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BRIEF NOTES

Temporal Pattern of Posterior Positional Identity in Mouse Limb Buds

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This study describes the temporal pattern of posterior positional identity in mouse limb bud cells. To do this wedges of tissue from the posterior edge of mouse limb buds at various stages (limb stages: Wanek *et al.*, 1989b. *J. Exp. Zool.* **249**, 41–49) were grafted to the anterior edge of a host chick embryo wing bud. Grafts of mouse posterior cells are able to induce the formation of supernumerary digits every time when they are taken from buds from stage 3 through stage 6. At stage 7, the frequency declines and by stage 8 the chick cells no longer respond. The results indicate a change in tissue properties at stage 7, which progresses by stage 8 to the point at which posterior positional identity is no longer detectable by this assay. These temporal changes in this aspect of limb pattern formation can be used as an additional criterion to guide the identification of genes involved in the specification of posterior positional identity. © 1991 Academic Press, Inc.

INTRODUCTION

A major conclusion from studies of pattern regulation in vertebrate limbs is that cells in different positions within the developing limb bud are nonequivalent (Lewis and Wolpert, 1976) in terms of their positional properties. This is most dramatically demonstrated when cells from different positions are brought into contact by grafting, the result of which is the stimulation of growth, the insertion of new pattern between the confronted cells, and the restoration of local continuity in the array of positional states or identities (French *et al.*, 1976; Bryant *et al.*, 1981).

The identification of the genes involved in the specification of positional identities and in the cell interactions leading to the formation of *de novo* pattern is a recognized goal of research in limb development and regeneration. Several recent studies have described the temporal and spatial expression domains of candidate pattern formation genes in the limb buds of developing mice (Oliver *et al.*, 1988, 1989; Dollé and Duboule, 1989; Dollé *et al.*, 1989a,b; Perez-Castro *et al.*, 1989). The relevance of particular genes to limb pattern formation could be determined if their expression patterns could be interpreted with reference to experimental data about the temporal and spatial distribution of cells that display (or respond to) a given positional identity. Un-

fortunately, very little is known about crucial aspects of mouse limb development due to the inaccessible mode of embryo development within the uterus. It has been possible using *exo utero* surgical procedures to demonstrate that mouse limb cells are able to respond to anterior-posterior confrontations by forming extra digit tips and to limb bud tip amputation by regenerating the distal parts of the peripheral digits (Wanek *et al.*, 1989a). However, a detailed functional determination of the temporal and spatial distribution of cells with particular positional identities (e.g., anterior or posterior) is not at present technically feasible.

In the chick, it has been possible to map limb bud cells with posterior qualities (the zone of polarizing activity or ZPA) by grafting cells from different stages and positions to a more anterior site (MacCabe *et al.*, 1973; Honig and Summerbell, 1985). Cells that stimulate the formation of extra digits are defined as showing posterior positional identity. Several years ago it was reported (Tickle *et al.*, 1976; Fallon and Crosby, 1977) that posterior cells from mouse limb buds are also effective in causing the formation of extra digits when grafted to the anterior of a chick wing bud. Other authors have reported similar results with posterior cells from the limb buds of other mammals and from reptiles (MacCabe and Parker, 1976; Fallon and Crosby, 1977; Honig, 1984). We have made use of the ability of mouse posterior cells to elicit a supernumerary response after heterospecific grafting to the chick wing bud to map the temporal pattern of active posterior positional identity in the developing mouse limb bud. The temporal pattern

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that we describe provides a means by which to evaluate the significance of genes thought to be involved in the specification of posterior positional identity.

MATERIALS AND METHODS

Swiss Webster mice (Simonson) were maintained at a constant temperature ($21 \pm 1°C$) with 12 hr of light from 7 AM–7 PM. Mated females were checked for vaginal plugs each morning, and plug day was designated Embryonic Day 1 (E1). E11–E13 embryos were staged according to Wanek et al. (1989b) after having been placed in Hanks' balanced salt solution on ice.

White Leghorn chicken eggs were obtained from K&R Industries (Westminster, CA) and incubated in a humidified incubator at 38°C prior to use. Shells were windowed, and embryos were staged according to Hamburger and Hamilton (1951). Three drops of antibiotic cocktail (0.25 mg/ml streptomycin sulfate and 0.25 mg/ml penicillin G) were added, and grafted embryos were incubated for a further 6 days.

A wedge of donor tissue (200–250 μm at the periphery) was removed from mouse limb buds of stages 3 to 8 using sharpened tungsten needles. Wedges were taken from the undifferentiated region immediately beneath the apical ectodermal ridge on the posterior side of the limb tip (i.e., the posterior edge of the progress zone). The location of donor pieces at each stage is shown in Fig. 1. Prior to excision, a spot of colloidal carbon was microinjected to facilitate orientation of the wedge during grafting.

The donor wedges were inserted into slits made in the anterior margin of stage 20–21 host chick embryos. No host tissue was removed; when necessary (20% of cases) a small tungsten tack was used to hold the graft in position. On the day after grafting, host embryos were examined for retention of the graft, and tacks, if any, were removed.

Six days after grafting, host limbs were collected, fixed in Bouin's, and stained for whole mount skeletal analysis with Victoria Blue (Bryant and Iten, 1974). Limbs were examined to determine the number and identity of any supernumerary digits.

The results were expressed in terms of both the frequency of limbs at a given stage that produced extra digits and the magnitude of the supernumerary response. The latter index is a modified version of the strength of activity index described previously (Honig et al., 1981; Honig and Summerbell, 1985). In our analysis, if the anteriormost supernumerary digit (excluding digits that formed as part of a secondary axis anterior to the graft itself) was a digit 4, 3, or 2 then the limb was scored as a 3, 2, or 1, respectively. For example, at the extremes, limbs with the digit pattern 4-3-2-2-3-4
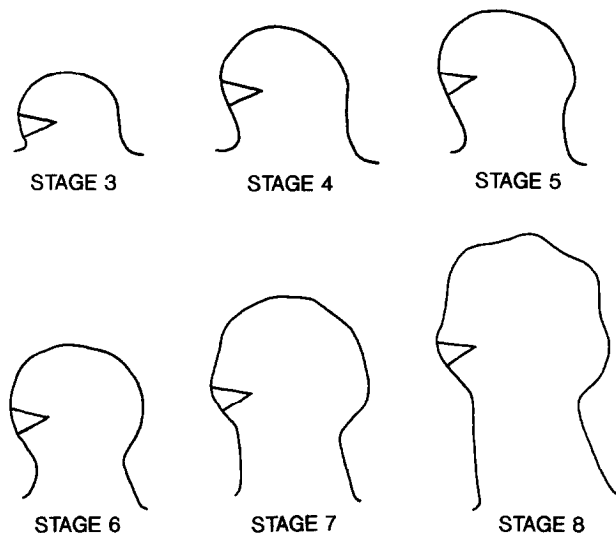

FIG. 1. Mouse limb buds at stages 3–8 showing the position of the donor posterior wedges. Drawings are of left limb buds with anterior to the right.

would score as 3; limbs with one supernumerary digit (2-2-3-4) were scored as 1. In those cases where the anteriormost digit was not clearly identifiable, the limb score was based on the adjacent identifiable digit plus 0.5 (e.g., ?-3-2-2-3-4 was scored as 2.5).

In some cases supernumerary structures formed anterior to the position of the graft and formed a secondary axis typically consisting of a humerus, a forearm element, and one or two digits. An additional 0.5 was added to the limb score for a secondary axis, since the formation of a secondary axis was also a consequence of the presence of adjacent graft cells with posterior positional identity. Scores of greater than 3 were not assigned. The strength of the stimulus to form supernumerary structures was calculated for each stage by dividing the sum of the scores for the individual limbs with extra digits by the sum of the maximum possible scores (number of limbs with extra digits × 3).

RESULTS

A total of 49 host chick limbs were analyzed for the presence of supernumerary digits induced by wedges of posterior cells from mouse limb buds grafted to the anterior margin. The positions from which the donor wedges were removed from stage 3 through 8 (Wanek et al., 1989b) limb buds are shown in Fig. 1. In all limbs with extra digits, the supernumerary digits were well differentiated and were always of chick phenotype. Examples of limbs with supernumerary digits are shown in Fig. 2.

The frequency and degree to which posterior mouse cells are able to stimulate a supernumerary response in anterior chick cells decline as a function of mouse limb
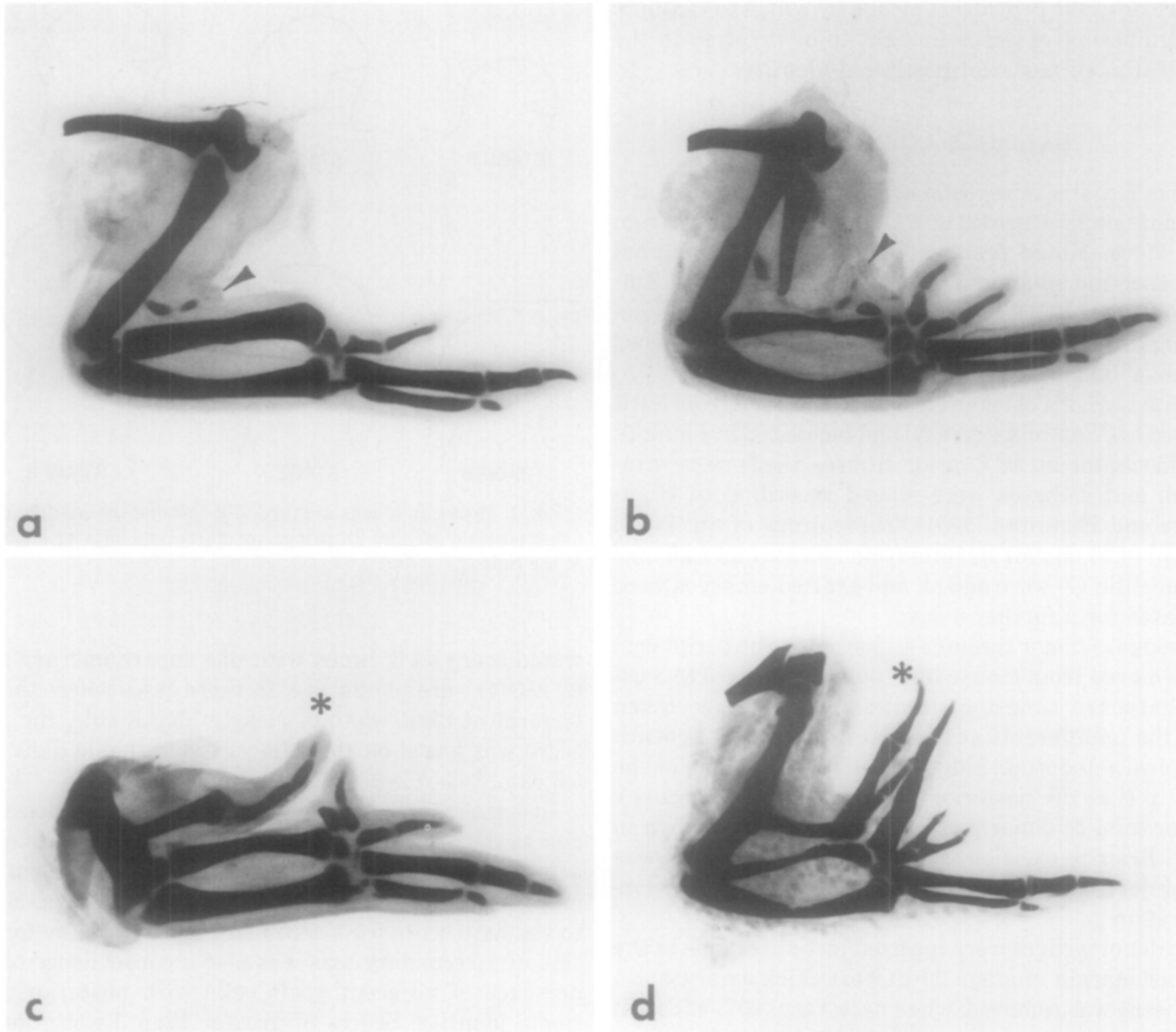
FIG. 2. Whole mount skeletal preparations of host chick limb buds 6 days after posterior mouse wedges were grafted to an anterior site. The score assigned to the limbs is a measure of the magnitude of the supernumerary response (see Materials and Methods). Arrowheads indicate the cartilages that we interpret as being derived from mouse tissue (presence of carbon marks) and asterisks indicate digits that formed anterior to the graft. (a) Limb that had received a stage 8 mouse posterior graft, showing a normal pattern of chick digits (2-3-4). The mouse tissue has formed small cartilages (arrowhead). (b) Limb with a single supernumerary digit (2-2-3-4; score = 1). Limb had received a stage 5 posterior mouse graft. (c) Limb with one supernumerary digit on the posterior side of the graft and one induced anterior to the graft (3/2-2-3-4; score = 1.5). Limb had received a stage 7 posterior mouse graft. (d) Limb with two extra digits posterior to the graft and two anterior to it (2-3/3-2-2-3-4; score 2.5). Limb had received a stage 4 posterior mouse graft.

bud stage (Table 1). All limbs receiving grafts from stage 3 to 6 mouse limbs developed one (38%) or more (62%) extra digits. The strength of the posterior stimulus declines slightly from 65% at stage 3/4 to 57% at stage 6. At stage 7, the number of cases forming extra digits decreases to 64%. However, in those limbs that form extra digits, the strength of the stimulus remains similar at 55%. Among those forming extra digits, 29% formed one extra and 71% formed two extra digits. By stage 8 none of the grafts were able to stimulate a supernumerary response.

Sixteen of the 49 limbs (33%) developed supernumerary chick structures on the anterior side of the graft, as illustrated in Figs. 2c and 2d. Such secondary axes, which usually consisted of a humerus, a forearm element, and one or two digits, were formed in response to grafts from all stages except stage 8. Even though it did not prove possible to unambiguously identify a digit 4 on the posterior edge of any graft, there were three cases in which a supernumerary digit forming anterior to the graft could be clearly identified as such.

As controls for the effect of cells with posterior posi-

TABLE 1
ASSAY FOR MOUSE POSTERIOR POSITIONAL IDENTITY

Mouse limb bud stage	n	% Supers	Score for magnitude of response						% Strength stimulus
			0	1	1.5	2	2.5	3	
3 + 4	11	100	—	3	1	2	4	1	65
5	7	100	—	2	1	2	2	—	59
6	11	100	—	3	2	4	2	—	57
7	11	64	4	2	2	2	1	—	55
8	9	0	9	—	—	—	—	—	0

tional identity, nine anterior wedges were grafted from mouse limb buds at stage 4, the stage at which posterior wedges were the most effective in stimulating supernumerary digits. None of these anterior wedges induced the formation of extra chick digits.

Regardless of the stage of the donor tissue, on the day after grafting the mouse tissue appeared to be well integrated into the host. The carbon marks in the grafted tissue were clearly visible for several subsequent days and in many cases were visible as an elongated trail of carbon particles after the limbs had been processed (Fig. 3). In the majority of cases (90%), the mouse grafts not only survived in the chick limb but apparently went on to differentiate into faintly staining rods or nodules of cartilage (Figs. 2 and 3).

DISCUSSION

Several previous studies have shown that cells from the posterior edge of limb buds of mice (Tickle et al., 1976; Fallon and Crosby, 1977) as well as other vertebrates (MacCabe and Parker, 1976; Fallon and Crosby, 1977; Honig, 1984) are able to stimulate a supernumerary response when grafted into the anterior of a host
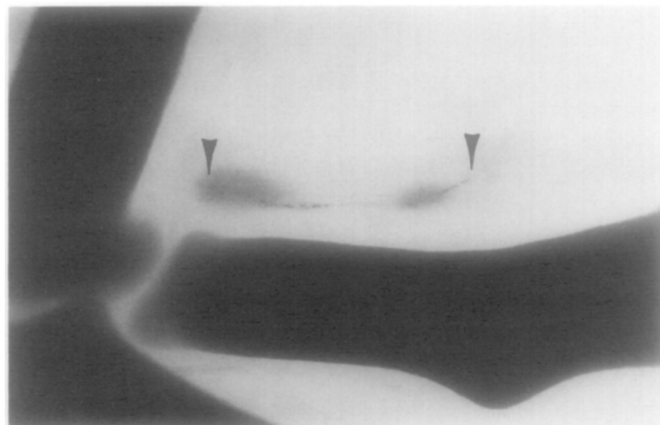

FIG. 3. Mouse cartilage differentiating in chick limb. This limb had received a stage 8 posterior mouse graft. The injected spot of carbon has become a trail elongated along the proximal–distal axis (arrowheads).

chick wing bud. In this study we have used such a heterospecific assay to detect cells at different stages of mouse limb bud development with active posterior positional identity, as judged by their ability to stimulate a supernumerary response in chick limbs.

Our results show that cells taken from limb buds ranging in stage from 3 to 7 (Wanek et al., 1989b) are able to stimulate a supernumerary response in host chick limbs. From stages 3 to 6, all grafted limbs formed extra chick digits, whereas the frequency of the response was lower at stage 7. Cells taken from stage 8 limbs were ineffective in this assay. We conclude from these results that posterior positional identity can be detected in mouse limb buds up to stage 7, becomes variable at stage 7, and can no longer be detected at stage 8.

The magnitude of the response at all stages tested was always less than the maximum achieved when chick posterior cells are grafted (Honig and Summerbell, 1985). It is likely that this reflects less than optimal cell interactions between the two species. Nevertheless, in our study mouse grafts were able to stimulate the formation of up to three extra digits between the graft and the normal host digits. The largest responses were generated by grafts of the younger stages. In the majority of stage 3–6 grafts, the formation of two or sometimes three extra digits between the graft and the normal host digits was stimulated. Also at these stages, when one or two digits formed anterior to the graft it was sometimes possible to identify one as a digit 4.

In addition to stimulating a response in chick limbs, in many instances the mouse tissue itself survived to the end of the experiment. The carbon mark made in the mouse wedge originally formed a spot, but by the end of the incubation period it had become proximal-distally elongated into a streak. This appearance is typical of the behavior of carbon marks placed in mouse limbs in vivo (Muneoka et al., 1989) and is indicative of tissue growth. The mouse tissue formed only a minor portion of the final limb, but it nevertheless differentiated into small, weakly stained cartilage elements.

Identification of the molecules that specify the positional identity of limb cells is necessary for an understanding of the way in which positional information is

used to construct the limb. Once identified, it is likely that ways could be developed to manipulate their expression, thus leading to the possibility of enhanced regenerative abilities in mammals. The results of the present study provide an additional criterion to guide the identification of posterior specifying molecules. Molecules likely to be directly involved in the specification of posterior identity are expected to exhibit temporal expression patterns that reflect the functional identification of posterior identity described here.

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