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FGF-4 regulates expression of *Evx-1* in the developing mouse limb

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SUMMARY

We describe here the temporal and spatial pattern of expression of *Evx-1*, a murine homolog of the *Drosophila even-skipped* gene, in the developing limb bud. *Evx-1* RNA is first detected in distal limb (progress zone) mesenchyme shortly after the formation of the apical ectodermal ridge. The level of *Evx-1* RNA increases during the next 24 hours of development, and then decreases in the subsequent 24 hours, such that by the time the ridge regresses *Evx-1* RNA is undetectable. At all these stages, *Evx-1* RNA is localized primarily to the posterior distal mesenchyme, in the region immediately underlying that portion of the ridge in which the *Fgf-4* gene is expressed. Using an in vitro culture system, we show that the ridge is required for both the induction and maintenance of *Evx-1* expression in the distal mesenchyme. We also demonstrate that in the absence of the ridge, FGF-4, as well as other FGF proteins, can induce *Evx-1* expression in the limb bud. However, this effect appears to be indirect, since it can be blocked by an inhibitor of protein synthesis. Additional studies demonstrate that the effect of FGF-4 on *Evx-1* expression is modulated by BMP-2. These data serve to identify *Evx-1* as a downstream gene in the FGF signal transduction pathway in the limb.

Key words: apical ectodermal ridge, FGF-4, fibroblast growth factor, *Evx-1*, limb development, progress zone mesenchyme, mouse limb

INTRODUCTION

Classical experiments on the embryonic chick limb have defined reciprocal signals that pass between the mesenchyme and epithelium and are required for normal limb outgrowth and differentiation. During early stages of limb development, signals from the mesenchyme induce ectoderm overlying the distal limb tip along the anteroposterior margin to thicken and form a morphologically distinct structure, the apical ectodermal ridge (AER or ridge) (Kieny, 1960, 1968; Saunders and Reuss, 1974). In turn, the ridge signals the mesenchyme immediately underlying it, termed the progress or proliferative zone, to proliferate, resulting in directed proximodistal (P-D) limb outgrowth (Reiter and Solursh, 1982; reviewed by Tabin, 1991). Furthermore, the ridge maintains the progress zone mesenchyme in an undifferentiated state (Globus and Vethamany-Globus, 1976). As mesenchyme cells leave the region influenced by the ridge they begin to differentiate. Pattern specification along the P-D axis is thought to occur in the progress zone, possibly as a function of the length of time a cell remains within the progress zone (Summerbell et al., 1973; Wolpert et al., 1975). Although most experimental studies have been performed in the chick because of the ease of limb manipulation in ovo, it is evident that the basic mechanism of limb development is similar in most vertebrates (reviewed by Tabin, 1991).

As a first step toward understanding limb morphogenesis, RNA in situ hybridization and immunolocalization studies have been carried out to identify genes that are expressed in the developing limb bud. Within the ridge, several genes that could play a role in controlling limb development are known to be expressed. These include *limb deformity* (*ld*; [Jackson-Grusby et al., 1992; Trumpp et al., 1992]), cellular retinoic acid-binding protein I (CRABP I; [Dollé et al., 1989; Ruberteet al., 1992]), presumed transcription factors related to the *Drosophila* homeobox-containing genes *engrailed* (*En-1* [Davis et al., 1991]), *msh* (*Msx-1* and *Msx-2*, formerly known by a variety of names including *Hox-7* and *Hox-8*, respectively [Coelho et al., 1991; Davidson et al., 1991; Robertet al., 1991; Suzukiet al., 1991]) and *distal-less* (*Dlx-1* and *Dlx-2* [Dollé et al., 1992; Bulfone et al., 1993]), and representatives of three different signaling molecule families, the FGF (*Fgf-4* [Niswander and Martin, 1992; Suzuki et al., 1992]), TGF-β (*Bmp-2* [Lyons et al., 1990] and *Bmp-4* [Jones et al., 1991]) and WNT (*Wnt-5a* [Gavin et al., 1990]) gene families. Recently, we have developed a culture system that facilitates the functional analysis of genes expressed in the ridge (Niswander and Martin, 1993). Data from that study indicate that FGF-4 plays a role in stimulating the proliferation of progress zone mesenchyme. Conversely, BMP-2 appears to inhibit proliferation of cells in this region.

Members of several different gene families have also been found to be expressed in limb mesenchyme. As one might expect from the finding that FGF stimulates limb mesenchyme proliferation, at least one member of the FGF receptor gene family, *Fgfr-1*, is expressed in devel-

oping limb mesenchyme (Orr-Urtreger et al., 1991; Peters et al., 1992). In addition, members of the *Hox* gene family, which are related to the homeotic genes of *Drosophila* and are known to play a role in the anterior-posterior (A-P) patterning of the vertebrate head and trunk (reviewed by McGinnis and Krumlauf, 1992), are expressed in the limb bud mesenchyme. Data on the expression patterns of genes of the *Hoxd* (formerly known as *Hox-4*) complex in normal and experimentally manipulated limbs have led to the suggestion that they play a role in A-P patterning of the developing limb (Dollé et al., 1989; Izpisúa-Belmonte et al., 1991; Nohno et al., 1991; reviewed by Izpisúa-Belmonte and Duboule, 1992). Evidence in support of this hypothesis has come from studies in which the mouse *Hoxd-11* gene has been ectopically expressed in the chick limb bud (Morgan et al., 1992). Genes in the other *Hox* complexes are also expressed in the limb mesenchyme (reviewed by Izpisúa-Belmonte and Duboule, 1992), including members of the *Hoxa* (formerly known as *Hox-1* [Yokouchi et al., 1991]) and *Hoxc* (formerly known as *Hox-3* [Oliver et al., 1988, 1989, 1990]) complexes. However, it is not yet clear what role these genes play in pattern formation in the limb. Several other non-*Hox* homeobox-containing genes are also known to be expressed in the limb bud mesenchyme. These include the *Msx-1* and *Msx-2* genes (Coelho et al., 1991; Davidson et al., 1991; Nohno et al., 1991; Robert et al., 1991), *goosecoid* (*gsc* [Gaunt et al., 1993]) and *Pax-3* (Goulding et al., 1991). However, as yet, the function of these genes in limb development is not known.

We have previously noted that another homeobox-containing gene, *Evx-1*, is expressed in the mesenchyme of the developing limb (Dush and Martin, 1992). This gene, which was cloned by virtue of its homology to the *Drosophila* homeobox-containing gene *even-skipped* (Bastian and Gruss, 1990; Dush and Martin, 1992), is also expressed in other regions during embryogenesis. *Evx-1* RNA is first detected in the pre-streak embryo, marking epiblast cells that will soon participate in primitive streak formation (Dush and Martin, 1992). Throughout gastrulation, *Evx-1* expression is limited to cells within the streak and just lateral to it. Within the *Evx-1*-positive region, the highest levels of RNA are found in cells in the proximal (posterior) portion of the embryo, with RNA levels decreasing more distally. Based on fate-mapping studies indicating that different types of mesoderm emerge from different regions of the primitive streak (Tam and Beddington, 1987), it has been speculated that *Evx-1* plays a role in the specification of mesodermal cell fate (Dush and Martin, 1992). Beginning at ~E10, *Evx-1* RNA is also detected in subsets of cells within the neural tube along the entire axis (Bastian and Gruss, 1990).

In this study, we provide a description of *Evx-1* expression in the developing limb bud in vivo. In addition, using cultures of intact mouse limb buds, we have explored the mechanism by which *Evx-1* expression is regulated in the progress zone mesenchyme. We demonstrate that signals from the ridge induce and maintain *Evx-1* expression. We also show that these functions of the ridge can be performed by FGF-4 and that the effect of FGF-4 on *Evx-1* expression can be modulated by BMP-2.

MATERIALS AND METHODS

Embryos at various stages of gestation were obtainedfrom random bred Swiss Webster mice (Simenson Laboratories, Gilroy, CA), with noon of the day on which the copulation plug was detected being considered E0.5. For the in vitro studies, limbs were isolated from embryos at E10.25-E10.5 and cultured in serum-free medium with and without added growth factors, as previously described (Niswander and Martin, 1993). RNA in situ hybridization was performed as previously described (Frohman et al., 1990). The probe for *Fgf-4* RNA was a cDNA containing the entire coding region (Hébert et al., 1990; Niswander and Martin, 1992), and the probe for *Evx-1* RNA was a 700 bp fragment from the 3′ end of the gene (Dush and Martin, 1992). The slides were developed and stained after an exposure of approximately 3 weeks.

Experiments to determine whether the effect of FGF-4 on *Evx-1* expression requires protein synthesis were carried out in medium with or without 100 ng/ml FGF-4, and containing 10 μg/ml cycloheximide. Hindlimb cultures were incubated for 6 hours then fixed and processed for RNA in situ hybridization. Control cultures were incubated in medium alone or medium containing 100 ng/ml FGF-4. To ensure that *Evx-1* expression had not been initiated prior to culture, stage-matched hindlimbs were fixed immediately and processed for RNA in situ hybridization at the same time as the cultured limbs.

RESULTS

Evx-1 is expressed in the progress zone mesenchyme

To determine the expression pattern of *Evx-1* in the developing limb bud, RNA in situ hybridization analysis was carried out on mouse embryos from E9.5 through E14.5 (Table 1, Fig. 1, and data not shown). The limbs were staged according to the system of Wanek et al. (1989). At each embryonic age, the hindlimbs were less developmentally advanced (by approximately one stage) than the forelimbs. *Evx-1* RNA is first detected in the late stage 3 limb, following ridge formation (e.g. in forelimbs at ~E10.5; Fig. 1A,B). Subsequently, *Evx-1* RNA levels increase and reach a maximum at ~stage 6, when condensation of digit 4 occurs (e.g. in forelimbs at ~E11.5), and then gradually decrease through stage 8 when the ridge regresses (Fig. 1D,E, and data not shown). By stage 9, when all digit condensations are prominent, *Evx-1* RNA is no longer detectable in the limb (e.g. in forelimbs at ~E12.5; data not shown), although expression in the neural tube and gut-associated mesenchyme is evident (as described by Bastian and Gruss, 1990, and our unpublished observations). Throughout this period of expression, *Evx-1* RNA is localized to the distal mesenchyme directly underlying the ridge (progress zone mesenchyme). Interestingly, *Evx-1* RNA does not appear to be uniformly expressed throughout this distal mesenchyme; instead, it is largely restricted to the posterior region (Fig. 1D,E, and data not shown). These data are summarized in Fig. 1G.

Evx-1 expression is regulated by signals from the ridge

The correlation between the presence of the ridge and expression of *Evx-1*, as well as the observation that expression of the gene is localized to the distal mesenchyme,

which is known to respond to signals from the ridge, suggests that *Evx-1* expression might be controlled by the ridge. To test this idea directly, we utilized an in vitro limb culture system that facilitates functional analysis of the ridge (Niswander and Martin, 1993). Briefly, trunk fragments with both left and right limbs attached are isolated from embryos at ~E10.5 with forelimbs at stage 3-4 (fully formed ridge) or hindlimbs at stage 2-3 (epithelium thickening). The ridge is removed from one limb bud (−AER limb), but kept intact on the contralateral one (+AER limb). The trunk fragments are then cultured in serum-free defined medium. Thus a direct comparison can be made between the −AER and +AER limbs from a given embryo.

Using this culture system, we examined *Evx-1* expression at various times after the start of the culture period, in limbs from a total of 35 individual embryos (Table 1). In all cases, we found that in hindlimbs, which do not express *Evx-1* at the time they are placed in culture, *Evx-1* RNA is not detected in the absence of the ridge at any time during the incubation period. In the contralateral +AER limb, it is expressed normally after 6-12 hours of culture (Fig. 2A,D, G and data not shown). In forelimbs, in which *Evx-1* expression has already been initiated at the time they are explanted, *Evx-1* RNA is still detected three hours after ridge removal but is no longer detected by six hours after removal. In contrast, *Evx-1* continues to be expressed normally in the contralateral +AER limb (data not shown). Thus, a signal(s) from the ridge is required both to initiate, and also to maintain *Evx-1* expression in limb mesenchyme.

Table 1. Analysis of *Evx-1* expression

(a) Expression in vivo

Embryonic ages examined	Number of embryos analyzed*
E9.5	15
E10.5	13
E11.5	7
E12.5	16
E13.5	3
E14.5	9

(b) Expression in cultured limbs

Growth factor added	Concentration (ng/ml)	Number of embryos analyzed*
no addition	–	35
FGF-4, 24-48 hours culture	1, 10, 100 or 500	14
FGF-4, 3 hours culture	50	5†
4 hours culture	50	11†
4-6 hours culture, with cycloheximide	50	6†
FGF-1	20 or 100	6
FGF-2	100	4
TGF-β1	1, 50 or 100	12
BMP-2	1, 50 or 100	14
PDGF	50 or 200	9
EGF	50 or 200	6
insulin	200 or 5,000	6
retinoic acid	1.25, 12.5 or 125	6
FGF-4 and BMP-2	100 (FGF-4):10 (BMP-2)	10
FGF-4 and BMP-2	50 (FGF-4): 50 (BMP-2)	7
FGF-4 and BMP-2	10 (FGF-4): 100 (BMP-2)	8

*both forelimbs and hindlimbs examined
†only hindlimbs examined

FGF-4 can induce *Evx-1* expression in the absence of the ridge

Since *Evx-1* expression in the normal limb is localized to the posterior distal mesenchyme, it is possible that the signal(s) that controls its expression is similarly localized in the posterior ridge. Although a number of signaling molecules that potentially could serve as regulators of *Evx-1* expression are produced by the ridge (Gavin et al., 1990; Lyons et al., 1990; Jones et al., 1991; Niswander and Martin, 1992; Suzuki et al., 1992), only one gene encoding such a molecule is presently known whose expression is localized in the posterior ridge. Expression of *Fgf-4*, a member of the fibroblast growth factor (FGF) gene family, is first detected shortly after ridge formation and within a few hours is observed at high levels in the posterior half of the ridge. Expression then gradually decreases and, by stage 8, when the ridge regresses, *Fgf-4* RNA is no longer detectable (Niswander and Martin, 1992). Examination of *Evx-1* and *Fgf-4* expression in alternate sections of individual embryos (Fig. 1), revealed that *Evx-1* RNA is detectable only after *Fgf-4* RNA is present, and *Evx-1* RNA is detected at highest levels in mesenchyme underlying the *Fgf-4*-positive portions of the ridge.

To test the possibility that *Evx-1* expression in limb mesenchyme is regulated by FGF-4, limbs were cultured in serum-free medium supplemented with FGF-4 protein (Table 1). In a previous study, we have shown that FGF-4 can substitute for the ridge in signaling the distal mesenchyme to proliferate, resulting in outgrowth and extrusion of the mesenchyme exposed to the growth factor(Niswander and Martin, 1993). Here we found that, at all concentrations tested (Table 1), *Evx-1* expression was induced in the distal mesenchyme of −AER hindlimbs and maintained in −AER forelimbs (Fig. 2B,E,H, and data not shown). Other FGF family members (acidic and basic FGF) are similarly capable of regulating *Evx-1* expression, whereas treatment with TGF-β1, BMP-2, PDGF, EGF, insulin or retinoic acid had no effect (Table 1, Fig. 2C,F,I and data not shown). *Evx-1* expression in the contralateral +AER limbs did not appear to be affected by any of the factors tested, presumably because the intact epithelium acts as a barrier, preventing them from reaching the mesenchyme. In most cases, the level of *Evx-1* expression in −AER limbs treated with FGFs appeared to be higher than in +AER limbs (see Fig. 2E), presumably because AER removal exposes the underlying mesenchyme to a higher concentration of exogenous FGF than is available from the endogenous source in the ridge. In addition, analysis of multiple sections indicated that *Evx-1* RNA is no longer restricted to the posterior distal part of the limb, but is now also detected in mesenchyme in the anterior distal region of the limb (Fig. 2H, and data not shown). This suggests that all distal mesenchyme is capable of expressing *Evx-1* upon exposure to FGF. Presumably, the restriction of *Evx-1* to the posterior distal mesenchyme during normal limb development is the consequence of localized production of FGF-4 in the ridge.

To explore the question of whether *Evx-1* expression is induced as a direct response to FGF treatment or whether it represents a downstream step in the cascade of gene regulation, we first examined the temporal relationship

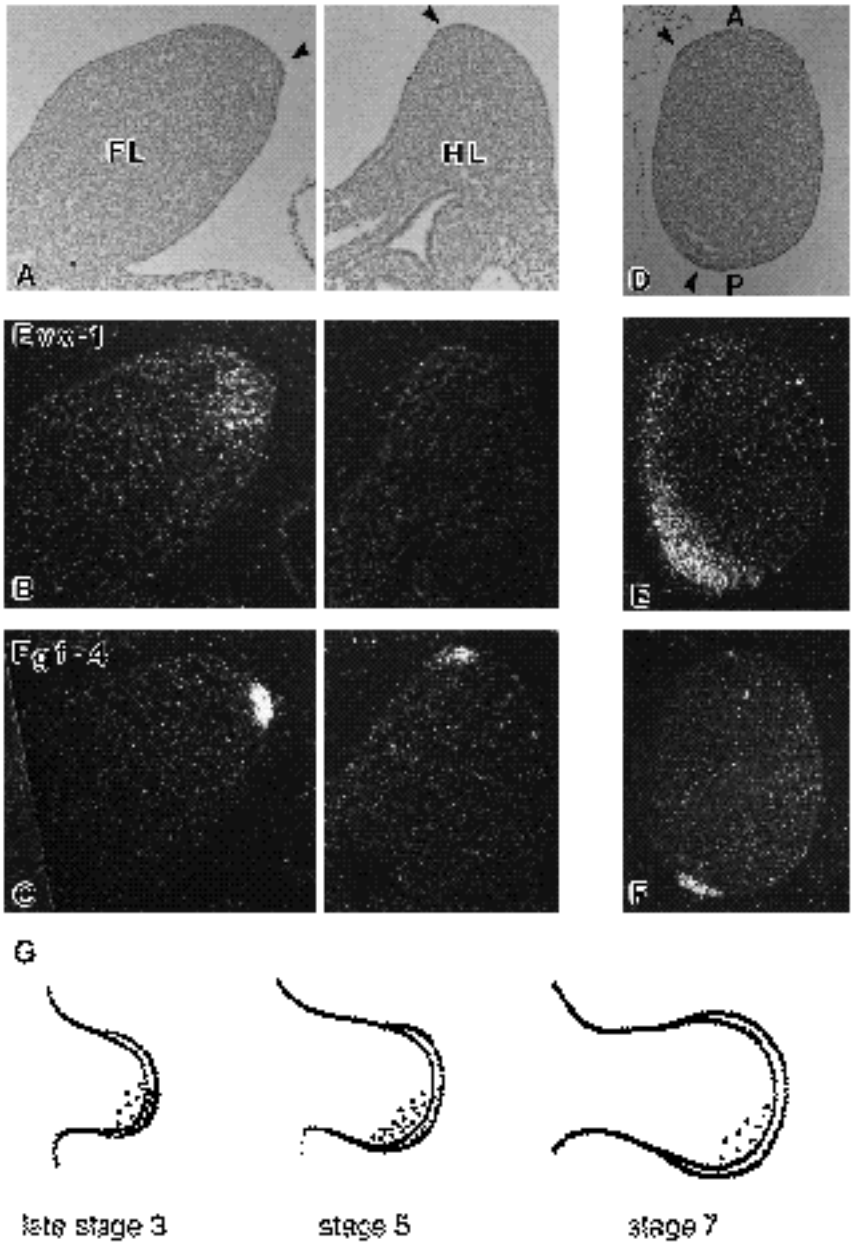

Fig. 1. Comparison of the normal expression patterns of *Evx-1* and *Fgf-4* in the limb. *Evx-1* RNA was first detected at E10.5 (A,B) in the distal mesenchyme of the forelimb (FL), which is at stage 4 of limb development, but was not yet detectable in the hindlimb (HL), which is at stage 3 of limb development. (A) Bright-field illumination; (B) dark-field illumination; (C) a near adjacent section hybridized with an antisense probe for *Fgf-4* RNA: dark-field illumination. Arrowheads in A point to the ridge, where *Fgf-4* is expressed at high levels in the more developmentally advanced forelimb and at moderate levels in the hindlimb in which the ridge has recently formed. (D) Section near the tip of a forelimb (stage 5) of an embryo at E11.5, in which only a portion of the posterior (P) and of the anterior (A) ridge are visible; bright-field illumination. Arrowheads point to the ridge. RNA in situ hybridization of near adjacent sections with an antisense probe for: (E) *Evx-1* RNA; (F) *Fgf-4* RNA: dark-field illumination. ~50×. Panel G illustrates the changes in expression of *Evx-1* within the mesenchyme and of *Fgf-4* in the apical ectodermal ridge during early limb development. *Fgf-4* RNA is most abundant at late stage 3, less abundant at stage 5 and undetectable at stage 7. *Evx-1* RNA is most abundant at stage 5. In the limbs shown here, posterior is at the bottom.

between FGF-4 stimulation and *Evx-1* expression in limbs from which the ridge was removed. Hindlimbs were fixed after various periods of culture in the presence of FGF-4 and analysed by RNA in situ hybridization (Table 1). After 3 hours of culture, no *Evx-1* RNA could be detected, but by 4 hours a low level was observed in all −AER limbs, even though the extrusion of the mesenchyme that occurs in response to FGF treatment was not yet evident. In all cases, *Evx-1* RNA could not be detected in the contralateral +AER limb at this time, presumably because the endogenous amount of FGF-4 in the +AER limb was not yet sufficient to induce the normal pattern of *Evx-1* expression. These results indicate a relatively rapid induction of *Evx-1* expression. They are consistent with our in situ hybridization analysis indicating that a similar period of time elapses between the onset of maximal *Fgf-4* expression and the detection of *Evx-1* transcripts during normal limb development. However, when the culture experiments were repeated in the presence of cycloheximide, a protein synthesis inhibitor, *Evx-1* RNA was not detected, indicating that the induction of *Evx-1* expression by FGF-4 requires protein synthesis (Table 1 and data not shown).

The effect of FGF-4 on *Evx-1* expression can be modulated by BMP-2

We have previously shown that the effect of FGF-4 on the proliferation of distal limb mesenchyme can be modulated by the addition of BMP-2 (Niswander and Martin, 1993). In the presence of 100 ng/ml BMP-2, 10 ng/ml FGF-4 has no effect on −AER limb outgrowth, even though this concentration of FGF-4 alone is sufficient to stimulate

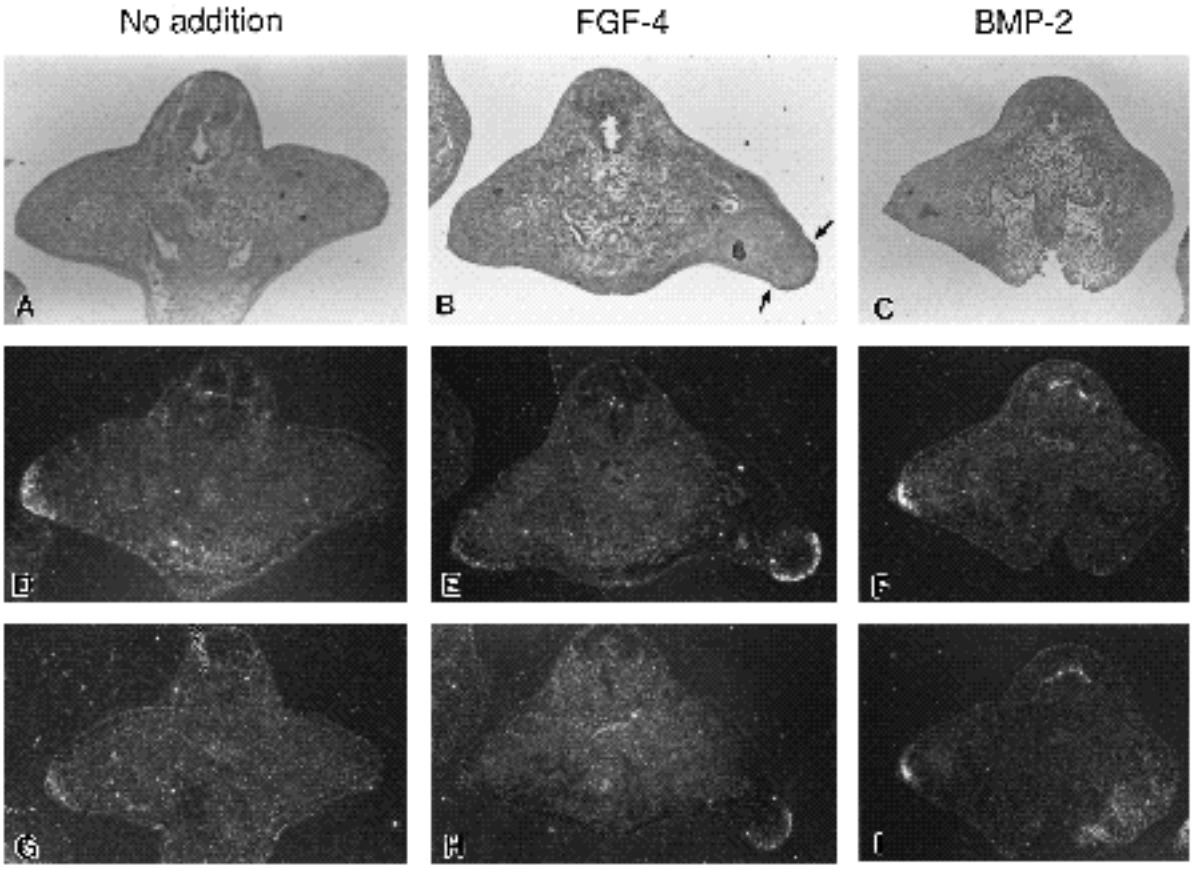

Fig. 2. Regulation of *Evx-1* expression in distal limb mesenchyme by signals from the ridge and by FGF-4. The upper row (A-C) shows bright-field images of transverse sections of trunk fragments with attached hindlimbs cultured for 48 hours in serum-free defined medium: (A) without growth factor supplementation; (B) containing 50 ng/ml FGF-4; (C) containing 50 ng/ml BMP-2. The control +AER limb is on the left side in each panel, the −AER limb is on the right side. These sections pass through the approximate middle of the limb buds. The middle row (D-F) shows dark-field images of the same sections hybridized with an antisense probe for *Evx-1* RNA. The lower row (G-I) shows *Evx-1* expression in the same embryos, in sections passing through a more anterior region of the limb buds. In the −AER limb, when FGF-4 is absent, *Evx-1* RNA is not detected in mesenchyme at either A-P level (D or G, F or I). However, when FGF-4 is added to the medium, *Evx-1* RNA is detected in mesenchyme of the −AER limb at both A-P levels (E and H). Similar effects on the pattern of gene expression were observed in forelimb cultures (not shown). Signal in the neural tube and gut-associated mesenchyme is a normal feature of the *Evx-1* expression pattern (Bastian and Gruss, 1990, and our unpublished observations). Arrows indicate the distal limit of epithelium covering the −AER limb. Extrusion of the mesenchyme is visible distal to the arrows. ~35×.

outgrowth. Conversely, a reciprocal combination of the growth factors (10 ng/ml BMP-2 and 100 ng/ml FGF-4) stimulates outgrowth of −AER limbs, to the same extent as that observed with FGF-4 alone, even though this concentration of BMP-2 alone is sufficient to inhibit outgrowth. When equal concentrations of FGF-4 and BMP-2 (50 ng/ml each) are used, P-D growth is neither stimulated nor inhibited. In essence, the effect of each growth factor is canceled by the other, and −AER limbs are similar to those cultured in medium without growth factor supplementation.

To determine whether BMP-2 similarly modulates the effect of FGF-4 on *Evx-1* expression, we cultured hindlimbs in media containing mixtures of FGF-4 and BMP-2 in different proportions, and assayed for *Evx-1* expression by in situ hybridization (Table 1). When the culture medium was supplemented with 100 ng/ml of FGF-4 and 10 ng/ml BMP-2, *Evx-1* expression was induced in the −AER limb (Fig. 3A,D), just as it was in −AER limbs cultured with FGF-4 alone (Fig. 2B,E,H). Thus, the presence of a relatively low concentration of BMP-2 does not prevent FGF-4 from stimulating limb outgrowth or inducing *Evx-1* expression. When the reciprocal combination of growth factors (10 ng/ml FGF-4 and 100 ng/ml BMP-2) was tested, no *Evx-1* expression was detected in the −AER limb (Fig. 3C,F), as was found in −AER limbs cultured with BMP-2 alone (Fig. 2C,F,I). Thus, a relatively high concentration of BMP-2 prevents FGF-4 from stimulating limb outgrowth and inducing *Evx-1* expression. Interestingly, when limbs were cultured in equal concentrations of FGF-4 and BMP-2 (50 ng/ml each), *Evx-1* expression was detected in the −AER limb (Fig. 3B,E), although limb outgrowth was not evident. This indicates that in appropriate combinations BMP-2 can inhibit the effect of FGF-4 on limb proliferation, without preventing the induction of *Evx-1* expression by FGF-4.

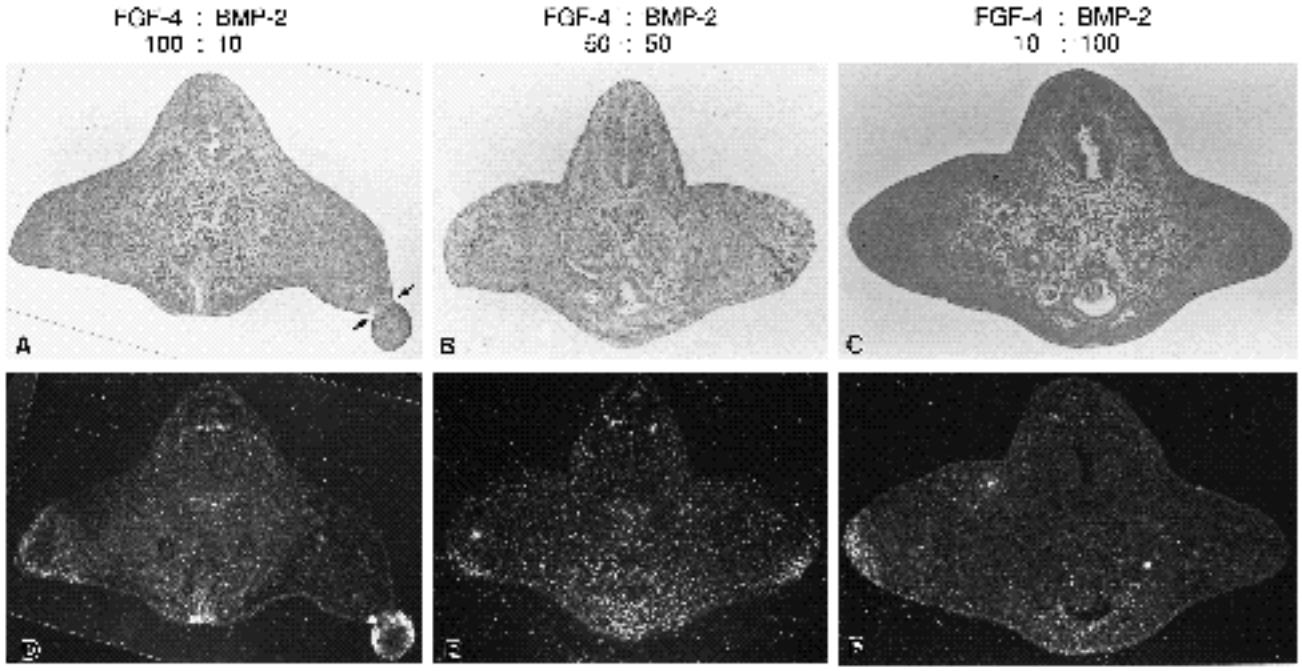

Fig. 3. Effects of mixtures of FGF-4 and BMP-2 on the expression of *Evx-1* in the distal limb mesenchyme. Transverse sections of trunk fragments with attached hindlimbs cultured for 48 hours in medium supplemented with: (A,D) 100 ng/ml FGF-4 and 10 ng/ml BMP-2; (B,E) FGF-4 and BMP-2, each at 50 ng/ml; (C,F) 10 ng/ml FGF-4 and 100 ng/ml BMP-2. For details, see legend to Fig. 2.

DISCUSSION

Our studies show that *Evx-1* RNA is first detected in progress zone mesenchyme shortly after the ridge is fully formed. The level of *Evx-1* RNA increases as the limbs develop to approximately the stage when condensation of digit 4 occurs, and by the time the ridge regresses *Evx-1* RNA is undetectable. At all these stages, *Evx-1* RNA is localized primarily to the posterior distal mesenchyme. By removing the ridge from limbs cultured in vitro, we have demonstrated that both the induction and maintenance of *Evx-1* expression in the progress zone mesenchyme is dependent on signals from the ridge. Furthermore, we have shown that members of the FGF gene family can perform the function of the ridge in regulating *Evx-1* expression. Since *Fgf-4* is a family member expressed in the posterior ridge just prior to *Evx-1* expression in the underlying mesenchyme, whereas at least three other FGF family members are not expressed in the ridge (Wilkinson et al., 1989; Haub and Goldfarb, 1991; Han and Martin, 1993), FGF-4 represents a good candidate for a normal regulator of *Evx-1* expression in the limb. Experiments showing that protein synthesis is required for such regulation further indicate that other molecules, as yet to be identified, are involved in this signaling pathway.

Several lines of evidence suggest that signals from the ridge also induce and maintain the expression of the *Msx*-homeobox-containing genes. For example, it was found that expression of both *Msx-1* and *Msx-2* is induced in mouse proximal limb mesenchyme following grafting beneath the ridge of the early chick wing bud (Davidson et al., 1991). In addition, de novo expression of both genes is induced in chick limb mesenchyme by grafting a ridge to the proximal dorsal surface of the wing bud (Robert et al., 1991). The finding that the expression of both genes is down-regulated following proximal placement of mouse limb mesenchyme in the chick wing bud further suggests that signals from the ridge are required for the maintenance of *Msx-1* and *Msx-2* expression (Davidson et al., 1991). The latter conclusion is consistent with the results of studies of chick embryos homozygous for the *limbless* mutation, showing that *Msx-1* and *Msx-2* expression in the distal mesenchyme decreases and eventually becomes undetectable in the absence of the ridge (Coelho et al., 1991; Robert et al., 1991). However, as yet there is no evidence as to which specific molecules produced by the ridge perform such regulatory functions.

It remains to be determined what role *Evx-1* plays in limb development. Since *Evx-1* is expressed in the undifferentiated mesenchyme at the distal tip of the limb and its expression is controlled by a growth factor known to stimulate proliferation of progress zone mesenchyme, one possibility is that *Evx-1* functions in eliciting the mesenchymal proliferative response. However, the observation that, in the presence of a 1:1 mixture of FGF-4 and BMP-2, *Evx-1* expression is induced but there is no concomitant limb outgrowth suggests that *Evx-1* expression alone may not be sufficient to stimulate limb mesenchyme outgrowth.

Another possibility is based on the proposal that pattern formation along the proximal-distal (P-D) axis is determined by the length of time a cell remains in the progress zone and that, as cells leave this region, their P-Dvalue becomes fixed (Summerbell et al., 1973; Wolpert et al., 1975). Since *Evx-1* RNA is detected in cells within the progress zone and its expression appears to be down-regulated as cells leave this

region, it is conceivable that *Evx-1* may play some role in the acquisition of positional value along the P-D axis. This idea would be generally consistent with the suggestion that during gastrulation in the mouse, *Evx-1* plays a role in patterning the mesoderm emerging from the primitive streak (Dush and Martin, 1992). Moreover, it has been suggested that *Xhox-3*, the *Xenopus* cognate of *Evx-1*, also plays a role in patterning of the mesoderm (Ruiz i Altaba and Melton, 1989a,c). In this context, it is intriguing that *Xhox-3* expression is activated by treatment of *Xenopus* animal cap cells with FGF, as part of their response to this mesoderm-inducing signal (Ruiz i Altaba and Melton, 1989b). This leads us to speculate that not only the genes themselves, but the regulatory elements that control the interaction between them have been conserved. Moreover, it suggests that the particular signal transduction pathway in which these two genes participate may be used repeatedly in a variety of developmental processes, in many vertebrate species.

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REFERENCES

Bastian, H. and Gruss, P. (1990). A murine *even-skipped* homologue, *Evx 1*, is expressed during early embryogenesis and neurogenesis in a biphasic manner. *EMBO J.* **9,** 1839-1852.

Bulfone, A., Kim, H.-J., Puelles, L., Porteus, M. H., Grippo, J. F. and Rubenstein, J. L. R. (1993). The mouse *Dlx-2 (Tes-1)* gene is expressed in spatially restricted domains of the forebrain, face and limbs in mid-gestation mouse embryos. *Mech. Dev.* **40**, 129-140.

Coelho, C. N. D., Krabbenhoft, K. M., Upholt, W. B., Fallon, J. F. and Kosher, R. A. (1991). Altered expression of the chicken homeobox-containing genes GHox-7 and GHox-8 in the limb bud of *limbless* mutant chick embyros. *Development* **113,** 1487-1493.

Coelho, C. N. D., Sumoy, L., Rodgers, B. J., Davidson, D. R., Hill, R. E., Upholt, W. B. and Kosher, R. A. (1991). Expression of the chicken homeobox-containing gene GHox-8 during embryonic chick limb development. *Mech. Dev.* **34,** 143-154.

Davidson, D. R., Crawley, A., Hill, R. E. and Tickle, C. (1991). Position-dependent expression of two related homeobox genes in developing vertebrate limbs. *Nature* **352,** 429-431.

Davis, C. A., Holmyard, D. P., Millen, K. J. and Joyner, A. L. (1991). Examining pattern formation in mouse, chicken and frog embryos with an *En*-specific antiserum. *Development* **111,** 287-298.

Dollé, P., Izpisúa-Belmonte, J.-C., Falkenstein, H., Renucci, A. and Duboule, D. (1989). Coordinate expression of the murine *Hox-5* complex homeobox-containing genes during limb pattern formation. *Nature* **342,** 767-772.

Dollé, P., Price, M. and Duboule, D. (1992). Expression of the mouse *Dlx-1* homeobox gene during facila, ocular and limb development. *Differentiation* **49,** 93-99.

Dollé, P., Ruberte, E., Kastner, P., Petkovich, M., Stoner, C. M., Gudas, L. J. and Chambon, P. (1989). Differential expression of genes encoding α, β and γ retinoic acid receptors and CRABP in the developing limbs of the mouse. *Nature* **342,** 702-705.

Dush, M. K. and Martin, G. R. (1992). Analysis of mouse *Evx* genes; *Evx-1* displays graded expression in the primitive streak. *Dev. Biol.* **151,** 273-287.

Frohman, M. A., Boyle, M. and Martin, G. R. (1990). Isolation of the mouse *Hox-2.9* gene; analysis of embryonic expression suggests that positional information along the anterior-posterior axis is specified by mesoderm. *Development* **110,** 589-607.

Gaunt, S. J., Blum, M. and De Robertis, E. M. (1993). Expression of the mouse *goosecoid* gene may mark mesenchymal cell lineages in the developing head, limbs, and ventral body wall. *Development* **117**, 769-778.

Gavin, B. J., McMahon, J. A. and McMahon, A. P. (1990). Expression of multiple novel *Wnt*-1/*int*-1-related genes during fetal and adult mouse development. *Genes Dev.* **4,** 2319-2332.

Globus, M. and Vethamany-Globus, S. (1976). An *in vitro* analogue of early chick limb bud outgrowth. *Differentiation* **6,** 91-96.

Goulding, M. D., Chalepakis, G., Deutsch, U., Erselius, J. R. and Gruss, P. (1991). Pax-3, a novel murine DNA binding protein expressed during early neurogenesis. *EMBO J.* **10,** 1135-1147.

Han, J.-K. and Martin, G. R. (1993). Embryonic expression of *Fgf-6* is restricted to the skeletal muscle lineage. *Dev. Biol.* (in press).

Haub, O. and Goldfarb, M. (1991). Expression of the fibroblast growth factor-5 gene in the mouse embryo. *Development* **112,** 397-406.

Hébert, J. M., Basilico, C., Goldfarb, M., Haub, O. and Martin, G. R. (1990). Isolation of cDNAs encoding four mouse FGF family members and characterization of their expression patterns during embryogenesis. *Dev. Biol.* **138,** 454-463.

Izpisúa-Belmonte, J.-C. and Duboule, D. (1992). Homeobox genes and pattern formation in the vertebrate limb. *Dev. Biol.* **152,** 26-36.

Izpisúa-Belmonte, J.-C., Tickle, C., Dollé, P., Wolpert, L. and Duboule, D. (1991). Expression of the homeobox *Hox-4* genes and the specification of position in chick wing development. *Nature* **350,** 585-589.

Jackson-Grusby, L., Kuo, A. and Leder, P. (1992). A variant *limb deformity* transcript expressed in the embryonic mouse limb defines a novel formin. *Genes Dev.* **6,** 29-37.

Jones, C. M., Lyons, K. M. and Hogan, B. L. M. (1991). Involvement of *Bone Morphogenetic Protein-4* (BMP-4) and *Vgr-1* in morphogenesis and neurogenesis in the mouse. *Development* **111,** 531-542.

Kieny, M. (1960). Rôle inducteur du mésoderme dans la differenciation précoce du bourgeon de membre chez l'embryon de Poulet. *J. Embryol. Exp. Morph.* **8,** 457-467.

Kieny, M. (1968). Variation de la capacité inductrice du mésoderme et de la competénce de l'ectoderme au cours de l'induction primaire du bourgeon de membre, chez l'embryon de Poulet. *Arch. Anat. Microsc. Morphol. Exp.* **57,** 401-418.

Lyons, K. M., Pelton, R. W. and Hogan, B. L. M. (1990). Organogenesis and pattern formation in the mouse: RNA distribution patterns suggest a role for *Bone Morphogenetic Protein-2A (BMP-2A)*. *Development* **109,** 833-844.

McGinnis, W. and Krumlauf, R. (1992). Homeobox genes and axial patterning. *Cell* **68**, 283-302.

Morgan, B. A., Izpisúa-Belmonte, J.-C., Duboule, D. and Tabin, C. J. (1992). Targeted misexpression of *Hox-4.6* in the avian limb bud causes apparent homeotic transformations. *Nature* **358,** 236-239.

Niswander, L. and Martin, G. R. (1992). *Fgf-4* expression during gastrulation, myogenesis, limb and tooth development in the mouse. *Development* **114,** 755-768.

Niswander, L. and Martin, G. R. (1993). FGF-4 and BMP-2 have opposite effects on limb growth. *Nature* **361,** 68-71.

Nohno, T., Noji, S., Koyama, E., Ohyama, K., Myokai, F., Kuroiwa, A., Saito, T. and Taniguchi, S. (1991). Involvement of the *Chox-4* chicken homeobox genes in determination of anteroposterior axial polarity during limb development. *Cell* **64,** 1197-1205.

Oliver, G., De Robertis, E. M., Wolpert, L. and Tickle, C. (1990). Expression of a homeobox gene in the chick wing bud following application of retinoic acid and grafts of polarizing region tissue. *EMBO J.* **9,** 3093-3099.

Oliver, G., Sidell, N., Fiske, W., Heinzmann, C., Mohandas, T., Sparkes, R. S. and De Robertis, E. M. (1989). Complementary homeoprotein gradients in developing limb buds. *Genes Dev.* **3,** 641-650.

Oliver, G., Wright, C. V. E., Hardwicke, J. and De Robertis, E. M. (1988). A gradient of homeodomain protein in developing forelimbs of *Xenopus* and mouse embryos. *Cell* **55,** 1017-1024.

Orr-Urtreger, A., Givol, D., Yayon, A., Yarden, Y. and Lonai, P. (1991). Developmental expression of two murine fibroblast growth factor receptors, *flg* and *bek*. *Development* **113,** 1419-1434.

Peters, K. G., Werner, S. and Williams, L. T. (1992). Two FGF receptor genes are differentially expressed in epithelial and mesenchymal tissues

during limb formation and organogenesis in the mouse. *Development* **114,** 233-243.

Reiter, R. S. and Solursh, M. (1982). Mitogenic property of the apical ectodermal ridge. *Dev. Biol.* **93,** 28-35.

Robert, B., Lyons, G., Simandl, B. K., Kuroiwa, A. and Buckingham, M. (1991). The apical ectodermal ridge regulates *Hox-7* and *Hox-8* gene expression in developing chick limb buds. *Genes Dev.* **5,** 2363-2374.

Ruberte, E., Friederich, V., Morriss-Kay, G. and Chambon, P. (1992). Differential distribution patterns of CRABP I and CRABP II transcripts during mouse embryogenesis. *Development* **115**, 973-987.

Ruiz i Altaba, A. and Melton, D. A. (1989a). Bimodal and graded expression of the *Xenopus* homeobox gene *Xhox3* during embryonic development. *Development* **106,** 173-183.

Ruiz i Altaba, A. and Melton, D. A. (1989b). Interaction between peptide growth factors and homoeobox genes in the establishment of antero-posterior polarity in frog embryos. *Nature* **341,** 33-38.

Ruiz i Altaba, A. and Melton, D. A. (1989c). Involvement of the *Xenopus* homeobox gene *Xhox3* in pattern formation along the anterior-posterior axis. *Cell* **57,** 317-326.

Saunders, J. W., Jr and Reuss, C. (1974). Inductive and axial properties of prospective wing-bud mesoderm in the chick embryo. *Dev. Biol.* **38,** 41-50.

Summerbell, D., Lewis, J. H. and Wolpert, L. (1973). Positional information in chick limb morphogenesis. *Nature* **224,** 492-496.

Suzuki, H. R., Padanilam, B. J., Vitale, E., Ramirez, F. and Solursh, M. (1991). Repeating developmental expression of G-Hox-7, a novel homeobox-containing gene in the chicken. *Dev. Biol.* **148,** 375-388.

Suzuki, H. R., Sakamoto, H., Yoshida, T., Sugimura, T., Terada, M. and Solursh, M. (1992). Localization of *Hst*1 transcripts to the apical ectodermal ridge in the mouse embryo. *Dev. Biol.* **150,** 219-222.

Tabin, C. J. (1991). Retinoids, homeoboxes, and growth factors: toward molecular models for limb development. *Cell* **66,** 199-217.

Tam, P. P. L. and Beddington, R. S. (1987). The formation of mesodermal tissues in the mouse embryo during gastrulation and early organogenesis. *Development* **99,** 109-126.

Trumpp, A., Blundell, P. A., de la Pompa, J. L. and Zeller, R. (1992). The chicken *limb deformity* gene encodes nuclear proteins expressed in specific cell types during morphogenesis. *Genes Dev.* **6,** 14-28.

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

Wilkinson, D. G., Bhatt, S. and McMahon, A. P. (1989). Expression pattern of the FGF-related proto-oncogene *int-2* suggests multiple roles in fetal development. *Development* **105,** 131-136.

Wolpert, L., Lewis, J. and Summerbell, D. (1975). Morphogenesis of the vertebrate limb. *Ciba Found. Symp.* **29,** 95-130.

Yokouchi, Y., Sasaki, H. and Kuroiwa, A. (1991). Homeobox gene expression correlated with the bifurcation process of limb cartilage development. *Nature* **353,** 443-445.

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