Retroviral expression of FGF-2 (bFGF) affects patterning in chick limb bud

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SUMMARY

To investigate the role of fibroblast growth factor-2 (basic fibroblast growth factor) in chick limb development, we constructed a replication-defective spleen necrosis virus to ectopically express fibroblast growth factor-2 in stage 20-22 chick limb bud. Because infecting cells in vivo proved to be inefficient, limb bud cells were dissociated, infected in vitro, and then grafted back into host limbs. This procedure caused duplications of anterior skeletal elements, including proximal humerus, distal radius, and digits 2 and 3. Eighty-nine percent of host wings receiving infected grafts at their anterior borders had duplications of one or more of these elements. The frequency of duplication declined dramatically when infected cells were grafted to progressively more posterior sites of host limb buds, and grafting to the posterior border had no effect at all. Several techniques were used to determine the role of infected tissue in forming skeletal duplications. First, staining with an fibroblast growth factor-2 specific monoclonal antibody showed higher than endogenous levels of fibroblast growth factor-2 expression associated with extra elements. Second, the host/donor composition of duplicated elements was determined by simultaneously infecting donor cells with viruses encoding fibroblast growth factor-2 or β-galactosidase; donor tissue was then visualized by X-gal staining. Patterns of ectopic fibroblast growth factor-2 expression and X-gal staining confirmed the presence of infected donor tissue near duplicated structures, but the duplicated skeletal elements themselves showed very little staining. Similar results were obtained in duplications caused by infected quail wing bud cells grafted to the chick wing bud. These observations suggest that fibroblast growth factor-2-expressing donor tissue induced host tissue to form normally patterned extra elements. In support of this conclusion, implanting beads containing fibroblast growth factor-2 caused partial duplications of digit 2. These data provide the first direct evidence that fibroblast growth factor-2 plays a role in patterning in the limb bud.

Key words: limb development, FGF-2, retrovirus, pattern formation, chick

INTRODUCTION

Little is known about the role of fibroblast growth factor-2 (FGF-2) in chick limb bud development. Although the limb bud contains significant quantities of FGF-2 (Seed et al., 1988; Munaim et al., 1988) and FGF receptors (Olwin and Hauschka, 1988; Orr-Urtreger et al., 1991; Peters et al., 1992), the pattern of FGF-2 signalling and its function(s) in the limb are unknown. Tissue culture studies conducted by Aono and Ide (1988) showed that exogenous FGF-2 stimulates division of cultured mesenchymal cells from the anterior of stage 22 limb bud but has no significant effect on posterior mesenchyme. Cells taken from intermediate positions show intermediate responses to FGF-2. These data suggest that there is an anteroposterior gradient of FGF-2-responsiveness in limb bud mesenchyme. In contrast, cells from anterior, posterior, or intermediate regions of the limb bud are equally responsive to exogenous PDGF or insulin, indicating that the putative gradient of responsiveness is specific for FGF-2.

Exogenous FGF-2 also affects in vitro differentiation of several mesoderm-derived cells and expression of components of the extracellular matrix (Seed and Hauschka, 1988; Kato and Iwamoto, 1990; Munaim et al., 1991). Mesoderm cells rapidly condense to form cartilage in vitro unless ectoderm is also present, in which case mesoderm cells continue to grow as a loose mesenchyme (Reiter and Solursh, 1982; Kosher et al., 1979). The identity of the ectoderm-derived growth factor(s) is unknown, but FGF-2 mimics its activity (Knudson and Toole, 1988). In cultures of limb bud mesoderm, exogenous FGF-2 inhibits condensation and chondrogenesis, as well as associated changes in gene expression. Moreover, FGF-2 induces accumulation of hyaluronan and other extracellular matrix components that are associated with maintenance of loose mesenchyme.

Reports of FGF-2 function in vivo have been varied and difficult to interpret. Hayamizu et al. (1991) reported that FGF-soaked beads had no detectable effects on chick limb bud development. In contrast, Frenkel et al. (1990) showed that injection of 1 µg of FGF-2 into day-11 eggs increased growth of chondrogenic tissue and stimulated synthesis of extracellular matrix. However, it is unclear from these studies whether FGF-2 is involved in chick limb development.

We therefore developed a system to ectopically express
FGF-2 by infection with replication-defective retrovirus (Riley et al., unpublished data). Our results show that ectopic expression of FGF-2 dramatically affects limb bud morphology.

**MATERIALS AND METHODS**

**Virus production**

Cell line DSDh (Hu and Temin, 1990) was used to package replication-defective spleen necrosis virus encoding FGF-2 or β-galactosidase (Riley et al., unpublished data). DSDh cells express viral proteins, gag-pol and env, from two different vectors to ensure against production of replication-competent virus, which is undetectable in DSDh culture media (Hu and Temin, 1990). Cells were grown in Dulbecco’s modified Eagle’s medium with 10% calf serum and 0.6 μM methotrexate at 37°C with 5% CO₂. Viral titers in culture media ranged from 5×10⁵ to 2×10⁶ infectious units/ml (IU/ml). To concentrate the virus, culture media were spun at 200 μl of M199 medium (Gibco) with 15% horse serum. This increased the titers of vlgFGF-2 and vFGF-2 by up to 20-fold, giving average titers of 1×10⁶ and 2×10⁵ IU/ml, respectively. The titer of vLac usually increased by more than 100-fold to 2×10⁶ IU/ml. The FGF-2 (Abraham et al., 1986) and IgFGF-2 (Rogel et al., 1988) cDNAs used for construction of vFGF-2 and vlgFGF-2 were provided by J. Abraham, Scios, Inc. and M. Klagsbrun, Children’s Hospital and Medical Center, Harvard Medical School, respectively. The construct for producing vLac was obtained from H. Temin, University of Wisconsin.

**Dissociation and infection of limb bud cells**

Eggs were obtained from white leghorn chickens maintained at the Poultry Science Department, University of Wisconsin-Madison. Anterior or posterior halves of wing buds were removed from stage 20-22 embryos with fine tip forceps and stored briefly in PBS, and fixed in 4% paraformaldehyde in PBS at 4°C. Staining cartilage and clarifying embryos were fixed overnight at 22°C in 10% formaldehyde and transferred to acid alcohol (1% HCl in 70% ethanol) for 24 hours. Wings were then dehydrated in a graded series of ethanol, embedded in paraplast X-TRA (Oxford Labware), and cut into 5 μm sections. After deparaffinizing in xylene and rehydrating, sections were incubated with a highly specific anti-FGF-2 monoclonal antibody followed by biotinylated secondary antibody (rabbit anti-mouse). Finally, sections were incubated with peroxidase-conjugated streptavidin and diaminobenzidine to visualize antibody binding. The primary antibody was made against human FGF-2 and binds FGF-2 from other species such as mouse and chick. However, the antibody does not bind other members of the FGF family. A detailed description of the characterization of the anti-FGF-2 antibody will be published elsewhere (Savage et al., unpublished data).

**RESULTS**

**Immunostaining**

Wings were removed from stage 27 embryos, rinsed in ice cold PBS, and fixed in 4% paraformaldehyde in PBS at 4°C for 2-3 hours. Wings were then dehydrated in a graded series of ethanol, embedded in paraplast X-TRA (Oxford Labware), and cut into 5 μm sections. After deparaffinizing in xylene and rehydrating, sections were incubated with a highly specific anti-FGF-2 monoclonal antibody followed by biotinylated secondary antibody (rabbit anti-mouse). Finally, sections were incubated with peroxidase-conjugated streptavidin and diaminobenzidine to visualize antibody binding. The primary antibody was made against human FGF-2 and binds FGF-2 from other species such as mouse and chick. However, the antibody does not bind other members of the FGF family. A detailed description of the characterization of the anti-FGF-2 antibody will be published elsewhere (Savage et al., unpublished data).

**β-galactosidase assays**

Day-9 to -10 wings (or whole embryos) were fixed for 2-3 hours at 4°C in PBS containing 2% formaldehyde and 0.2% glutaraldehyde. After rinsing in PBS, wings were incubated in X-gal cocktail (1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl b-D-galactopyranoside), 2 mM MgCl₂, 16 mM K₃Fe(CN)₆, 16 mM K₄Fe(CN)₆) for 18-24 hours at 22°C.

**Heterochromatin staining**

Whole wings were stained en bloc according to Carlson et al., 1986. Wings were removed from day-9 embryos and incubated on ice for 4-6 hours in Lavdowsky’s fixative (17:2:1 ratio of 95% ethanol, 37% formaldehyde, and glacial acetic acid). Wings were then rinsed in 70% ethanol, rehydrated, and incubated for 1 hour at 22°C in 5N HCl. After rinsing twice in water, wings were incubated at 22°C for 30-60 minutes with Feulgen reagent (0.5% basic fuchsin, 1% sodium metabisulfite, 1N HCl, mixed with 0.25 g neutral charcoal/100 ml and filtered with course paper). Wings were then incubated 3 times for 5 minutes each in 0.5% sodium metabisulfite, 50 mM HCl, and rinsed in running water for 15 minutes. Wings were dehydrated and sectioned as described above (see Immunostaining).

**Infection of limb bud cells**

A pilot study using a replication-defective virus expressing β-galactosidase (vLac) showed that direct injection of virus into stage 20 wing buds was unsuccessful because of low infection frequencies and infection of cells distant from the site of injection. We therefore developed an effective technique in which dissociated limb bud cells were infected in vitro and grafted to stage 20-22 host wing buds (usually to the anterior border; Fig. 1). This technique consistently gave high infection frequencies (up to 95% of donor cells) and better control over localization of viral expression.

In most experiments, ectopic expression of FGF-2 was achieved by infecting cells with a replication-defective virus expressing bovine FGF-2 fused to an immunoglobulin signal peptide (vlgFGF-2). Tissue culture experiments indicate that addition of a signal peptide greatly increases the rate of FGF-2 secretion (Rogel et al., 1988; Riley et al., unpublished data).
FGF affects limb pattern

Effects of infection on limb bud morphology

Infecting cells with vIgFGF-2 and grafting them to the anterior borders of stage 20-22 host wing buds caused duplications of digits 2 and 3, as well as proximal humerus and distal radius (Fig. 2 and Table 1). Proximal humerus and digit 2 were most frequently affected whereas digit 4 and the ulna were unaffected by such grafts. Of embryos receiving vIgFGF-2-infected grafts, 89% formed duplications (Table 1). In contrast, grafting uninfected tissue, or tissue infected with vLac, caused duplications in only 7-9% of host wings, and only digit 2 was affected (Table 1). In no case did vLac-infected or uninfected grafts affect any other skeletal elements. Thus, the high frequency and broad range of duplications associated with vIgFGF-2 are not caused by the grafting procedure or by retroviral infection per se.

Since IgFGF-2 is not a naturally occurring gene product, we tested whether infection with a virus expressing wild-type FGF-2 (vFGF-2) affects limb bud morphology. vFGF-2 caused the same range of duplications as did vIgFGF-2, although the duplication frequency was reduced slightly in vFGF-2-infected limbs (Table 1). This is despite the fact that the titer of vFGF-2 was usually twice that of vIgFGF-2 (see Materials and Methods). The lower duplication frequency obtained with vFGF-2 presumably reflects the fact that wild-type FGF-2 is not secreted as efficiently as is IgFGF-2 (Riley et al., unpublished data). The simplest explanation for these results is that ectopic expression of FGF-2 leads to duplications of affected structures. Further support for this explanation is presented below.

Examples of duplications caused by vFGF-2 and vIgFGF-2 are shown in Fig. 2. In a particularly striking specimen (Fig. 2A), duplications are evident in four different skeletal elements: all of digits 2 and 3, most of the radius, and the proximal half of the humerus. More typical specimens, in which only digit 2 and proximal humerus are affected, are shown in Fig. 2B,G. It was not uncommon for the duplicated portion of a humerus to separate from the parent humerus, forming a structure with mirror image symmetry (Fig. 2B,F). Duplications of the distal half of the humerus were not observed. Gradations in the duplication process are demonstrated by the extent of radius duplication in Fig. 2A,C and D. While most of the radius is affected in the specimen in Fig. 2A, only the distal tip is affected in the one in 2C. In Fig. 2D, the radius shows no discrete duplication but does show a marked anteroposterior broadening. The contralateral control (left wing) is shown in Fig. 2E for comparison. We view such broadening as an incipient state of duplication. The specimen in Fig. 2D also shows a duplication of digit 2.

For most experiments, ectoderm was not removed from the tissue to be grafted because the removal procedure reduces the subsequent ability of mesoderm to be infected with virus. Because mixing of mesenchymal and ectodermal cells complicates interpretation of the results, we tested different cell lines to replace mesocoele mesoderm in grafts to the anterior border (see Fig. 1). We used a retrovirus encoding the lacZ gene (vLac) to replace mesoderm from the anterior borders of stages 20-22 host limbs. Grafted tissue usually contained both mesoderm and ectoderm. Where indicated, ectoderm was removed prior to limb bud dissociation by incubation in PBS with 0.5% trypsin for 12-15 minutes at 37°C. Cells from posterior halves of limb buds (including the polarizing zone) were used only when grafting to the posterior borders of host limbs. This is because cells from the polarizing zone cause duplications when grafted to more anterior sites of host limbs (Saunders and Gasseling, 1968; Tickle et al., 1975).

Table 1. Effects of grafting infected donor cells to various sites in host wing buds

<table>
<thead>
<tr>
<th>Host graft site</th>
<th>Donor cell infection</th>
<th>Humerus</th>
<th>Radius</th>
<th>Digit 2</th>
<th>Digit 3</th>
<th>Total</th>
<th>Normal wings</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior border</td>
<td>No virus</td>
<td>0</td>
<td>0</td>
<td>3 (9%)</td>
<td>0</td>
<td>3 (9%)</td>
<td>32 (91%)</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>vLac</td>
<td>0</td>
<td>2 (7%)</td>
<td>0</td>
<td>2 (7%)</td>
<td>0</td>
<td>26 (93%)</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>vFGF-2</td>
<td>6 (26%)</td>
<td>4 (17%)</td>
<td>9 (39%)</td>
<td>0</td>
<td>13 (57%)</td>
<td>10 (43%)</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>vIgFGF-2</td>
<td>20 (71%)</td>
<td>4 (14%)</td>
<td>11 (39%)</td>
<td>1 (4%)</td>
<td>25 (89%)</td>
<td>3 (11%)</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>vIgFGF-2 (no ectoderm)</td>
<td>8 (42%)</td>
<td>1 (5%)</td>
<td>2 (11%)</td>
<td>1 (5%)</td>
<td>9 (47%)</td>
<td>10 (53%)</td>
<td>19</td>
</tr>
<tr>
<td>Apex</td>
<td>No virus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8 (100%)</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>vFGF-2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14 (100%)</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>vIgFGF-2</td>
<td>6 (40%)</td>
<td>2 (13%)</td>
<td>1 (7%)</td>
<td>7 (47%)</td>
<td>8 (53%)</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Posterior border</td>
<td>No virus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10 (100%)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>vIgFGF-2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>16 (100%)</td>
<td>16</td>
</tr>
</tbody>
</table>
whether removal of ectoderm affects the ability of vIgFGF-2-infected mesoderm to induce duplications. Even though the frequency of duplication was reduced by half (Table 1), the same elements were affected as when ectoderm was not removed (Fig. 2F,G). Fig. 2F shows a duplication of digit 3 and proximal humerus; Fig. 2G shows a duplication of digit 2 and proximal humerus. Thus, the presence of ectoderm in vIgFGF-2-infected grafts is not necessary for inducing duplications.

The lower frequency of duplication seen after ectoderm removal presumably reflects the concomitant decrease in infection frequency. This treatment does not reduce cell viability in vitro but reduces the percentage of infected cells by as much as 50% (Riley, unpublished observations). It seems likely that the reduced infection frequency is caused by proteolysis of viral receptors.

Effects of grafting to different sites in wing bud
To test for position-dependent effects on induction of duplications, we grafted infected cells to the apex or the posterior border of stage 20-22 host wing buds (Table 1). When grafted to the apex, anterior wing cells infected with vIgFGF-2 caused duplications of proximal humerus and digits 2 and 3. However, there were no duplications of the radius, and the overall duplication frequency was only half of the frequency obtained with vIgFGF-2-infected cells grafted to the anterior border. In contrast, grafting vFGF-2-infected cells to the apex caused no duplications or any other detectable changes in morphology. Uninfected grafts also did not affect morphology. As noted previously, the higher duplication frequency obtained with vIgFGF-2, particularly in apical grafts, most likely reflects the elevated rate of FGF-2 secretion. The different duplication frequencies obtained with anterior grafts and apical grafts are not due to experimental variation in virus titer because we routinely grafted pieces of the same pellet to both sites.

For posterior border grafts, we used posterior wing cells instead of anterior wing cells because the latter form a high background of cartilaginous rods and nodules when grafted to the posterior border (Stocker and Carlson, 1988). Tissue
culture studies showed that posterior wing cells can be infected with and express vLac or vIgFGF-2 with the same efficiency as anterior wing cells (Riley, unpublished data). Grafting posterior cells to the posterior borders of stage 20-22 wing buds produced no detectable changes in morphology, regardless of whether cells were infected with vIgFGF-2 or uninfected (Table 1).

**Visualizing ectopic FGF-2 expression**

To determine the role of infected cells in causing duplications, we used a monoclonal antibody specific for FGF-2 (Savage et al., unpublished data) to detect ectopic FGF-2 expression. Embryos were fixed and stained at stage 27 (only 2.5 days after grafting) because endogenous FGF-2 expression becomes more widespread and intense at later stages (Joseph-Silverstein et al., 1989) and obscures the viral expression pattern. The first response to grafting infected tissue was visible by 36 hours and appeared as a bulge near the site of the graft. The lag between grafting and bulge formation reflects the fact that at least two cell doublings (18-24 hours for chick limb bud cells, Searls and Janniers, 1971; White et al., 1992) are required for maximal expression of the viral sequence. It is important to note that no bulges formed in limbs with uninfected grafts (data not shown). Outgrowths in limbs with infected donor tissue continued to enlarge through stage 27 (approximately 2.5 days after grafting) when the limbs were fixed. Fig. 3A shows a slightly oblique cross section of a typical specimen that received a graft infected with vIgFGF-2. A broad crescent of FGF-2 staining is visible in the mesenchyme near the base of the extra outgrowth, as well as a band of staining along the proximal/ventral edge of the outgrowth. By comparison, staining in the contralateral control wing (Fig. 3B) is much lighter and more uniformly distributed. Thus, as expected, the formation of extra tissue in infected wings is associated with a high level of ectopic FGF-2 expression. However, the pattern of FGF-2 staining in the infected wing suggests that the majority of infected cells are concentrated around the base of the outgrowth and that the outgrowth itself is composed largely of uninfected tissue. This is consistent with the possibility that FGF-2-expressing donor cells induce host tissue to form an outgrowth.

**Tracing the fate of grafted tissue in day-9 wing buds**

Since endogenous FGF-2 precludes analysis of viral FGF-2 expression patterns in older wings, we infected donor cells with vLac and used expression of β-galactosidase to trace the fate of donor cells in 9-day hosts. Specifically, donor cells were infected with vLac (controls) or with both vLac and vIgFGF-2 and grafted to the anterior borders of stage 20-22 (day-3 to -3.5) host wing buds (Fig. 4). Under the conditions used here, up to 95% of the donor cells are infected with vLac, including cells that have also been infected with vIgFGF-2 (Riley et al., unpublished data). Host wings were then fixed and stained with X-gal on day 9. As shown in Fig. 4A,B, tissue adjacent to the humerus, especially distally, was the most frequent and intense site of X-gal staining. Regions anterior to and including the radius also stained positively in some specimens, as did digit 2 and, more rarely, digit 3. We detected no staining around the ulna or digit 4. The same staining pattern was evident in hosts receiving donor tissue infected with vLac alone or with both vLac and vIgFGF-2.

Several conclusions may be drawn from these data. First, infection with vIgFGF-2 does not detectably alter the migration or colonization patterns of donor cells. Second, hosts with vLac-infected tissue showed approximately the same degree of X-gal staining as hosts with both vLac- and vIgFGF-2-infected tissues, indicating that comparable numbers of donor cells survive in the two cases. Thus, the ability of vIgFGF-2 infected cells to cause duplications is not a simple consequence of survival of the graft. Finally, as demonstrated in Fig. 4F,G, duplicated structures were always associated with tissue that stained positively for X-gal. This suggests that infected donor tissue somehow participates in the formation of extra skeletal elements. However, some areas frequently stained positively but were not observed to form duplications. This is most notable near the elbow, including distal humerus and proximal radius (Fig. 4B,F and G). Clearly, not all skeletal elements are equally sensitive to the duplicating effects of infected donor tissue.

To study further the fate of donor cells at other sites in
the limb bud, cells were infected with vIgFGF-2 and vLac and grafted to the apex of host limb buds (Fig. 4C). As expected, the staining pattern was quite distinct from that obtained with anterior border grafts. Apical grafts resulted in extensive staining of posterior tissue, and the most frequent site of staining was the distal end rather than the proximal end of the humerus. In stage 20-22 limb bud, the proximal and distal ends of the humerus are actually located

Fig. 4. Use of vLac expression to detect donor tissue. (A-C) Pictorial representations of X-gal staining indices. Using skeletal elements and limb contours for landmarks, a coordinate system was developed to normalize data from different embryos. For every wing in the experimental and control populations, each region of the coordinate map was examined for X-gal staining and given a numerical value of 0 (no visible staining), 1 (light staining, with only a few widely scattered blue cells), or 2 (heavy, more widespread staining). Values were then added for each region and divided by the number of specimens to obtain a staining index between 0 and 2.0. In practice, staining indices did not exceed 1.1. (A) Pictorial composite of 22 specimens with anterior border grafts that were infected with vLac. (B) Pictorial composite of 36 specimens with anterior border grafts that were infected with both vLac and vIgFGF-2. (C) Pictorial composite of 24 specimens with apical grafts that were infected with both vLac and vIgFGF-2. (D, E) X-gal and cartilage staining, respectively, of a wing that received a graft to the anterior border of cells infected with vLac only. (F, G) X-gal and cartilage staining, respectively, of a wing that received a graft to the anterior border of cells infected with both vLac and vIgFGF-2. Note partial duplication of radius in 4G (short arrow). The long arrow, short arrow, closed arrowhead, and open arrowhead mark the humerus, radius, digit 2, and digit 3, respectively.
101FGF affects limb pattern anteriorly and posteriorly, respectively (Bowen et al., 1989). Thus, despite the increase in posterior staining, no duplications were observed in posterior limb structures. In contrast, the decrease in staining of anterior tissue in limbs with apical grafts (Fig. 4C) correlated with the decrease in the frequency of duplication of anterior structures (Table 1). These data support the conclusion that infected donor tissue causes duplications of anterior structures but that posterior structures are relatively insensitive to this effect.

To assess host/donor composition of duplicated structures, X-gal staining patterns were closely examined in individual specimens. Much of the staining associated with duplications of the radius and digit 2 occurred in adjacent tissue whereas the duplications themselves often showed very little staining (data not shown). Staining around the humerus was often too heavy to distinguish skeletal elements from surrounding tissue (data not shown). To analyze host/donor composition in more detail, quail cells were infected with vlgFGF-2 and grafted to chick wing buds. Quail cells were distinguished from chick cells in day-9 wings by staining with Feulgen reagent, which darkly stains heterochromatin in quail nuclei. Fig. 5A shows a sagittal section of a typical specimen with a well-defined duplication of digit 2 as well as a duplication of proximal humerus that lies dorsally and posterior to the parent humerus. The humerus duplication (Fig. 5B) and its parent (not shown) contained both chick and quail cells. This demonstrates that vlgFGF-infected donor cells can participate directly in forming duplications but does not indicate whether the role of donor cells is active or passive. In contrast, the duplication of digit 2 was composed primarily of chick cells. A small patch of quail cells was evident in the duplicated metacarpal 2 (Fig. 5D), and there were no quail cells visible in the duplicated phalanx (Fig. 5C). The parent digit 2 also contained a few widely scattered quail cells (data not shown). These data indicate that duplications need not contain large quantities of donor tissue per se. Instead, proximity to FGF-2-expressing donor cells may be sufficient to
beads incubated in 15 µM FGF-2 showed that, on average, amounts of FGF-2 within the beads are much lower; incorporation of radiolabeled FGF-2 into the beads incubated for 1 hour at 37°C in PBS containing 15 µM FGF-2 and was implanted at the anterior border of a stage 22 chick limb bud. At day 10, the wing was fixed, stained, and cleared. The bead is indicated by the arrow (B). Just proximal to the bead is a duplication of the metacarpal 2 of digit 2. Control beads incubated in buffer alone had no effect on limb morphology (data not shown). Partial duplications of digit 2 were observed in 20% (1/5) of limbs receiving beads incubated with 15 µM FGF-2 and 50% (4/8) of limbs receiving beads incubated with 50 µM FGF-2. No duplications were observed among 12 limbs receiving beads incubated with 5 µM FGF-2, nor among 19 limbs receiving beads incubated with FGF-2 at concentrations ranging from 0.5-2.5 µM.

**Effects of implanting beads containing FGF-2**

If, as suggested above, infected donor tissue serves as a source of FGF-2 that induces duplications of host tissue, then an acellular source of exogenous FGF-2 should also cause duplications. To test this hypothesis, we incubated Affigel Blue beads in FGF-2 and implanted them at the anterior borders of stage 20-22 limb buds. This treatment gave visible outgrowths within 12-24 hours (not shown) and resulted in partial duplications of digit 2 by day 10 (Fig. 6). Obtaining duplications required that the beads be incubated in a solution of ≥15 µM FGF-2. However, the amounts of FGF-2 within the beads are much lower; incorporation of radiolabeled FGF-2 showed that, on average, beads incubated in 15 µM FGF-2 for one hour take up only 0.5-1 pmol of FGF-2 and release the FGF-2 slowly over 4 days (Hayek et al., 1987; Fallon et al., unpublished data). Lower doses of FGF-2 initially gave outgrowths but failed to cause extra cartilage formation, presumably because of early exhaustion of the FGF-2 signal. This could explain the results of Hayamizu et al. (1991), where morphological changes were not observed in limbs implanted with FGF-soaked beads. The data shown here indicate that elevating extracellular FGF-2 levels, either from ectopic gene expression or from a slow release bead, is sufficient to induce host cells to form duplications.

**DISCUSSION**

**Mechanism of FGF-induced duplication**

We have shown that ectopic expression of FGF-2 in the chick limb bud causes duplications of (in order of decreasing frequency) proximal humerus, digit 2, distal radius, and digit 3. Only one other agent, retinoic acid (RA), has been reported to cause duplications in the limb bud (Tickle et al., 1982; Summerbell, 1983). However, two observations make it seem unlikely that FGF-2 affects patterning by the same mechanism as RA (i.e., by inducing a new axis). First, RA can respecify anterior tissue to form duplications of posterior elements whereas FGF-2 never gave duplications of posterior elements. Second, RA usually causes duplications of only the most distal elements and never causes duplications of the most proximal elements (Summerbell, 1983; Wilde et al., 1987; Tickle and Crawley, 1988). In contrast, FGF-2 frequently affects the humerus as well as digit 2. Thus, FGF-2, but not RA, can affect both proximal (older) tissue that has presumably undergone some differentiation and distal, relatively undifferentiated, tissue.

A mechanism that could accommodate these observations is one whereby FGF-2 stimulates growth of anterior limb tissue (Fig. 7). Specifically, ectopic expression of FGF-2 in the mesenchyme could lead to mitogenic expansion of prespecified skeletal primordia, which then regulate by splitting and forming two normally sized primordia. Occurrence of such a regenerative process is supported by the intermediate states of duplication of the radius shown in Fig. 2A,C and D. Evidence for involvement of a mitogenic pathway comes from several observations. First, regions of the limb bud showing the greatest mitogenic response to exogenous FGF-2 in vitro exhibit the highest duplication frequencies in vivo (Fig. 7 and Table 1; also cf. Aono and Ide, 1988). In both cases, FGF-2 responsiveness is strongest anteriorly and undetectable posteriorly. Second, vIgFGF-2-infected grafts caused host limbs to form prominent outgrowths within 36-48 hours (Fig. 3). The relationship between mitogenesis and skeletal duplications might be further tested by ectopically expressing other growth factors that are mitogenic for limb bud cells, such as IGF or PDGF (Aono and Ide, 1988; Ide, 1990), or possibly other members of the FGF family.

The differential responsiveness of anterior and posterior tissue to exogenous FGF-2 in vitro and in vivo suggests that a gradient or threshold of FGF signalling potential operates in the limb bud (Fig. 7). None of the FGF-binding proteins that have been examined to date, including two of four tyrosine kinase receptors (Peters et al., 1992, Orr-Urtreger et al., 1991), syndecan 1, a heparan sulfate proteoglycan (Solursh et al., 1990), or cysteine-rich FGF receptor (Burrus and Olwin, 1989; Olwin, unpublished observations) exhibit spatial distributions that might explain the observed pattern of FGF-2-responsiveness. However, post-translational modification of any of these proteins could generate a functional gradient. Alternatively, there could exist a concentration gradient of currently unknown extracellular, cytoplasmic, or nuclear factor(s) that are essential for FGF signal transduction.

While this study demonstrates the potential significance of an anteroposterior gradient or threshold of FGF-respon-
FGF affects limb pattern

Fig. 7. Distribution of FGF-responsiveness. (A) Duplication potential of skeletal primordia in stage 22 limb bud. Positions of primordia of the humerus (H), radius (R), ulna (U) and digits 2 (2), 3 (3), and 4 (4) are based on the fate map of Bowen et al. (1989). The dark superimposed curve marks the boundary between primordia that respond to FGF-2 by forming duplications (anterior) and primordia that do not (posterior). We propose that the boundary reflects a threshold or steep gradient of FGF signalling potential. (B) Mitogenic responsiveness of different regions of stage 22 limb bud. Tissue culture studies of Aono and Ide (1988) showed that cells taken from different anteroposterior levels of the limb bud are differentially responsive to exogenous FGF-2. Tissue from the anterior-most region shows a strong mitogenic response (++), middle regions show moderate responses (+), and the posterior-most region shows no response (−). The signalling threshold proposed in (A) accounts for the distribution of growth responsiveness. The intermediate mitogenic responses of middle regions of the limb bud reflect the existence of two subpopulations of cells, one that is fully responsive and one that is unresponsive to FGF-2.

...it does not rule out the possibility that FGF-2 plays a role in the posterior half of the limb bud. FGF might regulate development of posterior structures by a mechanism that is distinct from the anterior signalling pathway. Depending upon the nature of this regulation, over-expression of FGF-2 in the posterior half of the limb bud need not have any effect on limb morphology. Another way to test the role of FGF-2 in posterior regions of the limb bud would be to ectopically express sequences that disrupt FGF signal transduction (Burgess et al., 1990; Imamura et al., 1990; Amaya et al., 1991).

What is the role of FGF-2 in the limb bud?

It should be possible to deduce the function of FGF-2 in the limb bud by considering both the distribution of endoge-
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