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Pattern discontinuity, polarity and directional intercalation in axolotl limbs

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SUMMARY

Axolotl limb stumps with dorsal–ventral confrontations between digits **2** and **3** but with a normal anterior–posterior pattern were created by grafting between contralateral limbs. Graft and host differed in ploidy to permit a determination of the origin of cells in the regenerated limb. After regeneration, limbs were analysed for skeletal and muscle patterns and for the distribution of marked cells in the regenerate. Regenerated limbs showed varying degrees of abnormality in their dorsal–ventral organization. Following regeneration, the original dorsal–ventral discontinuities were in some cases maintained and in others resolved. The maintenance or resolution of pattern discontinuities occurred in a position-dependent manner. Cell marker analysis indicates a relationship between the resolution of discontinuities and the extent to which cells become displaced across the original graft–host interface. These data lend support to the suggestion that circumferential intercalation is directionally biased.

INTRODUCTION

Recent experiments on the regenerating urodele limb have demonstrated that regenerates with internal pattern discontinuities can be formed. Maden (1980) demonstrated that some supernumerary limbs resulting from 180° blastema rotation, while appearing outwardly to be normal limbs, were upon histological analysis found to possess internal dorsal–ventral discontinuities in their muscle patterns. Amputation of these unusual supernumerary limbs resulted in the regeneration of similar muscle patterns, that is the regeneration of limbs with discontinuities in the dorsal–ventral axis (Maden & Mustafa, 1982). Recently, Holder & Weekes (1984) have surgically created mixed-handed axolotl limbs containing dorsal–ventral discontinuities between digits **2** and **3**. After amputation of such mixed-handed upper and lower forelimbs, five different limb patterns were regenerated: normal, mixed-handed, part normal/part symmetrical, symmetrical, and limbs with a symmetrical region flanked on both sides by asymmetrical regions. It is clear from these two studies that discontinuities in the dorsal–ventral axis can persist during the regeneration process. To explore further the formation of these unusual muscle patterns, Maden & Mustafa (1984), using the triploid cell marker in axolotls, analysed the cellular contribution to supernumerary limbs,

Key words: limb regeneration, pattern discontinuities, cellular contribution, intercalation, axolotl.

formed as a result of 180° rotation of the blastema. They found that for the most part the muscle pattern was correlated with the cellular contribution from different regions of the stump or graft. Thus, cellular contribution from ventral or dorsal tissue coincides with the formation of ventral or dorsal muscle patterns, respectively.

The finding that dorsal–ventral discontinuities can persist during regeneration is in contrast to the results of experiments where blastemas are grafted contralaterally in such a way as to confront dorsal and ventral cells. In such experiments, supernumerary limbs were frequently formed at the sites of maximal positional disparity (Bryant & Iten, 1976; Tank, 1978; Maden, 1980), thereby eliminating any pattern discontinuity. The results of blastema-grafting experiments of this type in which pattern regulation occurs to restore pattern continuity have played an important role in the development of pattern formation models. In fact, the overwhelming majority of results from experiments on the regenerating urodele limb have demonstrated that the restoration of pattern continuity is the norm (reviewed in Tank & Holder, 1981). Hence, virtually all models of pattern formation during limb outgrowth are based on the assumption that pattern regulation results in pattern continuity. Experiments which demonstrate exceptions to this assumption represent an opportunity to characterize further the mechanisms controlling pattern formation during limb regeneration.

In the polar coordinate model (French, Bryant & Bryant, 1976; Bryant, French & Bryant, 1981) a specific mechanism for the achievement of pattern continuity, that is intercalation, is proposed. Cells on either side of a position disparity are proposed to interact and to divide to produce cells with positional values which are intermediate between those of the confronted cells. This process is thought to proceed until complete pattern continuity is established. The evidence that intercalary regeneration is an important mechanism in pattern regulation comes from numerous tissue grafting experiments (see Tank & Holder, 1981) as well as from cell marker studies using grafts between triploid and diploid axolotls (Pescitelli & Stocum, 1980; Muneoka & Bryant, 1984*a*,*b*).

One characteristic of intercalary regeneration that emerges from a number of studies is that it shows a directional bias. When blastemas are transplanted from a distal amputation site to a more proximal limb level, the cells which are intercalated to complete the limb pattern are derived only from the more proximal partner in the interaction (Pescitelli & Stocum, 1980). Furthermore, transplants along the proximal–distal axis show another interesting feature: when grafts of blastemas are made from a proximal site to a more distal limb level, graft and host fail to interact, leaving an unresolved discontinuity in the proximal–distal axis of the regenerated limb (Iten & Bryant, 1975; Stocum, 1975). Muneoka & Bryant (1984*a*,*b*), in analysing the cellular contribution to supernumerary limbs following contralateral limb bud or blastema grafts, found that cells of posterior origin contributed to posterior and dorsal regions of the supernumerary limbs, whereas cells of anterior origin contributed to anterior and ventral regions. Hence, a directional bias in circumferential intercalation exists in the transverse plane of the

limb and it is from posterior to dorsal and from anterior to ventral. It is possible that the demonstrated directionality of intercalation along the proximal–distal axis and around the circumference might be related to the inability of certain graft combinations to resolve pattern discontinuities, although the mechanism by which these phenomena could be related is at present obscure.

In this paper we analyse the cellular contribution to regenerates from mixed-handed limb stumps, in an attempt to investigate whether any relationship exists between directional intercalation and the failure to resolve dorsal–ventral discontinuities in the regenerate. Our results show that a specific class of such discontinuities is resolved during regeneration, and that a second class is not resolved. We propose that the different behaviour of these two classes of discontinuity is related to the known directional bias in circumferential intercalation in the transverse plane of the limb. These results have also been reported briefly in Muneoka, Holler-Dinsmore & Bryant (1986).

MATERIALS AND METHODS

All experiments were performed on axolotls, *Ambystoma mexicanum*, spawned at the University of California, Irvine. Animals used in this study ranged in length from 7·7 to 9·0 cm. Triploid animals were made by exposing newly fertilized eggs to hydrostatic pressure (Gillespie & Armstrong, 1979). Pressure-treated animals were screened for triploidy as described previously (Muneoka, Wise, Fox & Bryant, 1984). Experimental animals were maintained individually in 20% Holtfreters solution, and were changed and fed tubifex worms three times a week. Triploid and diploid sibling axolotls were maintained separately, but under identical conditions.

Grafting

The grafting operation is shown in Fig. 1. All grafts were made in the lower arm between similar-sized triploid and diploid sibling axolotls. Paired animals were anaesthetized in MS 222 (diluted 1:1000) and operated on simultaneously. Cuts were made along the proximal–distal axis between the radius and ulna, separating the lower arms into anterior and posterior halves. Transverse cuts were then made just distal to the elbow isolating either anterior or posterior half limbs. The isolated half limbs were then reciprocally exchanged within pairs (anterior to anterior or posterior to posterior) to the contralateral limb (right to left or left to right) of the sibling animal. Grafts were sutured into place with 8·0 silk and limbs were amputated immediately through the lower arm. A diagram of an amputated stump showing the dorsal and ventral regions of discontinuity is shown in Fig. 2A. Following the grafting operation, animals were observed daily for graft survival until the graft was well healed. Subsequent observations were made on a weekly basis.

Analysis

Regenerating experimental and control triploid limbs were harvested at the stage of late digits (Tank, Carlson & Connelly, 1976) and fixed in Carnoys fixative. Experimental limbs which regenerated normal-appearing 4-digit regenerates, and control triploid limbs, were skinned and processed for whole-mount dermal analysis (Muneoka *et al.* 1984). All skinned limbs were decalcified in versene, embeded in paraffin and serially cross sectioned. Sections and dermal preparations were stained with a nucleolus-specific bismuth stain (Muneoka *et al.* 1984).

Bismuth-stained cells in the dermis of whole mounts were counted to determine the frequency of trinucleolate cells (number of cells with three nucleoli divided by the number of cells with two and three nucleoli). In each dorsal and ventral dermal preparation, the cells of each digit were

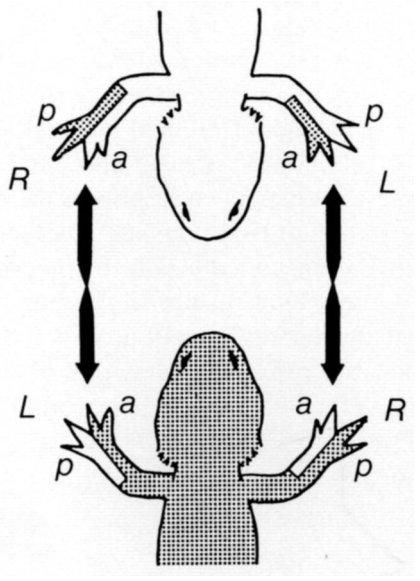


Fig. 1. Diagram of the grafting procedure as viewed from the dorsal aspect. Anterior or posterior lower half forelimbs were reciprocally exchanged between contralateral limbs from diploid (top) and triploid (bottom) axolotls. This grafting operation results in the construction of a limb which is normal in the anterior–posterior axis but with dorsal–ventral discontinuities between digits **2** and **3**. These surgically constructed mixed-handed limbs were amputated immediately through the lower arm. *L*, left; *R*, right; *a*, anterior; *p*, posterior.

counted. Actual trinucleolate frequencies were compared with individually matched control trinucleolate frequencies to determine the real cellular contribution to each digit (trinucleolate frequency/control frequency). Any real triploid frequency greater than 1·0 was truncated to 1·0 for subsequent quantitative analyses.

Maps of trinucleolate cells in dorsal and ventral dermal preparations were made using a light microscope fitted with a digitized stage (MD1 digitizer, Minnesota Datametrics) interfaced with an Apple IIe personal computer and a Houston Instruments DMP40 plotter. These maps were made by scanning a series of 100 μm wide regions of dermis from anterior to posterior and recording the location of trinucleolate cells. Samples were taken at 1000 μm intervals from the base of the digits towards the base of the regenerate. The maps give a general picture of the anterior–posterior and proximal–distal distribution of trinucleolate cells in the dorsal and ventral dermis.

Muscle patterns were determined from serial transverse sections of each experimental limb using the criteria of Maden (1980). The anterior–posterior pattern of digits for each experimental limb was analysed utilizing the criteria of Pescitelli & Stocum (1980). Phalangeal number was determined by direct observation of the skinned limbs, and the number and pattern of articulations of carpal elements were determined by serial reconstruction of transverse sections.

Terminology

The term discontinuity in this paper refers to the *abnormal* positioning of dorsal muscles adjacent to ventral muscles. Hence, the regions on the periphery of digits **1** and **4** where dorsal and ventral muscles are normally adjacent are not considered discontinuities. Grafts were made so as to create dorsal–ventral discontinuities between digits **2** and **3** on the dorsal and ventral sides of the host limb (see Fig. 2A), and these are called *central discontinuities*. Central discontinuities can be subdivided into two types, depending on the nature of the adjacent tissues: Type I are those with anterior–ventral tissue adjacent to posterior–dorsal tissue, and

Type II are those with anterior–dorsal tissue adjacent to posterior–ventral tissue (see Fig. 2A). Discontinuities which regenerate between digits **1** and **2** or between digits **3** and **4** are termed *peripheral discontinuities*.

RESULTS

A total of sixteen experimental limbs was analysed. Of these, four produced either 1 or 2 extra digits in a dorsal or ventral position on the grafted side of the limb (see Fig. 4). Limbs with supernumerary digits were analysed in sectioned tissue for muscle and cartilage pattern and for cellular contribution to the cartilage. However, it was not possible to analyse dermal preparations of these limbs.

The remaining twelve limbs formed 4-digit regenerates which from external appearance seemed normal. These twelve limbs were analysed for muscle and cartilage pattern and for cellular contribution to the dermis. All twelve limbs formed a normal anterior-to-posterior sequence of digits as judged by their skeletal morphology.

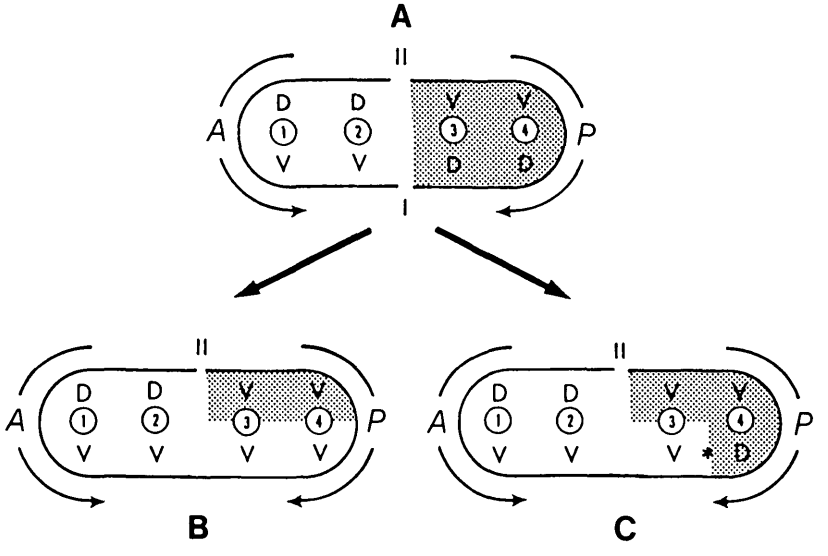


Fig. 2. Diagram showing the location of positional disparities in the limb stump prior to regeneration (A) and the major results following regeneration (B,C). (A) Limbs were surgically constructed to create dorsal–ventral positional disparities (Type I and Type II) between digits **2** and **3** in dorsal and ventral regions of the stump. In this example the grafted posterior half limb was triploid (stippled) and the host anterior half limb was diploid. The arrows in this diagram represent the predicted directionality of cellular interactions based on the results of cellular contribution to supernumerary limbs (Muneoka & Bryant, 1984*a*). (B,C) Following regeneration, the most frequent results were as follows: (1) the maintenance of the central Type II discontinuities, and (2) the elimination of the original Type I discontinuities by resolving the pattern discontinuity (B), or forming a peripheral discontinuity (*) between digits **3** and **4** (C). Alteration of the original limb pattern occurs in most cases in the original posterior–dorsal quadrant of the limb. *A*, anterior; *P*, posterior; *D*, dorsal muscle; *V*, ventral muscle. Circled numbers indicate placement of digits.

Muscle patterns

The muscle patterns of the twelve limbs that formed 4-digit regenerates are summarized in Table 1. One of the twelve experimental limbs (1-rt, Table 1) was completely normal with regard to the muscle pattern. Another limb (4-rt, Table 1) regenerated a pattern identical to that of the original stump, that is the regenerate contained both types of discontinuity. The remaining ten limbs regenerated patterns intermediate between completely normal and identical to the stump. Most of these maintained a single Type II central discontinuity (8/10). In these limbs, the original Type I central discontinuity was either resolved leading to a normal ventral muscle pattern or regenerated as a peripheral discontinuity. The two remaining limbs regenerated two peripheral discontinuities. In summary, of

Table 1. *Muscle patterns of regenerated limbs (other than those which formed supernumerary digits)*

Limb	Digit 1	Digit 2		Digit 3	Digit 4
Anterior grafts					
1-rt	DOR	DOR		DOR	DOR
	VEN	VEN		VEN	VEN
6-rt	VEN	VEN		VEN	VEN
	DOR	DOR	*	VEN	VEN
7-lt	VEN	VEN		VEN	DOR
	DOR	VEN		VEN	VEN
8-rt	VEN	VEN		VEN	DOR
	DOR	DOR	*	VEN	VEN
Posterior grafts					
1-lt	DOR	DOR	*	VEN	VEN
	VEN	VEN		VEN	VEN
2-rt	DOR	DOR	*	VEN	VEN
	VEN	VEN		VEN	VEN
3-lt	DOR	DOR	*	VEN	VEN
	VEN	VEN		VEN	VEN
4-rt	DOR	DOR	*	VEN	VEN
	VEN	VEN	*	DOR	DOR
5-rt	DOR	DOR	*	VEN	VEN
	VEN	VEN		VEN	VEN
6-lt	DOR	DOR	*	VEN	VEN
	VEN	VEN		VEN/DOR	DOR
7-rt	DOR	DOR		DOR	VEN
	VEN	VEN		VEN	DOR
8-lt	DOR	DOR	*	VEN	VEN
	VEN	VEN		VEN	DOR

Digits are indicated by number. DOR, dorsal muscle; VEN, ventral muscle; A, anterior; P, posterior; *, central discontinuities.

the twelve original Type I discontinuities, only one was maintained as a central discontinuity, while five regenerated as peripheral discontinuities and the remaining six were resolved to form regionally normal patterns of muscles. By contrast, of the twelve original Type II discontinuities, only one was resolved to form a regionally normal muscle pattern, two were regenerated as peripheral discontinuities and nine were maintained as central Type II discontinuities.

Cellular contribution

The observed trinucleolate frequencies in the dorsal and ventral dermal preparations are shown in Table 2. These data were used to construct Table 3 which shows the real triploid frequencies (observed frequency/control frequency)

Table 2. *Observed frequencies of trinucleolate cells*

Limb	Graft ploidy	Dermal prep.	Digit 1	Digit 2	Digit 3	Digit 4	Control
Anterior grafts							
1-rt	3N	d	0·03	0·06	0·03	0·01	0·84
		v	0·05	0·04	0·00	0·00	
6-rt	2N	d	0·04	0·08	0·23	0·27	0·82
		v	0·06	0·14	0·70	0·75	
7-lt	3N	d	0·71	0·63	0·49	0·12	0·61
		v	0·68	0·39	0·18	0·18	
8-rt	2N	d	0·03	0·02	0·34	0·61	0·61
		v	0·04	0·01	0·35	0·69	
Posterior grafts							
1-lt	3N	d	0·17	0·35	0·14	0·71	0·84
		v	0·00	0·01	0·03	0·09	
2-rt	2N	d	0·80	0·76	0·06	0·00	0·84
		v	0·86	0·69	0·77	0·78	
3-lt	3N	d	0·01	0·11	0·53	0·70	0·79
		v	0·02	0·05	0·04	0·12	
4-rt	2N	d	0·55	0·55	0·12	0·03	0·79
		v	0·46	0·58	0·05	0·02	
5-rt	3N	d	0·01	0·01	0·60	0·70	0·82
		v	nd	nd	nd	nd	
6-lt	2N	d	0·66	0·62	0·10	0·10	0·82
		v	0·71	0·59	0·27	0·06	
7-rt	3N	d	0·04	0·02	0·28	0·78	0·61
		v	nd	nd	nd	nd	
8-lt	2N	d	0·76	0·67	0·13	0·05	0·61
		v	0·59	0·70	0·34	0·02	

The data in this table represent the observed trinucleolate frequency for each digit in dorsal and ventral dermal preparations. Also included in this table are the observed trinucleolate frequencies in the matched control triploid dermal preparations. Digits are indicated by number. d, dorsal dermal preparation; v, ventral dermal preparation; nd, no data available.

Table 3. *Real frequencies of trinucleolate cells*

Limb	Graft ploidy	Dermal prep.	Real triploid frequency digit 1	2	3	4	TCV	Total CV A	P
Anterior grafts									
1-rt	3N	d	0·04	0·07	0·04	0·01	0·04	0·03	0·97
		v	0·06	0·05	0·00	0·00	0·02		
6-rt	2N	d	0·05	0·10	0·28	0·33	0·19	0·65	0·35
		v	0·07	0·17	0·85	0·91	0·50		
7-lt	3N	d	1·00	1·00	0·80	0·20	0·79	0·69	0·31
		v	1·00	0·64	0·30	0·30	0·58		
8-rt	2N	d	0·05	0·03	0·56	1·00	0·41	0·57	0·43
		v	0·07	0·02	0·57	1·00	0·44		
							$\bar{x}$ =	0·64	0·36
Posterior grafts									
1-lt	3N	d	0·20	0·42	0·17	0·85	0·41	0·77	0·23
		v	0·00	0·01	0·04	0·11	0·04		
2-rt	2N	d	0·95	0·90	0·07	0·00	0·48	0·70	0·30
		v	1·00	0·82	0·92	0·93	0·92		
3-lt	3N	d	0·01	0·14	0·67	0·89	0·42	0·75	0·25
		v	0·03	0·06	0·05	0·15	0·07		
4-rt	2N	d	0·70	0·70	0·15	0·04	0·39	0·37	0·63
		v	0·58	0·73	0·06	0·03	0·35		
5-rt	3N	d	0·01	0·01	0·73	0·85	0·40		
		v	nd	nd	nd	nd	nd		
6-lt	2N	d	0·80	0·76	0·12	0·12	0·45	0·47	0·53
		v	0·87	0·72	0·33	0·07	0·49		
7-rt	3N	d	0·07	0·03	0·46	1·00	0·46		
		v	nd	nd	nd	nd	nd		
8-lt	2N	d	1·00	1·00	0·21	0·08	0·66	0·67	0·33
		v	0·97	1·00	0·56	0·03	0·67		
							$\bar{x}$ =	0·62	0·38
							Total $\bar{x}$ =	0·63	0·37
							Type I $\bar{x}$ =	0·53	0·47
							Type II $\bar{x}$ =	0·72	0·28

The real triploid frequency data in this table were derived from the data in Table 2 as follows: the observed trinucleolate frequency for each dorsal and ventral dermal preparation of each digit was divided by the trinucleolate frequency of the matched control, to give a real triploid frequency. The triploid contribution value (TCV) for each dorsal and ventral dermal preparation was derived by dividing the sum of the real triploid frequencies for all four digits by the number of digits. Anterior half and posterior half contribution values (total CV) were derived as follows: the mean TCV for each limb was calculated from the TCVs of each dorsal and ventral preparation. This value is then the fraction of the cells in the regenerate contributed by the triploid half of the stump. The contribution from the diploid half is 1·00 minus the contribution from the triploid half. The mean values for contribution from anterior and posterior half limbs do not include limb 1-rt (see Results). d, dorsal dermal preparation; v, ventral dermal preparation; nd, no data available.

for each dermal preparation of each digit in the regenerated limb. These data were used to quantify the cellular contribution from triploid tissue to each dermal preparation by summing the real frequencies for all digits, then dividing by the number of digits. This analysis gives triploid contribution values (TCV) for each dermal preparation, which can range from 0·0 (no triploid contribution) to 1·0 (only triploid contribution). Contribution values of 0·5 indicate equal contribution from diploid and triploid tissues. The anterior and posterior contribution values (total CV) were determined by first calculating the mean TCV for dorsal and ventral, then subtracting this number, the contribution value of the triploid half limb, from 1·00 to obtain the contribution value from the diploid half limb. As can be seen from Table 3 there was a great deal of variation in contribution values from limb to limb, as well as from dorsal to ventral within a single limb. For example, limb 6-rt regenerated from an anterior half diploid (graft) posterior half triploid (host) stump. The dorsal TCV of 0·19 indicates that few posterior (triploid host) cells contributed to the dorsal region of the limb, whereas the ventral region of the same limb showed equal contribution (TCV = 0·5) from posterior (triploid host) and anterior (diploid graft) tissues in the limb stump. In this limb as a whole, the anterior (diploid graft) tissue was found to contribute 65% of the cells in the regenerate whereas the remaining cells (35%) were derived from the posterior (triploid host) half.

The only limb in this experiment which regenerated a completely normal limb pattern was also the only one which was found to be formed almost entirely (97%) of host tissue (Table 3, 1-rt), thus indicating failure of the graft to survive and participate. This limb will not be considered in further analyses. Considering the remaining limbs for which we have complete data, a clear asymmetry in contribution to the regenerated limb is apparent, with the anterior half tissue contributing overall about 63% of the cells, regardless of whether anterior was graft or host. Interestingly, limb 4-rt, which displayed a much reduced contribution from anterior tissue, was the only limb which regenerated both Type I and Type II central discontinuities (see Table 1). When the data in Table 3 are analysed separately for dermal preparations in which the original central discontinuity was Type I and for those in which it was Type II, a striking difference in anterior *versus* posterior contribution values is noted. From dermal preparations with originally Type I discontinuities, the majority of which are not maintained in the regenerate, anterior is found to contribute about 3/4 of the cells and posterior only 1/4. From dermal preparations with originally Type II discontinuities, the majority of which were maintained during regeneration, anterior and posterior halves contributed approximately equal numbers of cells (Table 3).

Cellular distribution

The final distribution of triploid and diploid cells (Table 4) was determined by normalizing the triploid contribution data in Table 3 as follows: the triploid frequencies of individual digits were divided by the sum of the frequencies of all 4 digits of each dermal preparation. The resulting values indicate the proportion of cells in each digit relative to all cells of that type (triploid or diploid) in the limb.

Table 4. *Distribution of cells*

Limb (G/H)	Dermal prep.	Digit 1 3N	Digit 1 2N	Digit 2 3N	Digit 2 2N		Digit 3 3N	Digit 3 2N	Digit 4 3N	Digit 4 2N
Anterior 3N										
2-rt	d	0·49	0·02	0·47	0·05		0·04	0·45	0·00	0·48
H	v	0·27	0·00	0·22	0·55		0·25	0·24	0·25	0·21
4-rt	d	0·44	0·12	0·44	0·12		0·09	0·35	0·03	0·40
H	v	0·41	0·16	0·52	0·10		0·04	0·36	0·02	0·37
6-lt	d	0·44	0·09	0·42	0·11		0·07	0·40	0·07	0·40
H	v	0·44	0·06	0·36	0·14		0·17	0·33	0·04	0·46
7-lt	d	0·33	0·00	0·33	0·00		0·27	0·20	0·07	0·80
G	v	0·45	0·00	0·29	0·20		0·13	0·40	0·13	0·40
8-lt	d	0·44	0·00	0·44	0·00		0·09	0·46	0·03	0·54
H	v	0·38	0·02	0·39	0·00		0·22	0·31	0·01	0·67
	3N $\bar{x}$ =	0·41		0·39		→	0·14		0·07	
	2N $\bar{x}$ =		0·05		0·13	←		0·35		0·47
Posterior 3N										
1-lt	d	0·12	0·34	0·26	0·25		0·10	0·35	0·52	0·06
G	v	0·00	0·26	0·06	0·26		0·25	0·25	0·69	0·23
3-lt	d	0·01	0·43	0·08	0·38		0·39	0·14	0·52	0·05
G	v	0·10	0·26	0·21	0·25		0·17	0·26	0·52	0·23
5-rt	d	0·01	0·41	0·01	0·41		0·46	0·11	0·53	0·06
G	v	nd	nd	nd	nd		nd	nd	nd	nd
6-rt	d	0·07	0·29	0·13	0·28		0·37	0·22	0·43	0·21
H	v	0·04	0·47	0·09	0·42		0·43	0·08	0·46	0·05
7-rt	d	0·04	0·38	0·02	0·40		0·29	0·22	0·64	0·00
G	v	nd	nd	nd	nd		nd	nd	nd	nd
8-rt	d	0·03	0·40	0·02	0·41		0·34	0·19	0·61	0·00
H	v	0·04	0·40	0·01	0·42		0·34	0·18	0·60	0·00
	3N $\bar{x}$ =	0·05		0·11		←	0·30		0·54	
	2N $\bar{x}$ =		0·36		0·33	→		0·21		0·10

The distribution data in this table were derived from those in Table 3 as follows. 3N values: the real triploid frequency for each dorsal and ventral half digit was divided by the sum of the frequencies of the four digits in each dermal preparation. The mean triploid distribution value for each digit (average of dorsal and ventral) is shown in the table (3N $\bar{x}$) and represents the fraction of all triploid cells present in a particular digit. 2N values: the distribution of diploid cells was determined by first calculating the real frequency as 1·00 minus the real triploid frequency for individual dorsal or ventral sides of each digit. The remainder of the calculation was the same as that described above for 3N values. The values at the bottom of the columns represent the overall mean distribution of 2N and 3N cells. Arrows indicate the direction of cellular displacement. d, dorsal dermal preparation; v, ventral dermal preparation; H, host; G, graft.

The overall results (summarized at the top of Table 5) indicate that most (79%) of the cells stayed in their original half of the limb during regeneration, although there is a great deal of individual variation when specific regions of each limb are analysed separately. The displacement of cells from grafted regions into host regions (21%) was overall virtually identical to the reciprocal displacement of host cells into grafted regions (20%). Furthermore, of the displaced cells about 67% were found in the digit directly adjacent to the tissue of origin, with the remaining 33% found in the most peripheral digit. The final distribution of cells in dermal preparations was determined separately for cases with central discontinuities, peripheral discontinuities, and no discontinuities (Table 5). This analysis was performed for bidirectional cell displacement in the case of central discontinuities and for unidirectional cell displacement (in the direction of pattern changes) in cases with peripheral discontinuities or no discontinuities. Central discontinuities showed a reduced displacement of cells ($\bar{x} = 16\%$) when compared to the cellular displacement occurring during normal limb regeneration ($\bar{x} = 24\%$; Muneoka, Holler-Dinsmore & Bryant, 1985). Peripheral discontinuities resulted in the displacement of 23% of the marked cells, and cases with no discontinuity resulted in the displacement of 48% of the cells into the opposite side of the limb. Hence, there was a clear relationship between the type of discontinuity observed

Table 5. *Summary of cellular distribution*

Type	n	Original half limb	Adjacent digit	Peripheral digit	Total displaced
Total	36	0·79	0·14	0·07	0·21
Host into graft	18	0·80	0·13	0·07	0·20
Graft into host	18	0·78	0·15	0·06	0·21
Discontinuity:					
Central	18	0·83	0·11	0·05	0·16
Peripheral	6	0·77	0·21	0·02	0·23
Resolved	4	0·52	0·25	0·23	0·48
Cellular displacement into limb quadrants:					
Ant–Dor	9	0·85	0·10	0·05	0·15
Ant–Ven	9	0·82	0·13	0·05	0·18
Post–Dor	9	0·67	0·21	0·12	0·33
Post–Ven	9	0·82	0·13	0·05	0·18

The data in this table were derived from the mean distribution data in Table 4. For the original half limb column, the fraction of cells in the two digits on the original side of the limb were summed. The cellular displacement into the digit adjacent to this side and into the most distant digit is shown. The total displaced column represents the sum of these two values. Data are also shown to allow a comparison between the behaviour of host and graft. The amount of cellular displacement which occurred in limb regions with different types of discontinuities were determined as follows: for central discontinuities, bidirectional cellular displacement was calculated, whereas for peripheral discontinuities and for no discontinuities unidirectional cellular displacement in the direction of the patterning change was calculated. The cellular displacement into the four quadrants of the originally grafted limb is also shown.

in the regenerate and the extent of cellular displacement across the graft–host interface.

The overall displacement of cells and the associated change in muscle pattern occurred in a directional manner (Table 1, Fig. 2). For the most part, cellular displacement occurred from anterior–ventral towards posterior–dorsal. Originally dorsal–posterior tissues were replaced during regeneration by tissues with characteristics of ventral muscle and with the ploidy of the anterior half limb regardless of whether the anterior half limb was host or graft. In ten of the twelve limb regions in which the originally central discontinuity was regenerated as a peripheral discontinuity or was resolved (no discontinuity) the pattern changes occurred in the posterior–dorsal quadrant (Table 1). Furthermore, the cellular displacement data (Table 5) indicate that on average about twice as many cells were displaced into the posterior–dorsal quadrant ($\bar{x} = 33\%$) as were displaced into the other three quadrants ($\bar{x} = 16\%$).

The anterior–posterior distribution of cells along the proximal–distal axis of the regenerated limbs was analysed by mapping the location of trinucleolate cells at different proximal–distal levels in dermal preparations of limb regions with central discontinuities ($n = 10$), peripheral discontinuities ($n = 5$) and no discontinuities ($n = 4$). Fig. 3 shows examples of maps of these dermal preparations. All ten limb regions with central discontinuities were found to have graft–host boundaries in the centre of the limb at all levels along the proximal–distal axis (Fig. 3A). Limbs with peripheral discontinuities in some cases (3/5) were found to have graft–host boundaries which were in the centre of the limb proximally but which deviated gradually to a position between the peripheral and central digits distally (Fig. 3B). In the remaining cases with peripheral discontinuities the boundary was found to be shifted to a peripheral position both proximally as well as distally. Most (3/4) of the preparations without discontinuities were seen to have a peripheral boundary between host and graft tissue at all proximal–distal levels, whereas in the remaining preparation there was a gradual shift of the boundary from a central location proximally to the edge of the limb distally (Fig. 3C). This particular preparation is of interest since it is a case in which grafted tissue displaced host tissue during regeneration.

Supernumerary digits

Fig. 4 summarizes the analysis of limbs which regenerated supernumerary digits. All four limbs with extra digits resulted from anterior grafts and the supernumerary digits formed either dorsally or ventrally on the graft side of the limb. Cell counts from sectioned cartilage indicate that the supernumerary digits arose predominantly (although not exclusively) from grafted tissue (Fig. 4). Most of the supernumerary digits branched in the region of the metacarpals. The analysis of the muscle patterns shows that of the four original Type I central discontinuities, none were maintained as central discontinuities. Two were resolved by the formation of supernumerary digits, one formed a peripheral discontinuity and a supernumerary digit, and the last formed a peripheral discontinuity without a

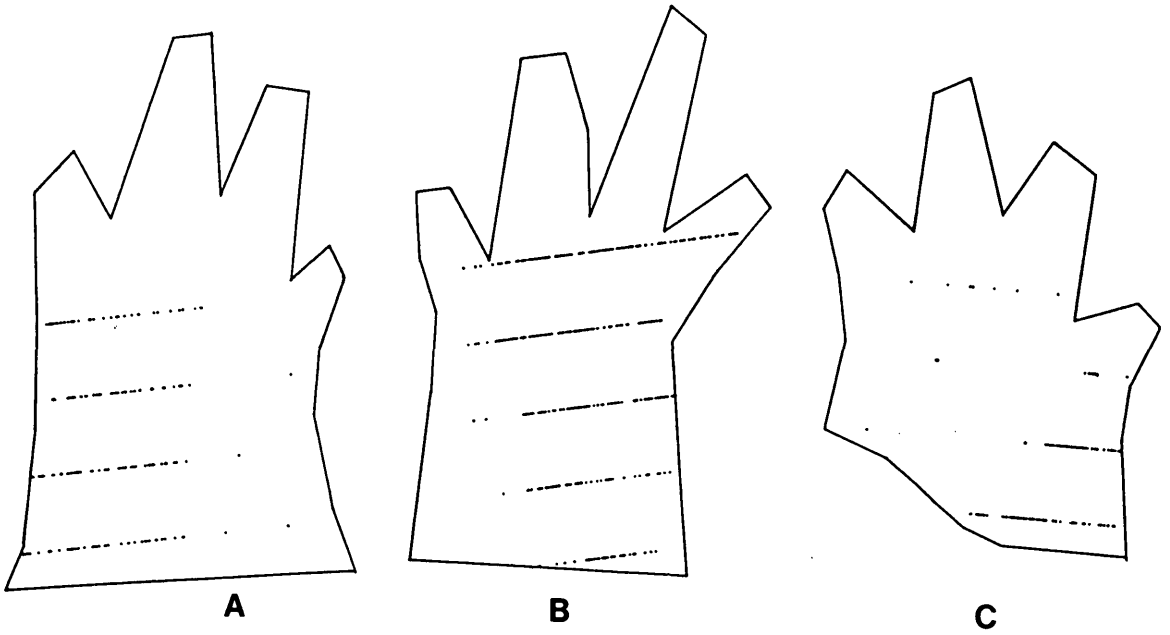


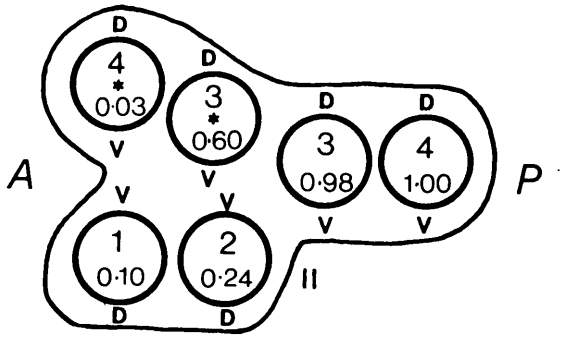
Fig. 3. Maps of dermal preparations to show the distribution of trinucleolate cells in the anterior–posterior dimension and at different proximal–distal positions. (A) Central discontinuity – the graft–host junction runs along the centre of the preparation at all proximal–distal levels. (B) Peripheral discontinuity – the boundary between graft and host shifts from a central position proximally to a peripheral position distally. (C) No discontinuity – the position of the boundary is central proximally and shifts to the edge of the limb distally. Preparation in (A) from side of limb with an originally Type II discontinuity; preparations in (B,C) from side of limb with an originally Type I discontinuity.

supernumerary digit. Of the four original Type II central discontinuities, two were maintained as central discontinuities, one regenerated with a supernumerary digit, and the last formed a peripheral discontinuity and no supernumerary digits.

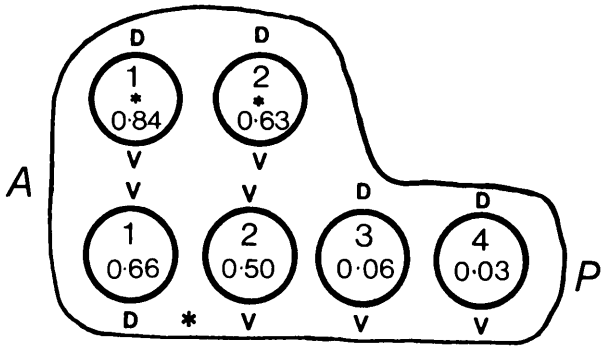
DISCUSSION

In this experiment we have analysed regeneration from surgically constructed, chimaeric limb stumps, each containing two anatomically different central discontinuities where dorsal and ventral tissues were opposed. At the Type I discontinuity anterior–ventral tissue is opposed to posterior–dorsal tissue, and at the Type II discontinuity anterior–dorsal tissue is opposed to posterior–ventral tissue (see Fig. 2). Although the spectrum of regenerated limb patterns obtained in this study is similar to that described by Holder & Weekes (1984) from comparable surgeries (i.e. lower arms amputated immediately after grafting), our conclusions about underlying mechanisms are not. Holder & Weekes (1984) concluded that little cellular interaction takes place between confronted dorsal and ventral tissues during regeneration. In our study, we not only analysed the skeletal and muscle patterns of the regenerated limbs, but also the cellular contribution

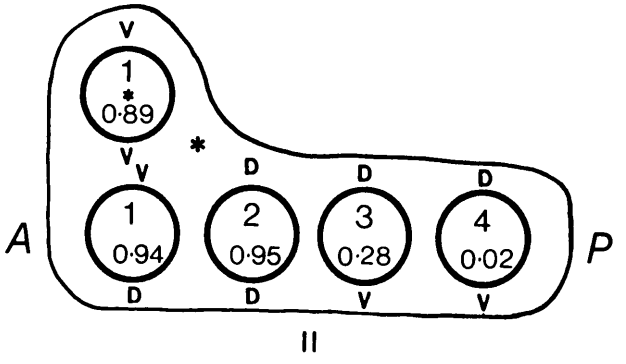
2-lt ant 2N



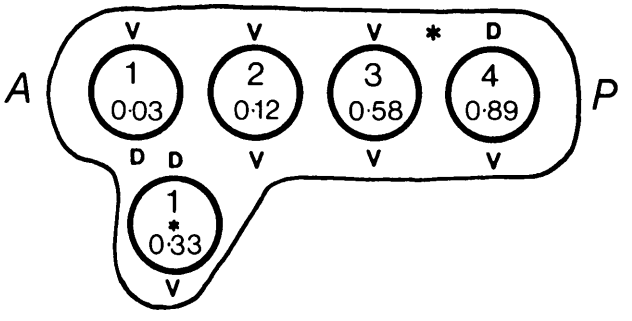
3-rt ant 3N



5-lt ant 3N



4-lt ant 2N



and distribution during regeneration from each half of the limb stump. In addition, we have analysed the behaviour at Type I and Type II discontinuities separately. The evidence we discuss below suggests that Type I and Type II discontinuities are not only anatomically but also behaviourally different from one another, such that Type I discontinuities tend to show interactive, regulative behaviour leading to pattern continuity, whereas Type II discontinuities tend to show non-interactive behaviour, leading to the persistence of pattern discontinuities. In the following sections we will discuss the behaviour of each type of discontinuity separately and offer interpretations based on intercalation as proposed in the polar coordinate model (French *et al.* 1976; Bryant *et al.* 1981).

Interaction at Type I discontinuities

Several lines of evidence support the idea that dorsal–ventral interactions, leading to pattern regulation, occur at Type I discontinuities. First, three of the four supernumerary outgrowths which developed in this experiment formed on the side of the limb which originally possessed a Type I discontinuity (Fig. 4). In two of these cases, intercalation between opposed dorsal and ventral cells, as shown in Fig. 5A–C, can readily account for the final pattern, and for the establishment of pattern continuity. In the third case (Fig. 5A,D,E), intercalation with some loss of positional values around the plane of symmetry created by the intercalatory event can account for the final pattern. Such loss of positional values around a plane of symmetry has previously been shown to account for a variety of experimental results (Bryant *et al.* 1981).

Second, when the cell contribution data are examined, they are found to differ markedly from those reported to occur from half diploid/half triploid limb stumps with normal anatomy, i.e. without dorsal–ventral discontinuities (Muneoka *et al.* 1985). The cellular contribution to regenerates from such anatomically normal chimaeric limbs was found to be 45% from anterior halves and 55% from posterior halves. In the present experiment, contribution from anterior *versus* posterior limb halves was calculated separately from limb regions with Type I and Type II discontinuities. At Type II discontinuities, which are mostly maintained during regeneration, the values are closest to the data from anatomically normal limbs, with anterior halves contributing 53% of the cells and posterior 47%. At Type I discontinuities, which are not maintained during regeneration, the contribution data differ markedly from those in normal limbs, with anterior halves contributing 72% of the cells and posterior halves 28%. We believe that these differences in contribution values between anterior and posterior limb halves in

Fig. 4. Diagrammatic representation of the limbs which formed supernumerary digits. Circles represent digits, identified by number. Digits labelled with a small asterisk (*) are supernumerary digits. Muscle pattern is indicated as D (dorsal) or V (ventral). The real triploid frequency of each digit was determined from cartilage counts, and is indicated within each digital outline. In each case, the specimen identity and the location and ploidy of the inverted graft are indicated at the left. Persistent Type II discontinuities are indicated, and peripheral discontinuities are marked with a large asterisk (*).

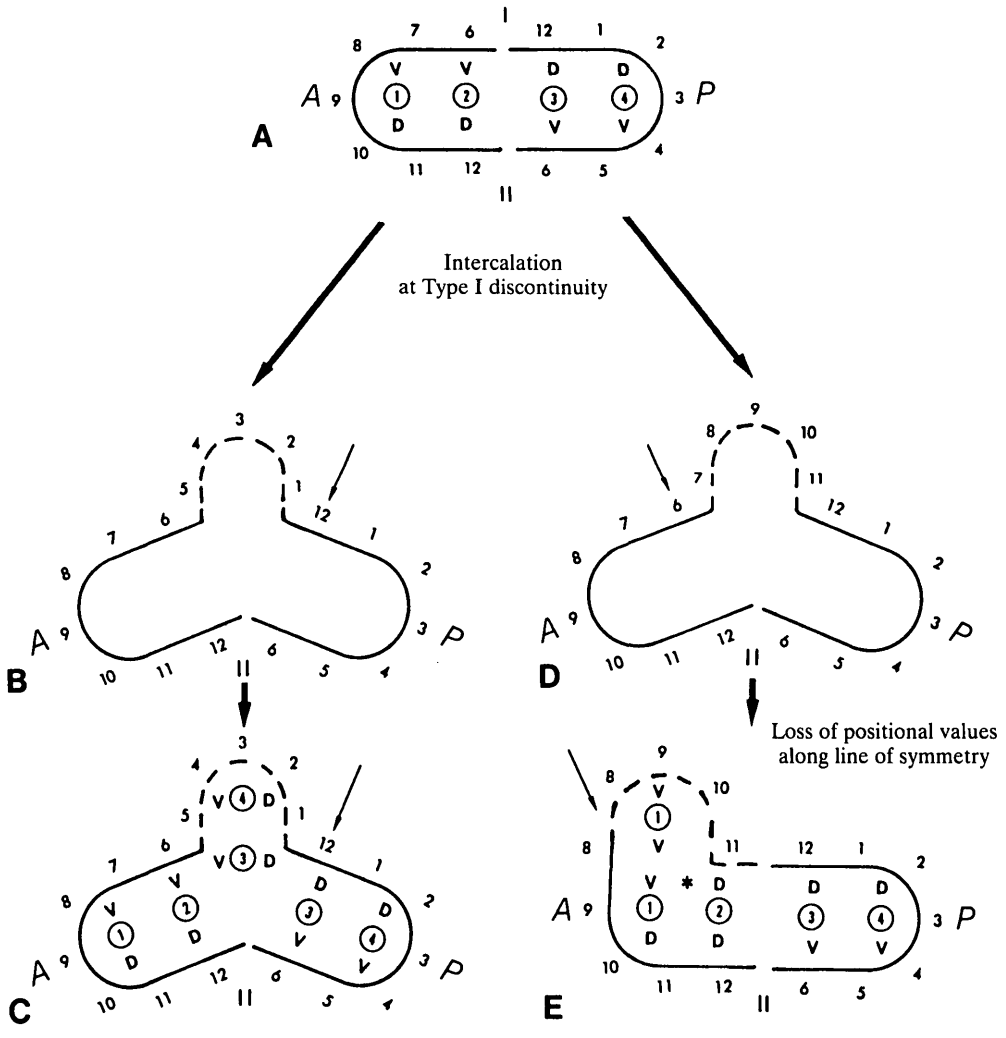


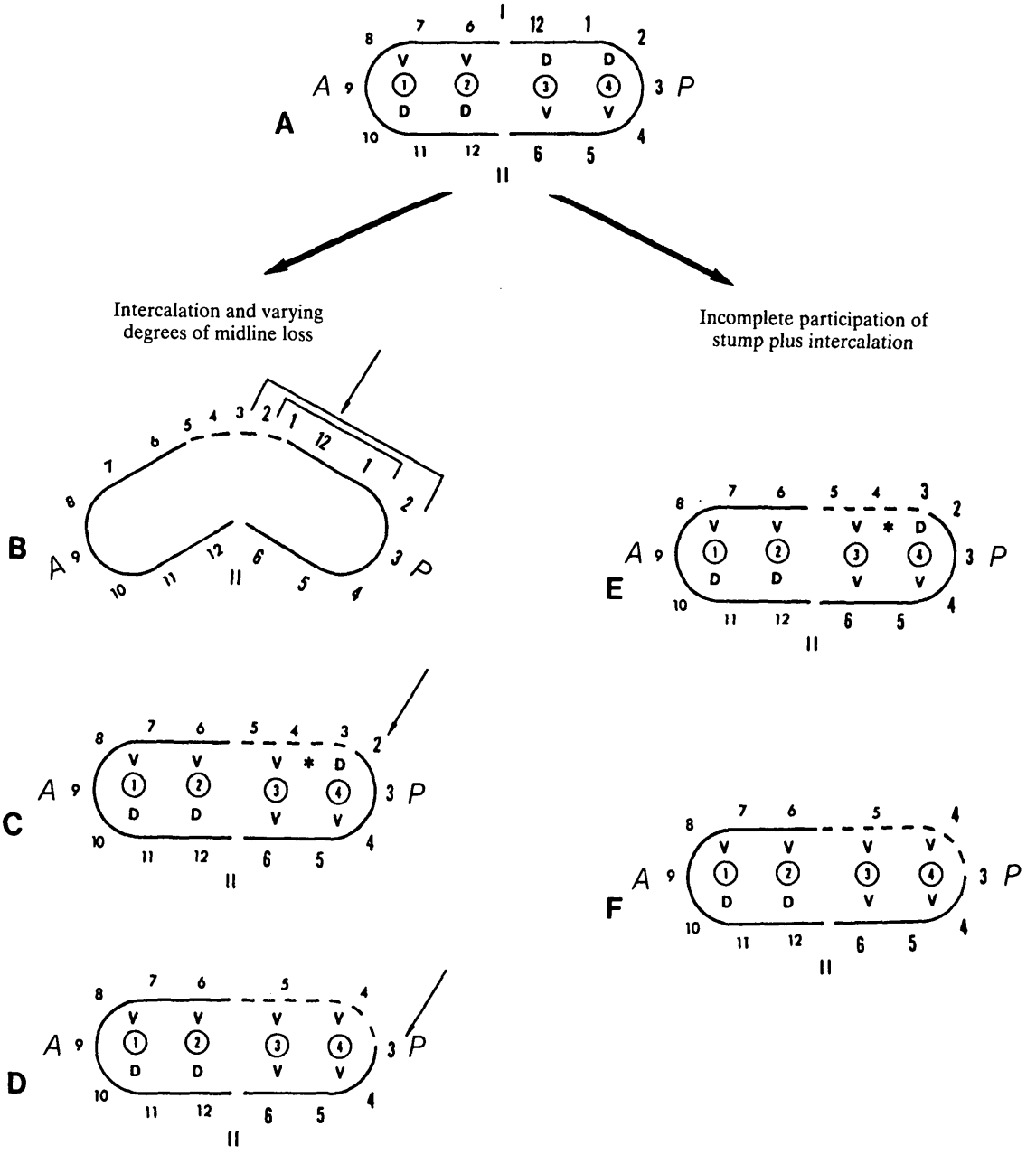
Fig. 5. Interpretation of limbs which developed supernumerary outgrowths. Circumferential positional values are indicated around the periphery of each diagrammatic cross section. Intercalated positional values are connected by dashed lines. Digits are indicated by circled numbers inside the outlines. Muscle patterns are indicated as D (dorsal) or V (ventral). Anterior (*A*) and posterior (*P*) are indicated, as are Type I and Type II central discontinuities. (A) Diagram of a section through a limb stump with an anterior graft. (B,D) Diagrams to show intercalation to remove the discontinuity in positional values at the Type I interface. A plane of symmetry is generated (arrow) in each case. (C) Diagram of the final limb pattern if no loss of positional values around the plane of symmetry occurs during distal outgrowth. This diagram represents limb 2-lt illustrated in Fig. 4, with two supernumerary digits. (E) Diagram of the final limb pattern assuming some loss of positional values around the plane of symmetry during outgrowth. In this example, positional values 6 and 7 have been lost. This diagram represents limb 5-lt illustrated in Fig. 4, with one supernumerary digit.

the absence of dorsal–ventral discontinuities (Muneoka *et al.* 1985) and in the presence of Type I or Type II discontinuities are related to the degree to which cellular interactions are stimulated in the graft–host junction in the different cases.

The third line of evidence concerns the degree to which cells originating on one side of the limb stump become mixed with cells on the opposite side during regeneration. When no central discontinuity is present in the original stump, Muneoka *et al.* (1985) found that 24% of the cells become displaced to the opposite limb half during regeneration. In this study, we have shown that cellular displacement varies according to whether an originally central discontinuity is maintained, regenerated in a more peripheral position or lost entirely. When discontinuities are maintained (as is the case at most Type II discontinuities), cellular displacement is reduced to an average of 16%. Almost all Type I discontinuities are either eliminated or become peripheral. When discontinuities are eliminated, cellular displacement is twice that of a normal limb regenerate (48%). Cases in which the discontinuity is located peripherally show about the same amount of cellular displacement as in normal limbs, i.e. 23% (Table 5). Our interpretation of these differences in the degree of cellular displacement when compared to regeneration from chimaeric limb stumps with normal anatomy is that they reflect different degrees of cellular interaction stimulated by the juxtaposition of dorsal and ventral tissue at Type I and Type II discontinuities.

The final line of evidence that cellular interactions leading to pattern continuity occur at Type I discontinuities is the fact that of the 16 such discontinuities examined after regeneration, only one was maintained as a central discontinuity. The remainder either (1) were lost, thereby restoring pattern continuity, (2) formed a supernumerary outgrowth, thereby restoring pattern continuity (see Fig. 5), or (3) formed a peripheral discontinuity, which, as we argue below, also restores pattern continuity. In Fig. 6 we illustrate two possible routes by which Type I discontinuities can become resolved. Regardless of mechanism, it can be seen that although appearing as discontinuities in muscle pattern, peripheral discontinuities can in fact be viewed as continuous patterns when positional values are assigned to different regions of the limb circumference (French *et al.* 1976; Bryant *et al.* 1981) (see Fig. 6C,E). One mechanism by which pattern continuity would be achieved is shown in Fig. 6A–D. Intercalation at a Type I discontinuity occurs during early outgrowth, and midline loss of positional values along the plane of symmetry generated by this intercalation accompanies outgrowth (Fig. 6B). Depending on the degree of midline loss, the final continuous pattern will shown either a peripheral discontinuity (Fig. 6C), or no discontinuity (Fig. 6D). Without midline loss, supernumerary digits would form as shown in Fig. 5. Plots of the position of the diploid–triploid boundary at different proximal–distal levels in the regenerate support the idea that the process of change from a central to a peripheral discontinuity occurs gradually as would be predicted if midline loss occurred during distal outgrowth. As can be seen from Fig. 6, if the intercalated region were contributed to equally by diploid and triploid tisues, after midline loss, there would be an appearance of invasion of one region

by the adjacent region. Hence, our results which show that changes in the pattern are accompanied by changes in ploidy of that region can be understood as a consequence of intercalation and midline loss. Turning to the second possible mechanism, the final pattern can also be accounted for if not all parts of the original circumference participate in blastema formation. The cells which do participate could intercalate to form either a peripheral discontinuity or no discontinuity (see Fig. 6A,E,F). Hence, resolution of the pattern discontinuity by



this means would also involve interactions. As in the previous example, changes in the pattern would be accompanied by changes in ploidy, such as we have observed in our results. The fact that the position of the boundary at different proximal–distal levels within the regenerate in some cases is peripheral from the outset, suggests that this second mechanism can account for some of the results. Regardless of whether one or both of these mechanisms are operative, it is clear that the final pattern in limbs with either a peripheral discontinuity or with no discontinuity can be considered to be continuous.

Lack of interaction at Type II discontinuities

Despite the evidence for regulation and the restoration of pattern continuity at Type I discontinuities, little or no cellular interactions appear to take place at Type

Fig. 6. Interpretation of the resolution of Type I discontinuities either by intercalation and varying degrees of loss of positional values around the plane of symmetry (left) or by incomplete participation of stump cells followed by intercalation (right). In all diagrams, circumferential positional values are indicated around the outlines. Positional values of host are larger than those of graft. Digits are indicated by circled numbers inside the outlines. Muscles are indicated as dorsal (D) and ventral (V). Anterior (*A*) and posterior (*P*) positions are also indicated. Intercalated positional values are connected by dashed lines, and both host and graft are assumed to participate equally to the intercalated sequences. Planes of symmetry generated by intercalation are indicated by arrows. (A) Diagram of a section through a limb stump with an anterior graft. (B) Diagram to show intercalation at the Type I discontinuity. Only one possible route of intercalation is illustrated. The graft is assumed to provide positional values 5, 4 and 3; host, positional values 1 and 2. Inner bracket indicates extent of midline loss leading to (C) and larger bracket the extent of midline loss leading to (D). (C) Diagram to show pattern resulting from a small amount of midline loss during outgrowth. This amount of loss would result in resolution of the Type I discontinuity, and the appearance of a peripheral discontinuity (*) between digits **3** and **4**. A consequence of this interpretation is that the majority of cells in the region where the pattern has changed (i.e. over digit **3**) will be primarily of the ploidy of the opposite limb half. (D) Diagram to show the pattern resulting from a larger amount of midline loss of positional values during distal outgrowth. This amount of loss would result in elimination of the Type I discontinuity, and the change in the muscle pattern over digits **3** and **4** from dorsal to ventral. As in the previous case (C), the majority of cells in the region of the pattern change (i.e. over digits **3** and **4**) will be primarily of the ploidy of the opposite limb half. (E) Diagram to show the pattern resulting from the failure of some parts of the stump to participate in blastema formation (represented here by failure of host positional values 12 and 1 to participate), accompanied by intercalation between confronted values (in this case, positional value 6 of graft and 2 of host). Graft and host are assumed to contribute equally to the intercalated sequence. The resulting sequence of positional values is continuous, and leads to a peripheral discontinuity (*) between digits **3** and **4**. This mechanism would lead to the region of pattern change (over digit **3**) being contributed to primarily by cells from the opposite limb half. (F) Diagram to show pattern resulting from failure of some parts of the stump to participate in blastema formation (represented here by the failure of host positional values 12, 1 and 2 to participate), accompanied by intercalation between confronted values (in this case, positional value 6 of the graft and 3 of the host). Graft and host are assumed to contribute equally to the intercalated sequence. The resulting sequence of positional values is continuous, and leads to the elimination of the original Type I discontinuity. This mechanism would lead to the region of pattern change (i.e. over digits **3** and **4**) being contributed to primarily by cells from the opposite limb half.

II discontinuities. Only one case formed a normal pattern of muscles, and this limb was subsequently found to consist only of host cells, indicating graft failure. A further three cases regenerated peripheral discontinuities, and one formed a supernumerary outgrowth, but the remaining eleven discontinuities were maintained centrally in the regenerate. We believe this result, in combination with the behaviour at Type I discontinuities, argues for the existence of circumferential tissue polarity which results in a directional bias to intercalation.

In a previous study on the cellular origin of supernumerary limbs (Muneoka & Bryant, 1984*a*) we argued that the results indicated a directionality to circumferential intercalation, such that anterior tissue tends to form ventral tissue, and posterior tissue tends to form dorsal tissue. When this directionality is indicated by arrows on the experimental limbs in the present study, with the arrowheads pointing in the direction in which contribution tends to occur (Fig. 2), it can be seen that at Type I discontinuities, either anterior–ventral or posterior–dorsal tissue should be able to intercalate. Our evidence suggests that circumferential intercalation does occur at Type I discontinuities and that anterior–ventral and posterior–dorsal tissues contribute to the intercalated region. In addition, since in the majority of cases it is the originally dorsal tissue which tends to be 'replaced' by ventral tissue, the intercalary events depicted in Fig. 6 must also have a directional bias, with intercalation between opposed dorsal and ventral at Type I discontinuities involving posterior rather than anterior positional values. However, at Type II discontinuities, neither anterior–dorsal nor posterior–ventral is positioned so as to intercalate, and our evidence suggests that circumferential intercalation usually does not occur at Type II discontinuities. We do not at present know what cellular properties could underlie this proposed directionality, but we feel that experiments such as the one described here clearly point to some tissue polarity properties which tend to favour or inhibit intercalation. It seems likely that the behaviour of cells along the proximal–distal axis, where grafts of distal to proximal lead to intercalation but grafts of proximal to distal do not (Iten & Bryant, 1975; Stocum, 1975; Pescitelli & Stocum, 1980), also illustrates a similar relationship between directional intercalation (in this case, proximal to distal) and tissue polarity, such that cells at some specific graft–host junctions fail to interact with one another.

Taken as a whole, the results of this study support the idea that circumferential intercalation can have a directional bias. This idea was first suggested by Muneoka & Bryant (1984*a*) to account for the pattern of cellular contribution to supernumerary limbs following contralateral grafts of blastemas or limb buds. Both of these studies differ from the situation during normal limb outgrowth from a half diploid, half triploid limb stump, where we did not detect a directional bias in cellular contribution, and where the proportion of cells crossing from one limb half to the other was the same in both directions (Muneoka *et al.* 1985). The major experimental difference between the normal limb study, the present paper and that of Muneoka & Bryant (1984*a*) is that in the latter two instances, major positional disparities were created, whereas in the former they were not. Hence, it

is possible that a strong directionality to intercalation is only triggered by the existence of major discontinuities. We suggest that this behaviour could account for the original establishment of the limb pattern, starting with only anterior and posterior boundaries of the prospective field. Directional intercalation between confronted anterior and posterior values could result in the unambiguous formation of dorsal and ventral positional values, and hence in the formation of all the circumferential values of the limb base.

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