Position Specific Growth Regulation of 3T3 Cells in Vivo

CHRISTOPHER TREVINO, ANNE CALOF,* AND KEN MUNEOKA

Department of Cell and Molecular Biology, Tulane University, New Orleans, Louisiana 70118; and *Department of Biology, University of Iowa, Iowa City, Iowa 52242

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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 exo 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.

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 \textit{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 derived 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, \textit{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 \textit{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 \textit{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 \textit{3T3} cell proliferation. Fragments from the distal region demonstrated the greatest ability to stimulate \textit{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 \textit{3T3} cells introduced into the developing mouse limb. To do this we marked \textit{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 \textit{ex utero} surgery (Muneoka et al., 1990). The results from this study provide strong evidence that the proliferative rate of \textit{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 \textit{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 (Madden 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 (GIRCO, 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 μg/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 immunochemical 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 reveal the location of 3T3CD8 cells and processed for cryosectioning as follows. 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 exo utero surgery. Experimental embryos were located and 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 exo utero surgery. Experimental embryos were located and 10 μCi of [3H]thymidine (sp act = 33-64 Ci/mmole) 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 μm) of experimental limbs were collected at 60 μm 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-μm 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 $[^3]H$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 $[^3]H$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 $\mu$m 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 $\mu$m, 16 adjacent sections). Variation in the size of the injection site (based on the total number of 3T3CD8 cells scored) did not influence 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
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 ($P < 0.001$, $\chi^2$, $2 \times 2$ 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
find that the labeling index of 3T3CDS cells located in
when the injection site is fragmented into fewer equally

... that are localized within the limb environment, possibly

... correlation observed between 3T3CD8 and limb cell labeling indices. The dash line indicates

... being regulated in a position-specific manner by signals

... the zeugopodium and corresponds positionally with the

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... cell density within the limb environment and

... the fact that individual injection sites, or sub-

... 3T3CD8 cell growth in vivo may be influenced by differences in [3H]thymidine uptake.

An interesting aspect of the data is that in general the

... refined for a perfect 1:1 correlation.

... further supported by the observation that similar cell

... completely normal morphology (Trevino et al.,

... a positive manner (Fig. 3c). In fact, we find that with increasing injection size the

... the limb in a position-specific manner. This conclusion is

... 3b support the idea that the proliferation of 3T3 cells is

... 3T3CD8 cell growth in vivo may be influenced by differences in cell density within the limb environment and
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 μm², middle (intermediate growth) density was 5.7 cells/1000 μm², and distal (high growth) density was 5.5 cells/1000 μm². 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 μm², middle tissue (intermediate growth) was found to have a density of 6.6 cells/1000 μm², and distal tissue (high growth) displayed a density of 6.4 cells/1000 μm². 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
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 proliferation 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 (Saiizawa 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\(^{ck}\)) has been implicated as being the first phosphorylated protein subsequent to CD3 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\(^{ck}\) 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 exo utero 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 exo utero 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 (Treviso, unpublished data). Thus, the utilization of cell lines to experimentally monitor embryogenesis may be restricted to systems for which cell lines are available.

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