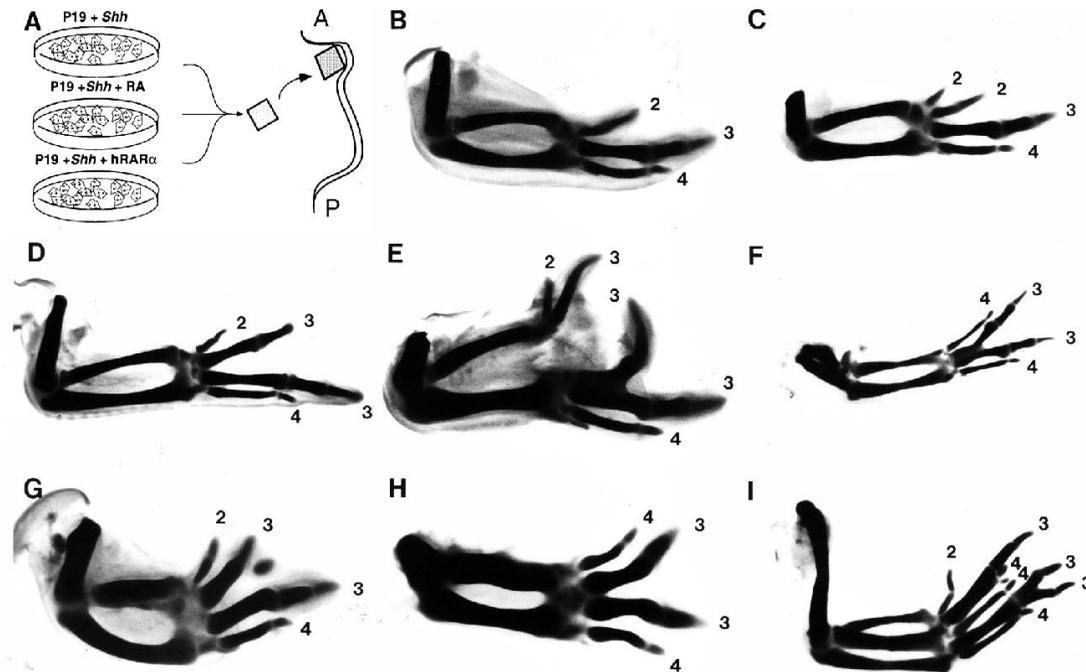


Fig. 2. Whole-mounts of embryonic chicken wings stained with alcian green to show digit patterns following grafts of the various cell lines. (A) Scheme of the grafting procedure of the different cell lines into the anterior of the chicken limb bud. (B) Digit pattern obtained following a graft of the parental cell line P19. (C) Digit patterns following grafts of P19 expressing *Shh*; (D,E,F), following grafts of P19 cells expressing *Shh* that had been cultured in the presence of 1 μ m RA for 48 hours, and (G,H,I) following grafts of P19 cells expressing *Shh* cotransfected with VP16-hRAR α , to the anterior margin of a young chick wing bud. (B) Wing that developed no additional digit with normal digit pattern **234**. Since there is no change with respect to the non-operated embryo, this specimen could be considered as a wild-type pattern when compared to the rest of the grafts. (C) Duplicated wing with digit pattern **2234**; (D) duplicated wing with digit pattern **2334**; (E) duplicated wing where two humeri and two ulnas developed. The digit pattern is **23334**; (F) duplicated wing where the radius with digits 2 and 3 is disconnected from the wrist. The ulna is associated with a split digit 3 and digit 4. The digit pattern is **4334**; (G) duplicated wing with digit pattern **23?34**. The radius in this embryo that died early is thickened and shortened; (H) duplicated wing with a duplicated ulna. The digit pattern is **4334**. (I) This wing developed a radius and two ulnas with a digit pattern **2344334**.

logical analysis at different time points after grafting showed that the implanted cells do not proliferate, nor disperse within the limb tissue (data not shown). None of the control grafts, non-transfected P19 cells, produced digit alterations (Fig. 2B).

Proteolytic processing of the chicken *Shh* product in P19 cells

Bumcrot et al. (1995) and Chang et al. (1994) have shown that chicken and mouse *Shh* protein undergo proteolytic processing

(from an original polypeptide of approximately $46 \times 10^3 M_r$) to yield two peptides with relative molecular masses of approximately 19×10^3 (amino terminus) and 27×10^3 (carboxy terminus), both of which are secreted. To study translation and processing of chicken *Shh* protein in P19 cells we used a rabbit antiserum against an amino terminus fragment of mouse *Shh*. This antiserum has been shown to cross react with the chicken *Shh* protein (Bumcrot et al., 1995). As a positive control we used a CV-1 cell line stably transfected with chicken *Shh*. CV-1 cells

Table 1. *Shh* expression, digit pattern and polarizing activity obtained with cell line grafts

Cell lines	<i>Shh</i> expression					Digit pattern				Polarizing activity (%)	Number of grafts for digit pattern	Total number of grafts
	Northern blot	Grafts after 4 hours		Grafts after 30 hours		Normal	Extra digit 2	Extra digit 3	Extra digit 4			
		Whole mount	Number	Whole mount	Number							
P19	–	–	2	–	2	10				0	10	14
#3	+	+	2	+	3	3	2			13	5	10
P <i>Shh</i> #27	+++	+	2	+	3	3	2			13	5	10
#28	+	+	2	+	2	3	1			8	4	8
Total P <i>Shh</i>			6		8	9	5			12	14	28
P19+RA	–	–	2	–	2	9	1			3	10	14
P <i>Shh</i> +RA	+	+	3	±	4	5	7	7	1	40	20	27
P19-VP16hRAR α			n.d.			10				0	10	10
P <i>Shh</i> -VP16hRAR α			n.d.			2	4	2	2	47	10	10

Line describing the grafting of P19 cells in the presence of RA (P19*Shh* + RA) is a compilation of data using the three different cell lines P19*Shh*3, 27 and 28, while the RAR cotransfections were made with line P19*Shh*27.

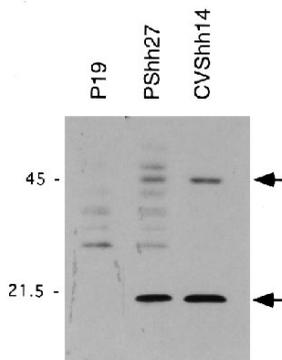


Fig. 3. Proteolytic processing of chicken *Shh* in P19 cells. Cell extracts prepared from parental P19, P*Shh*27 and CV-1 expressing chick *Shh* gene were separated by SDS-PAGE and electro blotted. *Shh* proteins were detected with an anti-*Shh* antibody that recognizes the N-terminal half of *Shh* protein. Parental P19 cells do not express *Shh* protein. Full length (approximately $45 \times 10^3 M_r$) and N-terminal (approximately $19 \times 10^3 M_r$) forms of *Shh* in P*Shh*27 and CV*Shh*14 are indicated by arrows. Positions of molecular mass markers are shown on the left side of the figure.

are the parental cell line of COS cells which are able to efficiently process *Shh* protein (Bumcrot et al., 1995). As shown in the immunoblot in Fig. 3, CV-1 and P19 cells transfected with chicken *Shh* produce a polypeptide of approximately $45 \times 10^3 M_r$ which could correspond to the full length *Shh*. In addition, an approximately 19kDa polypeptide is detected, which corresponds to the amino terminus region of *Shh*. Neither of these two peptides are detected in P19 untransfected cells. Expression of processed $19 \times 10^3 M_r$ amino-terminal product was higher than the full length product in both CV-1 and P19 *Shh* transfected cells. Overall, these results suggest that production and processing of *Shh* protein in P19 cells occurs in a similar fashion to that shown in a variety of other cell types as well as in vivo (Bumcrot et al., 1995 and Lopez Martinez et al., 1995).

Retinoic acid and *Sonic hedgehog* are required for ZPA activity

It has been reported that a retinoic acid bead, when implanted at the anterior margin of the limb, is able to induce the expression of *Shh* (Riddle, 1993; Niswander et al., 1994). Although P19 cells transfected with *Shh* were unable to induce complete digit duplications it is possible that cooperation between these two signaling molecules, RA and *Shh*, could elicit ZPA-like limb duplication in our assay. To test this, we incubated *Shh* transfected cells in the presence of RA for 2 days. To rule out the possibility of RA carryover in the grafted cells we cultured the P19 *Shh* transfected cells for a further 24 hours in the absence of RA to allow for clearing RA out of the cells. After aggregation, a small pellet of cells was grafted into the anterior margin of stage 19-20 limb buds. Table 1 and Fig. 2 show a summary of the results obtained. In contrast to the cell lines cultured in the absence of RA, duplications of digit 3 or 4 were obtained (Fig. 2D,E,F and Table 1). As had been done for P19 *Shh* cells cultured in the absence of RA, RA cultured cells were monitored for *Shh* expression at various time points after grafting. The results showed strong expression of *Shh* in the grafted cells but not in the host tissue four hours after grafting (Fig. 4C and Table 1). *Shh* transcripts were downregulated, but still weakly present,

in the graft 30 hours after the operation (Fig. 4D and Table 1). Similar results were observed after grafting P19 *Shh* cells cultured in the absence of RA (Fig. 4A,B). When parental P19 cell pellets cultured with RA were grafted, no *Shh* expression was detected in the graft or in the host (data not shown) and the limbs obtained were normal (Table 1). Northern blot data show that the transcript levels for *Shh* remained relatively constant after RA treatment of P19*Shh* cells (Fig. 1B).

A constitutively active retinoic acid receptor interacting with *Shh* induces ZPA activity

It is believed that the biological effects of RA are primarily modulated by its different receptors. One response pathway is mediated by a family of RA receptors (RARs) consisting of three isoforms, RAR α , RAR β , RAR γ (Evans, 1988). To test whether the ZPA activity displayed in the P19 cells treated with RA is mediated through this family of receptors we made use of a constitutively active receptor. A human RAR α derivative cDNA was fused downstream of the VP16 transactivation domain driven by the CMV promoter. This construct, VP16-hRAR α , is constitutively active through RAREs upon transfection (Perlmann et al., 1993). We transiently transfected the P19*Shh* stable lines with the VP16-hRAR α construct and cultured them for two days in the absence of RA. Grafts of these cells placed at the anterior margin of the chick wing had the highest level of polarizing activity observed with any graft (47% including, in two cases, the appearance of an extra digit 4 (Fig. 2G,H,I and Table 1). An extra digit 4 is considered to be the result of the

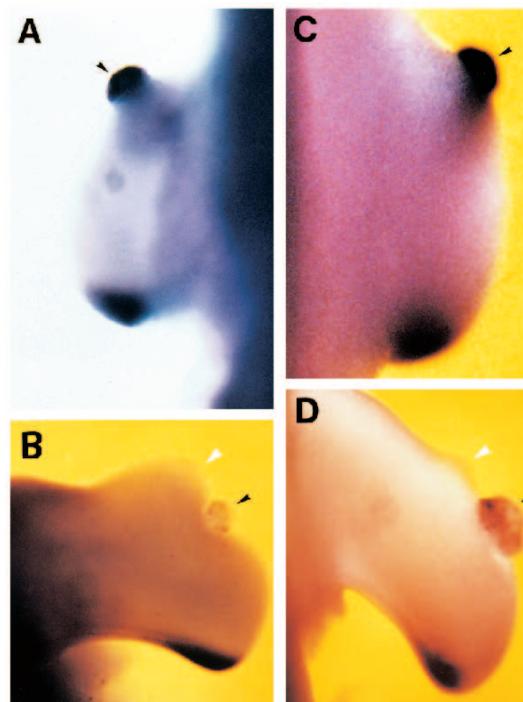


Fig. 4. Distribution of *Shh* transcripts in whole-mount preparations of wing buds after cell grafting. The transcript levels of *Shh* in P19*Shh*27 cells cultured in the presence (A) or in the absence (C) of RA are downregulated 30 hours after grafting (B, P19*Shh*27+RA; and D, P19*Shh*27). Black arrowheads point to the grafted tissue or cells. White arrowheads in B and in D point to the extra growth consequence of the graft. The normal domain of *Shh* expression is always at the posterior of the buds.

highest levels of polarizing activity. Control grafts showed no change in the skeletal pattern. Northern data showed no effect of P19*Shh* VP16-hRAR α in the transcript levels of *Shh* (data not shown).

Effect of P19*Shh*, P19*Shh*-RA and P19*Shh*-VP16-hRAR α on *HoxD* gene activity in the limb tissue

We have previously shown that the organization of limb pattern involves the sequential activation of the *Hox* genes under the control of a signal released by the ZPA (Izpisúa Belmonte et al., 1991a). This signal is now believed to be mediated by the product of the gene *Shh*. We then hypothesized that the P19*Shh* cell lines, cultured in the presence or in the absence of RA, or transiently transfected with the constitutively active RA receptor, would activate, in a sequential fashion, *HoxD* gene expression.

To check whether this was the case we performed *in situ* hybridization of limb buds after grafting the different cell lines described above. Both P19*Shh* +RA and P19*Shh*+ VP16-hRAR α cell grafts induced the sequential appearance of the more posteriorly expressed *HoxD* genes in the limb tissue near the graft implant. Fig. 5 shows the ectopic anterior expression of *hoxd-13* (5A) and *hoxd-11* (5B) 30 hours after the grafts were made. P19 *Shh* cells cultured in the absence of RA, although able to ectopically activate *hoxd-11* did not turn on the last two members of the *HoxD* complex (Fig. 5C and data not shown). These results demonstrate that the digit inducing activity of the *Shh* transfected cell lines, either cultured with RA or transiently transfected with a constitutively active retinoic acid receptor, like the ZPA grafts, leads to the sequential activation of the *HoxD* genes.

DISCUSSION

The morphogenic properties of retinoids have been based on a variety of experiments over the last 10 years, including bead implants, which at physiologic RA levels lead to dramatic limb duplications. The recent discovery of *Shh* led to the conclusion that the protein product of this gene is the mediator of the

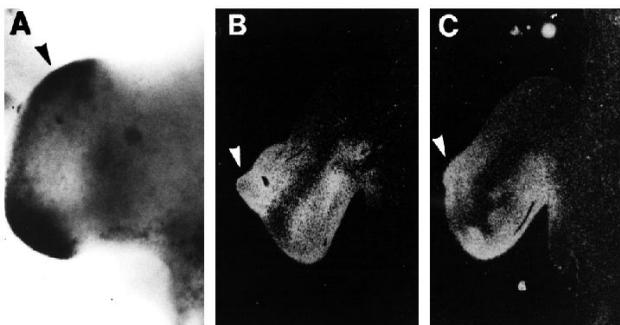


Fig. 5. *HoxD* gene expression after cell grafting. (A) Ectopic *hoxd-13* mRNA expression in chicken wing buds 30 hours after grafting P19*Shh* cells cotransfected with the constitutive active human retinoic acid receptor α . (B) Ectopic *hoxd-11* mRNA expression in chicken wing buds 30 hours after grafting P19*Shh* cells treated with RA. (C) Ectopic *hoxd-11* mRNA expression in chicken wing buds 30 hours after grafting P19*Shh* cells cultured in the absence of RA. The normal domain of expression for both *hoxd-11* and *hoxd-13* is always at the posterior of the limb. The ectopic domain of expression in the host limb buds is indicated with an arrowhead. Although not able to elicit complete duplications, P19*Shh* cells induce ectopic expression of *hoxd-11* in the absence of RA.

ZPA signaling pathway. The goal of this work was to evaluate the role of RA, its receptors, and *Shh* in ZPA activity. We show here that an embryonal carcinoma cell line, P19, transfected with *Shh* has low polarizing activity, but when cultured with RA, digit duplications arise. Complete duplications are also obtained by cotransfecting P19 *Shh* cells with a constitutively active human retinoic acid receptor. Both types of graft induced, in a sequential fashion, *HoxD* gene expression at the anterior margin of the developing limb bud.

The development of cell lines with ZPA activity supports the hypothesis that *Shh* is required for limb digit duplications. However, these cell lines also reveal that RA and *Shh* do not act independently, arguing for the existence of a cascade with RA initiating the process. This would be consistent with the observations that implantation of an RA bead at the anterior margin of a limb bud induces ZPA activity in the tissue distal to the bead and activates *Shh* in the same region as the induced ZPA activity (Wanek et al., 1991; Riddle et al., 1993). This suggests that RA acts upstream of *Shh*.

Our results seem to contradict the established view that *Shh* expression is sufficient to mediate polarizing activity. Overexpression of *Shh* at the anterior limb margin, via recombinant retrovirus or with purified protein, is able to cause digit duplications (Riddle et al., 1993; Lopez Martinez et al., 1995). However, it has also been shown that anterior limb mesenchyme tissue expressing *Shh* after RA application fails to induce a polarizing region (Helms et al., 1994). Since other regions of normal (Wagner et al., 1990; Hornbruch and Wolpert, 1991; Izpisúa Belmonte et al., 1992 and our own unpublished results) and mutant embryos (Francis-West et al., 1994) are able to induce digit duplications without expressing *Shh*, taken together, these results suggest that additional factors, besides *Shh*, are involved in the polarizing activity phenomenon.

It is possible that P19 *Shh* transfected cells fail to induce complete digit duplications because translation of *Shh* protein is repressed or its processing blocked in P19 cells, and this is relieved by RA treatment. Our results show that this is not the case because *Shh* protein is translated and correctly processed in P19 cells (Fig. 3). Since *Shh* has been shown to be induced by RA, a potential explanation is that the effect of RA is to induce *Shh* expression above a threshold that is required for prolonging activity. This is unlikely since one of the stable cell lines (P19*Shh*27) expressed more than ten times the physiological levels of *Shh* found in the ZPA at stage 20. Furthermore, the transcript levels for *Shh* remained relatively constant after RA treatment of P19*Shh* cells or cotransfection with VP16-hRAR α . An alternative hypothesis is that RA and *Shh* activate different signaling pathways. This is unlikely for two reasons: (a) RA-induced digit duplications are preceded by activation of *Shh* and (b) parental P19 cells, not expressing *Shh*, but cultured in the presence of RA, do not have any morphological effect when grafted at the anterior limb bud margin. A third, more likely explanation is that RA and *Shh* produce different signals that form part of a common pathway. RA could induce a signaling molecule (X) that cooperates with *Shh* to produce ZPA activity. This is reinforced by the observation that P19*Shh* cells are not able to activate the full complement of *HoxD* genes (only up to *hoxd-11*) while culturing the same cells in the presence of RA has a synergistic effect and activates the late appearing and more posteriorly expressed *HoxD* genes (*hoxd-12* and *hoxd-13*). This, in a way, is reminiscent of expression patterns following grafting

of different embryonic tissues to the anterior margin of the limb bud. For example, tissue from the mouse polarizing region and Hensen's node induce complete digit duplications and lead to activation of all the genes of the *HoxD* complex. In contrast, mouse neural tube and genital tubercle grafts, which give much less extensive changes in pattern, do not activate the last members of the complex (Izpisua Belmonte et al., 1992). Similarly to the P19 cell line system, Hensen's node and floor plate cells from the neural tube can produce RA and express *Shh* (Wagner et al., 1990; Hogan et al., 1992; Echelard et al., 1993).

In this model, ZPA activity would be RA dependent but the RA effect would be restricted to the graft and not the surrounding tissue. The results with the constitutively active RAR expression vector strongly support this proposal. Since these cells are not treated with RA and because the receptor variant is confined to the transfected cells, ZPA activity cannot be due to the release of RA from the graft. The data shown here suggest that RA would be morphogenic but not a morphogen. Because an RA permissive event is required for *Shh* action, it remains to be determined whether *Shh* is itself a morphogen or, like RA, a component in a morphogenic pathway. In this respect the distribution of *Shh* protein is of considerable interest. Martí et al. (1995) and Lopez Martinez et al. (1995) have shown that *Shh* peptides remain tightly localized to the posterior distal mesenchyme arguing that the mode of action of *Shh* during limb development would imply a chain of inductive interactions rather than being based in a gradient mechanism in which different concentrations of protein dictate different cellular outputs.

Together these results indicate a role for *Shh* and RA in generating ZPA activity from a well characterized cell line. They provide direct evidence that *Shh*, while essential, may not act alone in this process. Thus, polarizing activity may represent a two stage or a two component process. The data also suggest that RA lies upstream of *Shh* and that these cell lines will provide a simple approach to identifying and cloning the RA inducible co-factor. Finally, these cells should be useful in identifying whether other *Shh*-sensitive inductions are also co-factor dependent.

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