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Journal

Developmental biology, 150(1)

ISSN

0012-1606

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Publication Date

1992-03-01

DOI

10.1016/0012-1606(92)90008-5

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Position Specific Growth Regulation of 3T3 Cells *in Vivo*

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Accepted November 26, 1991

In this study we have investigated the mechanism by which spatial growth is regulated by monitoring 3T3 cells, introduced into the developing mouse limb using *ex utero* surgery. The 3T3 cells were labeled with a human cell surface glycoprotein, CD8, and injected into stage 7-9 mouse limbs. At 24 and 48 hr after injection embryos were labeled with [³H]thymidine and processed for immunohistochemistry and autoradiography. The labeling index of CD8 positive cells was compared to that of neighboring limb bud cells and also to the position of the injection site within the limb. We find that the labeling index of 3T3 cells is in accord with that of the limb cells that immediately surround them; 3T3 cells display a high labeling index in limb regions of high growth and a low labeling index in limb regions of low growth. In addition, we find that both limb bud cells and injected 3T3 cells display a general proximal (low) to distal (high) gradient of growth at the stages analyzed. We conclude from these results that position-specific regulation of growth occurs in a non-cell autonomous manner and is likely to be mediated by mitogenic signals that are localized within the limb environment. In addition, our results demonstrate the usefulness of utilizing established cell lines as *in vivo* probes to monitor developmental mechanisms. © 1992 Academic Press, Inc.

INTRODUCTION

Much of vertebrate embryogenesis is characterized by well-defined spatial patterns of cell proliferation important for the formation and normal functioning of a wide variety of tissues. The developing limb, for example, is characterized by a highly reproducible pattern of cell proliferation; limb outgrowth occurs by distal growth, thus a general distal to proximal gradient of growth is observed with distal cells possessing a higher proliferation index than proximal cells (Summerbell and Wolpert, 1972). Such position-specific growth is observed in both developing and regenerating limbs, although little is known about how developmental growth patterns are regulated. Evidence from limb bud tissue grafting experiments suggests that cellular interactions, based solely on position, may play a role in the regulation of proliferation during limb development (French *et al.*, 1976; Cooke and Summerbell, 1980; see Bryant and Muneoka, 1986). The importance of positional interactions in the regulation of limb outgrowth is clearly illustrated by grafting experiments performed on the chick limb bud where supernumerary limbs form following tissue grafts of the posterior region of the limb (called the zone of polarizing activity, ZPA) into the anterior region (see Tickle *et al.*, 1975). A direct effect on the growth pattern of the limb bud has been demonstrated by Cooke and Summerbell (1980) based on [³H]thymidine uptake studies following ZPA grafts. Thus, the formation of supernumerary limbs following ZPA grafts is preceded by a local enhancement of the

normal pattern of limb bud growth. While a direct analysis of growth has not been carried out in other vertebrate limbs, similar grafting experiments to generate supernumerary limbs is documented in both regenerating and developing limbs of amphibians (for review see Bryant *et al.*, 1987) and also following interspecific ZPA grafts from mammals, reptiles, and other birds into the chick embryo (see Fallon and Crosby, 1977). In addition, experiments show that both a regenerative response and positional interactions resulting in the formation of supernumerary digits can occur *in situ* following embryo manipulations in the mouse (Wanek *et al.*, 1989a). These latter experiments show that the developmental window within which limb cells are able to regulate their pattern extends into later stages of limb development in the mouse embryo. Inherent in the observation that growth is stimulated as a result of positional interactions is the possibility that growth is inhibited or slowed as limb cells resolve positional discontinuities during development. Thus, one potential factor in considering the regulation of spatial growth patterns during normal limb development is the role of positional interactions.

The importance of growth regulation in the formation of the limb pattern has been recognized by some theoretical models for limb formation. For example, the progress zone model (Summerbell *et al.*, 1973) proposes that the apical ectodermal ridge maintains the distal tip of the limb bud in a positionally labile state (thus creating a "progress zone") and that positional identity along the proximal-distal axis is specified by a timing mechanism

where limb cells are thought to count cell divisions. As cells exit the progress zone proximally their positional value becomes fixed along the proximal-distal limb axis based on the number of divisions a cell incurred; few cell divisions specify more proximal structures whereas many cell divisions specify more distal structures. The maintenance of the progress zone itself is dependent on interactions with the apical ectodermal ridge; however, the way in which the proximal-distal pattern is specified in this model is with reference to an internal autonomous clock (Summerbell and Lewis, 1975).

An alternative view of limb formation is proposed in the polar coordinate model (French *et al.*, 1976; Bryant *et al.*, 1981). The important conceptual proposal of this model is the idea that cells interact with one another based on their positional identity or value and that cell proliferation is regulated by such interactions. Positional interactions that regulate growth is called intercalation. In this model limb cells are proposed to possess positional information within a polar coordinate system whereby a continuous gradient of positional information exists within the developing bud. Growth stimulation occurs as a result of interactions between cells with different positional values, with daughter cells adopting intermediate values. Growth inhibition occurs when cells with similar positional values interact. A variety of studies suggest that not all limb cells possess positional information; fibroblastic cells have been singled out as having the ability to interact in a positional manner (see Bryant *et al.*, 1987). These results have led to the proposal that fibroblastic cells within the limb bud initially establish a connective tissue scaffold of the limb pattern important for regulating subsequent growth and morphogenesis of the limb tissues (Bryant *et al.*, 1987). An important aspect of this view is that local positional interactions, of the type resulting in intercalary growth following tissue grafting, continue into later stages of development until the limb pattern is complete.

Although alterations in the spatial patterns of growth precede experimental changes in the pattern of differentiated cell types, the problem of how growth of the limb bud is spatially regulated during development has been largely ignored. This is in part due to the widespread belief that growth of limb bud cells is regulated in a cell-autonomous manner (see for example, Wolpert and Stein, 1984), and also because experimental approaches to study patterned growth have not been adequately developed. What little we do know about the role that position plays in the regulation of cell proliferation is derived from *in vitro* studies and the evidence to date suggests that there exist both cell autonomous and cell interactive regulatory mechanisms. Shi (1991) has shown that primary cultures of mouse limb bud cells exhibit position-specific growth differences; cells de-

rived from the posterior region of the limb bud proliferate at a much reduced level when compared to cells derived from the rest of the limb bud (anterior and central limb bud regions). Similarly, Aono and Ide (1988) demonstrated that fibroblast growth factor was more mitogenic for primary cultures of anterior chick cells than posterior cells. One possible interpretation of these results is that limb bud cells from different positions differ either qualitatively or quantitatively in receptors responsible for receiving mitogenic signals. This interpretation would argue for a cell autonomous component for the regulation of position-specific growth; growth could be regulated by the autonomous regulation of available receptors.

Evidence for a role for cell-cell interactions in the regulation of growth patterns is derived from studies in which cells from one limb region (e.g., the ZPA) can be shown to provide a mitogenic stimulus for cells from a different limb region. As described above, *in vivo* ZPA grafting studies demonstrate the presence of an anterior specific mitogenic signal produced by posterior limb bud cells. Similar growth enhancement of limb cells has been shown *in vitro* by medium conditioned by posterior tissue (Aono and Ide, 1988) and in experiments where anterior and posterior cells are cocultured (Shi, 1991). In addition, Bell and McLachlan (1985) have demonstrated that 3T3 cell proliferation can be influenced in a position-specific manner in coculture experiments with fragments of the early chick limb bud. In their study they tested fragments from four different positions of the limb (distal, proximal, anterior, and posterior) for their ability to stimulate 3T3 cell proliferation. Fragments from the distal region demonstrated the greatest ability to stimulate 3T3 cell proliferation, followed by posterior fragments, anterior fragments, and finally proximal fragments. Together these studies suggest that limb cells produce position-specific mitogenic activity that may be involved in the regulation of patterned growth of the limb bud.

In this paper we have extended the observations of Bell and McLachlan (1985) by assessing the proliferative activity of 3T3 cells introduced into the developing mouse limb. To do this we marked 3T3 cells with a replication-defective retrovirus carrying the cDNA for human CD8 (Calof and Jessell, 1986) and performed site-directed cell injections into the mouse limb using *ex utero* surgery (Muneoka *et al.*, 1990). The results from this study provide strong evidence that the proliferative rate of 3T3 cells becomes regulated by the limb environment in a position-specific manner that is in accordance with limb bud cells that immediately surround the injected 3T3 cells. We interpret these results to suggest that position-specific growth of limb cells themselves is regulated, at least in part, in a non-cell autonomous

manner by mitogenic signals localized within the limb environment. In addition, these data demonstrate that cells derived from a cell line that has been maintained *in vitro* for an extended time are still competent to respond appropriately to growth related signals present *in vivo*.

MATERIALS AND METHODS

Outbred Swiss Webster mice (CFW, Charles Rivers) bred at Tulane University were used in this study. Timed-pregnant mice were generated and developmental stages of mouse embryos were ascertained according to Wanek *et al.* (1989b). Embryos were operated on Embryonic Days (E) 13 and E14, corresponding to limb stages 7/8 and 9, respectively. *Exo utero* surgery was performed following procedures outlined in Muneoka *et al.* (1990). Embryo survival following cell injections was approximately 50%.

Cell lines used in this study included NIH 3T3 cells (ATCC) and a psi-2 virus producing cell line (psi-2-T8) derived by transfection of the pMV7-T8 expression construct into psi-2 cells (Maddon *et al.*, 1986, Calof and Jessell, 1986). In addition, a CD8 expressing 3T3 cell line (3T3CD8) was established by viral infection (see below). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. G418 (GIBCO, 1 mg/ml) was added to the medium for neomycin selection. The 3T3CD8 cell line was established by viral infection as follows. Conditioned medium derived from subconfluent cultures of psi-2-T8 cells was used to infect a subconfluent culture of 3T3 cells for 12–24 hr. The 3T3 cells were tested for expression of CD8 based on immunocytochemistry 24–48 hr after infection. All infections were performed in the presence of polybrene (8 μ g/ml, hexadimethrine bromide, Sigma). All experiments reported here were performed using cells derived from a single infection in which the percentage of cells labeled after infection was greater than 99%. This cell line is called 3T3CD8. There is no indication of down regulation of CD8 expression 3T3CD8 cells *in vitro*.

The 3T3CD8 cells were identified based on immunocytochemical staining using a monoclonal antibody OKT8 (American Type Culture Collection, Hoffman *et al.*, 1980), specific for the human CD8 surface protein. No cross-reactivity with either uninfected 3T3 cells or with mouse embryonic tissue was observed. Cultured cells were stained using the avidin/biotin immunoperoxidase method (ABC kit, Vector Laboratory) following the manufacturer's recommended protocols and using 3-amino-9-ethylcarbazole as the chromagen. Tissue sections were stained using biotinylated OKT8 to avoid nonspecific background staining associated with the indirect method. For biotinylating OKT8, antibodies were col-

lected in ascites fluid, purified using a protein A column (Bio-Rad), and biotinylated with long arm biotin (Vector Laboratory), in all cases following protocols recommended by the manufacturers. Prior to staining, cryosections (10 μ m) were fixed in 2.5% paraformaldehyde containing 10% picric acid for 10 min. Antibody staining was carried out at 37°C for 30 min (cultured cells) or at 4°C overnight (tissue sections).

Cells were prepared for injection into developing limb buds in the following manner. 3T3CD8 cells were collected using 1.0 mM EDTA in Ca^{2+} / Mg^{2+} -free Hanks' balanced salt solution (HBSS) and concentrated to 5×10^6 cells/ml in HBSS containing 40 mg/ml bovine serum albumin. Fluorescent beads (Fluoresbrite, 0.88–2.0 μ m, Polyscience) were added to the cell suspension to identify the injection site both during the operation and during cryosectioning. This cell suspension was loaded into a glass needle which was used to perform the site-directed injection into the limb bud. Injections were made from the dorsal aspect into soft tissue at various proximal-distal positions of the zeugopodial region of the hind limb. Experimental limbs were collected 24 and 48 hr following cell injection and cell proliferation was measured based on the incorporation of [^3H]thymidine (Cooke and Summerbell, 1980). For collecting limbs, the female mouse was anesthetized and prepared for *ex utero* surgery. Experimental embryos were located and 10 μ Ci of [^3H]thymidine (sp act = 33–64 Ci/mmol) was administered by intraperitoneal injection into each embryo 30 min prior to the collection of the tissue. During the incorporation period the female mouse remained anesthetized and embryos were kept moist with lactated ringers. After the incorporation period embryos were checked to ensure viability and the experimental limb was collected and processed for cryosectioning as follows. Experimental limbs were immediately frozen by submersion into liquid nitrogen. Frozen limbs were embedded in O.C.T. compound (Miles, Inc.) at -20°C and either sectioned immediately or stored at -20°C for no longer than 24 hr.

Serial transverse sections (10 μ m) of experimental limbs were collected at 60 μ m intervals, stained with OKT8 to reveal the location of 3T3CD8 cells and processed for autoradiography. Sections were counterstained with hematoxylin to identify individual nuclei. Cell counts were made on every section which contained 3T3CD8 cells. For each injection site ($n = 19$) all 3T3CD8 cells were counted and the labeling index based on [^3H]thymidine incorporation was determined. To determine the labeling index of the neighboring limb cells, all limb cells that were found within a 40- μ m perimeter surrounding the 3T3CD8 injection site were analyzed.

To control for the cell autonomy of the replication-defective retrovirus labeling method employed, we per-

formed three separate biological tests for gene transfer in our 3T3CD8 cell line. First, we assayed conditioned medium from 3T3CD8 cells for their ability to cause CD8 expression in uninfected 3T3 cells using protocols identical to that used for our original infection. In three separate experiments we found no evidence of CD8 expression in our uninfected 3T3 cells treated with 3T3CD8 conditioned medium. In a second series of control experiments, uninfected 3T3 cells were prelabeled with [^3H]thymidine and cocultured with 3T3CD8 cells at either micromass densities (Ahren *et al.*, 1977) or in monolayer cultures. Cocultures were done in 10% fetal bovine serum with either 90% 3T3CD8 cells/10% 3T3 cells or 80% 3T3CD8 cells/20% 3T3 cells. At 48 hr, cultures were trypsin-dissociated into single cells and replated for 1 hr at a lower density prior to immunocytochemical staining and processing for autoradiography. Replating cells at a lower density allowed cell counts to be done at a density where no ambiguity existed as the result of multiple layers of cells. For micromass cocultures a total of 5842 cells in 5 different trials was counted with no evidence of a single double-labeled cell. Similarly, for monolayer cultures a total of 4811 cells was counted in 4 different trials and in all cases there was no evidence of double-labeled cells. We conclude from these biological assays that it is highly unlikely that the marker CD8 gene is being transferred to limb cells in our *in vivo* studies.

RESULTS

A total of 19 injection sites from 5 experimental limbs were analyzed for [^3H]thymidine incorporation into 3T3CD8 cells as well as neighboring limb cells (Figs. 1a-1g). Most of the sites (16) were analyzed 24 hr postinjection with the remaining 3 sites analyzed 48 hr after injection. Nine injections were made into stage 7/8 limb buds and the remaining 10 injections were made into stage 9 limbs. Differences in terms of position-specific growth were not observed either between the two time points or between the limb stage injected, thus the data were compiled into a common data base for analysis. The 3T3CD8 cells were found in clusters of varying size located in the soft (muscle-forming) tissues between the peripheral ectoderm and the central chondrogenic region. In this study cell counts were made only in this soft tissue and not in chondrogenic or ectodermal regions. The total number of 3T3CD8 cells counted per injection site varied from a low of 40 cells spanning 120 μm along the proximal-distal limb axis (2 adjacent sections) to a high of 2047 cells spanning the entire proximal-distal length of the zeugopodium (960 μm , 16 adjacent sections). Variation in the size of the injection site (based on the total number of 3T3CD8 cells scored) did not in-

fluence positional growth differences within the limb (see below, Fig. 3c).

When the growth rates of 3T3CD8 cells are compared to their respective neighboring limb bud cells for all 19 injection sites, we find a relationship which demonstrates that 3T3CD8 cells are responding to the limb environment in a manner that is identical to that of endogenous limb cells (Fig. 2). In other words, the labeling index of 3T3CD8 cells is in accordance with that of the limb cells that immediately surround them; 3T3CD8 cells injected into regions of high limb cell growth have a high labeling index, whereas 3T3CD8 cells injected into regions of low limb cell growth have a low labeling index. This correlation covers more than a threefold variation in labeling index and is statistically significant. The labeling index of 3T3CD8 cells scored within the 19 injection sites varied from a low of 7.6 to a high of 36.6%. Similarly, the labeling index of the limb cells surrounding the 3T3CD8 injection sites varied from 8.2 to 23.6%. These data show that within 24 hr after their introduction into the limb bud, 3T3CD8 cells alter their growth rates to correspond to those of their neighboring limb cells. We conclude that when 3T3CD8 cells are placed into limb regions of high or of low growth they appear to regulate their own growth in accordance with the limb environment.

While the above analysis clearly demonstrates that 3T3CD8 cells are responsive to the limb environment, it does not directly address whether their growth is regulated by positional signals that might control the reproducible patterns of cell proliferation witnessed during limb development. Since variation in growth rate along the proximal-distal limb axis has been reported for earlier staged chick limb buds (Summerbell and Wolpert, 1972), it seemed logical to investigate whether the growth differences we observed for 3T3CD8 cells correlated with position along the proximal-distal limb axis. In order to address this issue, we have further analyzed a subset of our data in which the relative position of injection sites within a single limb could be accurately determined. This was carried out in two different ways, first, by the analysis of a single limb which received multiple injections into different regions along the proximal-distal limb axis and, second, by the analysis of a single limb in which the injection site spanned the entire proximal-distal length of the zeugopodium.

Our results from an analysis of multiple injection sites within a single limb provide evidence that the variation of 3T3CD8 cell growth is indeed related to their position along the proximal-distal axis of the limb. Figure 3a shows the growth rates of 3T3CD8 cells and neighboring limb cells from a limb in which eight injection sites were analyzed. The data are plotted relative to the position of the midpoint of each injection site along the

FIG. 1. Bright field (b, d) and phase contrast (a, c) micrographs of 3T3CD8 cells (c, d) and uninfected 3T3 cells (a, b) stained with mAb OKT8 ($\times 186$). (e) Micrograph of an E13 mouse embryo prepared for *exo utero* surgery. The left hindlimb (stage 7–8) is shown protruding through an incision in the extraembryonic membranes and the zeugopodium has received three injections of 3T3CD8 cells. Fluorescent polystyrene beads (Fluoresbrite, Polyscience) were coinjected with the 3T3CD8 cells to help visualize the injection site ($\times 18$). (f, g) Bright field micrographs of the same transverse section of limb tissue at slightly different focal planes showing CD8 labeled cells (f) and [^3H]thymidine labeled cells (g). The section was counterstained with hematoxylin to identify individual nuclei. The arrows in f and g indicate an example of a double-labeled 3T3CD8 cell ($\times 744$).

proximal–distal axis and ignoring differences in the position of injection sites along the other limb axes (which are not necessarily constant). The data show a general (but certainly not perfect) trend in which a proximal to distal increase in labeling index of both limb and 3T3CD8 cells is observed. A comparison of labeling index between 3T3CD8 cells located in the most proximal and the most distal limb regions show that they are statistically significant ($P < 0.001$, χ^2 , 2×2 contingency table) with proximal regions displaying a lower labeling index than distal. Similarly, a comparison of labeling

index between the limb cells surrounding the most proximal and most distal 3T3CD8 injection site show that they are statistically different ($P < 0.001$, χ^2 , 2×2 contingency table) with proximal regions having a lower labeling index than distal. Thus, we observe a similar proximal–distal difference in labeling index within the zeugopodium for both limb cells and 3T3CD8 cells.

To further assess the position-specific growth response of 3T3 cells, we have analyzed in greater detail a limb which received a single large injection. This injection site spanned the entire proximal–distal extent of

FIG. 2. The labeling index of 3T3CD8 cells from 19 separate injection sites is shown plotted versus the labeling index of limb cells surrounding each injection site. The solid line indicates correlation observed between 3T3CD8 and limb cell labeling indices. The dash line indicates the predicted response for a perfect 1:1 correlation.

the zeugopodium and corresponds positionally with the injection sites shown in Fig. 3a. Since our analysis was carried out on transverse sections of the zeugopodium, we were able to analyze separate subpopulations of 3T3CD8 cells that were located at different proximal-distal levels. We have arbitrarily broken this injection site into 8 equally spaced regions of 120 μm although the statistical significance of our results is not altered by this choice. In other words, the proximal-distal difference in labeling index is also significantly different when the injection site is fragmented into fewer equally spaced regions along the proximal-distal extent of the zeugopodium (not shown). When position-specific subpopulations of this large injection site are compared we find that the labeling index of 3T3CD8 cells located in the most distal limb region are significantly different than that of the most proximal region ($P < 0.001$, χ^2 , 2×2 contingency table) with proximal regions displaying a lower labeling index than distal. Similarly, a comparison of labeling index between the limb cells surrounding the most proximal and the most distal 3T3CD8 injection sites shows that they are statistically different ($P < 0.001$, χ^2 , 2×2 contingency table) with proximal regions having a lower labeling index than distal. Thus, we find that even within a single contiguous population of 3T3CD8 cells, the growth rates of smaller subpopulations display a position-specific variation that correlates with a general proximal-distal pattern of growth (Fig. 3b). Taken together, the data shown in Figs. 3a and 3b support the idea that the proliferation of 3T3 cells is being regulated in a position-specific manner by signals that are localized within the limb environment, possibly the same signals that act on endogenous limb cells. In

addition, the fact that individual injection sites, or subpopulations of a large injection site, within a single limb display positional variation in labeling index eliminates the possibility that variation in growth observed among injection sites in different embryos is an artifact due to individual differences in [^3H]thymidine uptake.

An interesting aspect of the data is that in general the 3T3CD8 cell growth response is higher than the growth rate of the neighboring limb cells. The fact that the vast majority of data points shown in Fig. 2 lie above a line indicative of perfect regulation between limb bud and 3T3CD8 cell growth (dashed line) reflects this general trend. This enhanced response is also evident in the position related growth stimulation shown in Figs. 3a and 3b. These data suggest that 3T3CD8 cells are more responsive to positional growth signals than are limb cells. One reason for this difference may be related to the fact that terminal differentiation of limb cells (at least chondrogenic and myogenic cells) are occurring during these limb stages (7-8), thus not all limb cells are growth competent whereas 3T3CD8 cells are expected to be uniformly growth competent.

To investigate the possibility that injected 3T3 cells might be affecting the normal growth pattern of limb cells, we have compared the growth rate of limb cells in relation to the relative size of each injection site. This analysis is based on the assumption that the effect of 3T3 cells on limb cell growth would increase with increasing numbers of 3T3 cells; thus, a negative effect should give lower limb cell growth rates as the number of injected 3T3 cells increases, and a positive effect should result in higher growth rates with increasing injection size. Instead, we find no evidence that the injected 3T3 cells alter the growth of endogenous limb cells in either a positive or a negative manner (Fig. 3c). In fact, we find that with increasing injection size the labeling index of limb cells tend not toward extremely low or high values but toward intermediate values, a result that was predicted given the fact that subpopulations within a single injection site display position-specific growth rates (Fig. 3b). These results are consistent with the idea that the introduced 3T3CD8 cells are not modifying the normal pattern of limb cell growth, but are merely receptive to growth signals distributed in the limb in a position-specific manner. This conclusion is further supported by the observation that similar cell injections result in 3T3CD8 cell integration into neonate limb of completely normal morphology (Trevino *et al.*, unpublished data).

Although the nature of position-related growth regulatory signals present in the developing limb is unknown, one possible explanation for our results is that 3T3CD8 cell growth *in vivo* may be influenced by differences in cell density within the limb environment and

FIG. 3. (a) The labeling index of 3T3CD8 cells and surrounding limb cells is plotted versus the position along the proximal-distal (zeugopodium) limb axis for a single limb which received eight injections. Error bars are provided for both limb cells labeling index (down) and for 3T3 cells labeling index (up). (b) Histogram showing the labeling

are therefore displaying an *in vivo* form of contact inhibition. A role for contact inhibition during chick limb development has been suggested by Summerbell and Wolpert (1972) based on studies demonstrating an inverse relationship between cell density and mitotic index. For this reason we determined the density of 3T3CD8 and limb cells for proximal ($n = 1$), middle ($n = 3$), and distal ($n = 2$) sites which display low, intermediate, and high levels of growth, respectively. The results from this sampling showed that the measured 3T3CD8 cell density was invariant; proximal (low growth) density was 5.6 cells/1000 μm^2 , middle (intermediate growth) density was 5.7 cells/1000 μm^2 , and distal (high growth) density was 5.5 cells/1000 μm^2 . Similarly, the density of limb cells surrounding these sites was determined and found to be invariant; proximal tissue (low growth) was found to have a density of 6.7 cells/1000 μm^2 , middle tissue (intermediate growth) was found to have a density of 6.6 cells/1000 μm^2 , and distal tissue (high growth) displayed a density of 6.4 cells/1000 μm^2 . We conclude from this study that the position-specific growth we have observed cannot be attributed to differences in cell density.

DISCUSSION

In the present study we have developed a novel approach to probe for developmental signals present during embryogenesis and we provide evidence for the existence of spatially localized signals that can regulate the proliferative rate of exogenously introduced 3T3 cells during the development of the mouse limb. The strength of this conclusion lies in the observation that 3T3 cell proliferation correlates with the proliferation rates of neighboring limb bud cells for a number of injection sites into different positions within the limb bud (Fig. 2). Further analyses revealed general trends along the proximal-distal limb axis with distal injection sites displaying a higher proliferative index than proximal sites. We interpret these results to suggest that the growth signals regulating 3T3 cells are indeed involved in the *in vivo* control of position related growth observed in the developing limb. The fact that we observed this regulation of 3T3 cell growth within 24 hr following their injection suggests that this effect is likely to be mediated by mitogenic activity present in the developing limb tissue rather than by some indirect mechanism. Indeed, *in*

index of 3T3CD8 cells and surrounding limb cells for eight proximal-distal levels (120 μm intervals) of a single large injection site which spanned the entire proximal-distal extent of the zeugopodial region (approximately 1 mm). (c) The labeling index of limb cells surrounding each injection site is shown plotted against the relative size of the corresponding 3T3CD8 injection site.

vitro studies of 3T3 cell growth stimulation by medium conditioned by region-specific explants of early chick limb buds support this conclusion (Bell and McLachlan, 1985). In addition, we have observed a similar proximal-distal difference in mitotic activity of dissociated limb cells on 3T3 cells in cultures of later stage mouse limbs (stages 7–8); position specific stimulation of 3T3 cell growth occurs in limb/3T3 cocultures in defined medium (Trevino, unpublished data). Thus, we conclude that position-specific growth in developing limb cells is, at least in part, regulated by the availability of localized mitogenic signals present within the limb environment.

Obvious candidates for a role in the regulation of patterned growth during limb development are peptide growth factors. A number of known factors have been shown to be present within the limb during development. Of these, basic fibroblast growth factor (bFGF) and the transforming growth factor- β (TGF- β) superfamily of factors have been characterized (Lyons *et al.*, 1989; Manaim *et al.*, 1988; Pelton *et al.*, 1989, 1990; Seed *et al.*, 1988). The presence of FGF within the developing limb has been reported by a number of investigators (Manaim *et al.*, 1988; Seed *et al.*, 1988) and there is some evidence indicating temporal fluctuations in FGF levels during limb development although the meaning of these fluctuations remains unclear (Manaim *et al.*, 1988). In addition bFGF has been spatially localized to muscle-forming regions based on immunohistochemical analyses of the developing chick limb (Joseph-Silverstein *et al.*, 1989). bFGF is also known to be a potent mitogen of myoblasts *in vitro* and thus also has the ability to inhibit or delay myogenesis (Gospodarowicz, 1974; Linkhart *et al.*, 1980, 1981). Since bFGF is known to be a strong mitogen for 3T3 cells as well, one possible interpretation of our results is that the availability of bFGF is graded along the proximal-distal axis. This interpretation deserves serious consideration since our injection sites were localized in tissues within which muscle morphogenesis was occurring in a proximal to distal sequence (Shellswell, 1977). Although a detailed analysis of the distribution of bFGF along the proximal-distal axis has not been performed, we can estimate relative bFGF levels required to account for our fourfold change in labeling index (Fig. 2) based on the mitogenic activity of bFGF on 3T3 cells *in vitro*. Based on the published effective range of bFGF on 3T3 cell proliferation *in vitro* (Gospodarowicz, 1974), we estimate that a five- to eightfold change in bFGF levels between proximal (low) and distal (high) regions of the zeugopodium alone could account for our results. Whether such a gradient exists within the developing limb is at present unknown.

Regardless of the specific mitogens that are affecting 3T3 cell growth in our studies, the observed position-specific growth patterns lead us to suggest that cell pro-

liferation within specific tissues of the limb are regulated by quantitative rather than by qualitative differences in factor production and/or availability. This can be concluded because 3T3 cells represent a constant monitoring system for mitogenic activity and are not expected to possess receptor levels that would vary with the position of injection sites. Thus, it is not necessary to evoke qualitative or quantitative variation in mitogenic receptor levels of limb cells within a specific tissue to explain proximal-distal growth patterns during development. Further, there is evidence to suggest that different tissue types that develop side by side utilize qualitatively different factors to regulate growth and differentiation. For example, whereas bFGF is immunolocalized to muscle (Joseph-Silverstein *et al.*, 1989) and is a potent regulator of myoblast growth and differentiation (Olwin and Hauschka, 1988), there is evidence that the TGF- β -like factors are localized to chondrogenic regions (Pelton *et al.*, 1989, 1990) and similarly regulate chondrogenesis (Kulyk *et al.*, 1989a; Hayamizu *et al.*, 1991). We suggest that in later stages of limb formation tissue-specific growth is regulated qualitatively by different mitogens, and that within a specific tissue forming region (e.g., along the proximal-distal axis), patterned growth is regulated by quantitative differences in mitogen availability.

An alternative interpretation of our results is that 3T3 cell proliferation patterns result not from specific mitogenic stimulation but rather from possible nonspecific effectors of growth such as cell density, proximal-distal differences in nutrient availability (i.e., differential vascularization), or cell substrate (i.e., extracellular matrix). Of these we have investigated the simple explanation is that 3T3 cells are responding to position-related variations in cell density that has been reported by Summerbell and Wolpert (1972) and thus are displaying an *in vivo* form of contact inhibition, a well-established characteristic of these cells *in vitro* (Todaro, 1963). However, we do not observe any relationship between growth and cell density in our analysis of either 3T3CD8 cells or limb cells, thus we cannot attribute the position-related variation in the observed labeling index of 3T3CD8 cells or limb cells to differences in cell density within the developing limb. The possibility that the patterned growth of 3T3CD8 cells we observe may be attributed to other nonspecific factors (i.e., vascularization or extracellular matrix) seems unlikely because similar position-related stimulation of 3T3 cell growth has been demonstrated *in vitro* (Bell and McLachlan, 1985; Trevino, unpublished data) under conditions where nutrient availability and substrate are kept constant.

In this study we employed the use of a replication defective retrovirus as a vehicle to label 3T3 cells with the CD8 human cell surface glycoprotein (Perlmutter,

1989). Although it seems unlikely, the possibility exists that CD8 itself may be influencing the positional growth response of 3T3 cells. CD8 is normally expressed on human T cells and is associated with MHC class I protein recognition (Perlmutter, 1989). The CD8 protein is believed to form part of the antigen recognition complex that includes the α/β heterodimer of the T-cell receptor and the CD3 complex (Saizawa *et al.*, 1987). The involvement of CD8 in the T-cell-signaling pathway is still unclear, however, a lymphocyte-specific membrane-associated protein tyrosine kinase (p56^{lck}) has been implicated as being the first phosphorylated protein subsequent to CD8 activation (Perlmutter, 1989). With regard to our use of CD8 as a cell marker for 3T3 cells, we point out that 3T3 cells do not express the α/β heterodimer or the CD3 complex of the T-cell receptor (Marth *et al.*, 1988). In addition, 3T3 cells do not express p56^{lck} which is important for signal transduction. Thus, we conclude that it is highly unlikely that the constitutive expression of CD8 is affecting in any way the *in vivo* response of 3T3 cells.

The system we have developed takes advantage of the efficiency of retroviral gene transfer *in vitro* combined with the ability to introduce modified cells into the embryonic environment using *ex vivo* surgical procedures. In this study we found no indication that normal limb growth patterns are adversely affected by the presence of 3T3 cells; therefore, we conclude that 3T3 cells are behaving in a developmentally neutral manner. Thus, for studies of limb development in the mouse, 3T3 cells represent potential probes to monitor or modify cell-cell interactions *in vivo*. Although we employed *ex vivo* surgical procedures to make use of a well-characterized cell line, this approach is equally valid for experimentally probing other systems. However, our attempts to expand this approach by performing xenotypic cell injections (i.e., 3T3 cells into the chick embryonic limb bud) were unsuccessful (Trevino, unpublished data). Thus, the utilization of cell lines to experimentally monitor embryogenesis may be restricted to systems for which cell lines are available.

We thank Dr. Richard Axel for the pMV7T8 vector. We thank Rosalie Anderson, Philip Brylski, Susan Bryant, David Gardiner, Terry Hayamizu, Arthur Lander, Changping Shi, and Nancy Wanek for their helpful discussions. This work was supported by grants from the NIH (HD20662, HD23921), Louisiana State Board of Regents (BRSG S07RR07040), and the Monsanto Co.

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