233

The cellular contributions of blastema and stump to 180° supernumerary limbs in the axolotl

By M. MADEN AND K. MUSTAFA

Division of Developmental Biology, National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA, U.K.

SUMMARY

By inverting triploid blastemas onto diploid stumps (and vice versa) the cellular contributions to supernumerary limbs so generated have been assessed. The four classes of 180° supernumerary limbs each had a different mixture of stump and blastemal cells. The mesoderm of normal supernumeraries was composed entirely or almost entirely of stump cells and were always of stump handedness. The mesoderm of symmetrical supernumeraries was of variable composition, it could be mostly stump, mostly blastema or half and half. In part normal/part symmetrical supernumeraries the normal part was usually of stump origin and the abberant symmetrical part of blastemal origin. In part normal/part inverted supernumeraries the normal part came from the stump and the inverted part from the inverted blastema. The handedness of each part of these supernumeraries corresponded with its cellular origin. The epidermis of the supernumeraries was not of the same relative composition as the mesoderm, it tended to have a larger stump component. The black/white marker was also used and this too tended not to conform to the mesodermal contribution patterns. These results are discussed in terms of rules for generating supernumeraries and it is concluded that with the exception of symmetrical supernumeraries the cellular contributions of stump and blastema determine their structure.

INTRODUCTION

Supernumerary limbs in amphibians can be produced either by grafting blastemas from one limb stump to the contralateral side (Iten & Bryant, 1975; Tank, 1978; Maden, 1982) or by turning blastemas upside down and replacing them on the same stump (Bryant & Iten, 1976; Maden & Turner, 1978; Wallace & Watson, 1979; Maden, 1982). The former operation results in the reversal of a single transverse limb axis – either the anteroposterior (AP) or the dorsoventral (DV) axis – and supernumerary limbs appear at the points of maximum discontinuity. Analyses of their morphology by whole-mount cartilage staining and then serial sectioning to examine the muscle patterns have revealed that AP and DV supernumeraries are of normal structure and of stump handedness (Maden, 1982). The latter operation results in the reversal of both transverse limb axes and produces supernumerary limbs (180° or APDV supernumeraries) which are not so consistent. They are normal in the anteroposterior axis, as revealed by cartilage staining, but often abnormal in the dorsoventral axis, as revealed by muscle patterns in sections (Maden, 1980;

Tank, 1981; Maden, 1982; Papageorgiou & Holder, 1983). Four classes of structure have been found (Maden & Mustafa, 1982). These are:– normal, symmetrical (double dorsal or double ventral), part normal/part symmetrical and part normal/part inverted. An example of each class of limb is shown in Figs 5, 6, 7 & 9.

Most contemporary models of pattern formation are quite incapable of explaining this diverse behaviour, particularly the last two classes of structure. These contain gross discontinuities within the limb, thereby contravening the 'principle of continuity' which underlies many models. Furthermore, not only do such limbs develop in the first place, but they also regenerate and in doing so regenerate the same discontinuities (Maden, 1982). One model which has encompassed most of these features is the boundary model of Meinhardt (1983) and this is discussed in more detail later.

In order to continue testing these models of pattern formation and to advance our understanding of complex supernumerary limbs, one question clearly needs answering. This concerns the precise contributions of blastemal cells and stump cells to such limbs. In particular, do the lines of discontinuity in pattern coincide with boundaries between cellular contributions? If so then supernumerary limb production would seem to be a relatively mosaic phenom-enon with the particular class of structure produced determined solely by its relative contributions. On the other hand, the contributions could be the same or random in all supernumeraries. This situation would thus involve changes in the fate of the contributing cells imposed upon them by pattern-forming mechanisms arising *de novo* after blastemal rotation.

This is the subject of the work reported below. After exchanging blastemas between diploid and triploid animals relative contributions to supernumerary limbs can be assessed by staining for nucleoli. Grafts have also been exchanged between black and white animals. This genetic colour marker depends upon the presence (in black tissue) or absence (in white tissue) of melanophores. It cannot identify the origin of individual cells as the nucleolar marker can and so its reliability as a tissue marker can be assessed when the two are used in combination. It is shown that cellular contribution boundaries do coincide with pattern discontinuities in most of the types of supernumeraries, but not necessarily in the symmetrical ones. Thus the generation of supernumerary limbs seem to be, in general, a mosaic behaviour of blastemal cells.

MATERIALS AND METHODS

All experiments were performed on larval axolotls, *Ambystoma mexicanum*, 70–120mm in length. Triploid animals were produced as described by Slack (1983) with a pressure shock of approximately 6,000 psi for 6 minutes. This treatment was administered to naturally fertilized eggs 1 hour after fertiliza-tion. Surviving animals were checked for triploidy with tail tip squashes.

Triploid induction was performed on the eggs from a mating of two white parents, triploids were thus white larvae. A normal batch of diploid, dark larvae of the same age were reared for grafting between the two. When the animals had reached the size of about 70mm all four limbs were amputated through the mid-stylopodium and blastemas allowed to develop. At the late cone/palette stage blastemas were rotated between white triploid and black diploid larvae, forelimb to forelimb and hindlimb to hindlimb, so that 180° ipsilateral (APDV) rotations resulted. This produced 180° white 3n blastemas on black 2n stumps and vice versa. After 1 h at 4 °C to facilitate healing of the grafts the larvae were returned to individual tanks. They were allowed to develop for about 8 weeks until well-formed supernumerary limbs were apparent. All limbs with supernumeraries present were drawn with a camera lucida and the position of the supernumeraries noted along with their digital sequences. They were then removed from the larvae and fixed in 5ml of 37% formol, 5ml of glacial acetic acid and 90ml of 80% ethanol for 3|-|4 days.

After fixation the limbs were transferred to 70% alcohol and the area in which melanophores were present in the supernumeraries was plotted on the camera-lucida drawings. Fixation causes the epidermis to become transparent and the dermis opaque. Thus the melanophores which mostly accumulate at the dermal/epidermal boundary can easily be seen. The multiple limbs were then separated into individual components and processed for sectioning. They were embedded in wax and cut at 10μm. Transverse sections were cut starting at the tips of the digits and continuing up to the mid-carpal or mid-tarsal level. These sections, when stained could then be scored for muscle patterns (to identify the class of supernumerary) and the presence of triploid cells in the epidermis, dermis and the cartilage. To identify triploid cells in muscle, longitudinal sections are needed and so at this point the remaining tissue was re-embedded and cut in L.S.

Sections were stained with an improved silver stain elaborated by Rager, Lausmann & Gallyas (1979). The procedure is as published, the only modifications concern the mounting and storage of slides. After developing, the sections were dehydrated through alcohols, xylene and mounted in XAM. They were immediately placed in a dark box at 4 °C to prevent fading. Our normal technique of drying out coverslipped slides in an oven causes fading of stained nucleoli, which can be prevented for many months by the above treatment. Such staining results in nerve fibres and nucleoli appearing black against a yellow to pale brown background. Thus triploid cells can easily be identified (Fig. 1).

The sections were studied in several ways. Firstly the presence of melanophores was recorded and correlated with the maps drawn from camera-lucida representations of whole limbs. Secondly the muscle patterns were drawn and the class of supernumerary structure identified. Thirdly areas of triploid cells were plotted. Fourthly counts of percent triploid cells were made on several

control diploid limbs, grafted triploid limbs and supernumeraries. The following is an analysis of a total of 64 limbs so treated.

RESULTS

### Staining in normal diploid limbs

The Rager stain was devised for the reliable staining of nerve fibres in embryonic material (Fig. 1D), but we also discovered during the course of other experiments that it consistently stains nucleoli. Thus diploid and triploid cells can easily be identified in all tissues of the limb (Fig. 1).

At least 50 sections from three diploid limbs were searched to see if any nuclei contained three nucleoli. In each of the tissues scored – epidermis, muscle and cartilage, a uniformly low frequency of 1–2% was recorded in counts of 5000–7000 cells from each tissue (Fig. 2). This figure agrees with that reported by others (Pescitelli & Stocum, 1980; Muneoka, Wise, Fox & Bryant, 1984). Whether these represent errors due to staining of heterochromatin, as others have suggested (Pescitelli & Stocum, 1980) is not known, but such cells do look remarkably like triploids (Fig. 1A).

Counts of triploid limbs

The percentages of nuclei with three nucleoli were determined in the epidermis (Fig. 1B), cartilage (Fig. 1C) and muscle (Fig. 1D) of unamputated triploid limbs. Values of 25% (epidermis), 25% (cartilage) and 27% (muscle) were recorded (Fig. 2). These are in agreement with most of the percentages obtained by other investigators who have used the triploid marker, but with different stains. Pescitelli & Stocum (1980) recorded 22% 3n in triploid cartilage, Namenwirth (1974) recorded 20% in cartilage and 21% in epithelium and Dunis & Namenwirth (1977) recorded 18% in epidermis and 25% in cartilage. However corresponding values for muscle seem to differ somewhat: 34–40% (Namenwirth, 1977) and 38–56% (Dunis & Namenwirth, 1977). Occasional individual values of this magnitude were observed here, but the average was significantly lower (27%). Perhaps the difference is due to the smaller number of cells that Namenwirth counted (836) compared to 5000–7000 counted here. More recently, Muneoka, et al (1984) have obtained

Fig. 1. Rager-stained sections of diploid (A) and triploid (B, C & D) tissues. (A) diploid (2n) epidermis with most cells having one or two dark-staining nucleoli except for one cell (arrow) which has three. These cells constitute the low percentage of triploid-like cells in diploid limbs (see Fig. 2). (B) Triploid (3n) epidermis . (C) 3n cartilage. (D) 3n muscle. This section also shows that the method stains nerve fibres (arrows). Bar = 50μm.
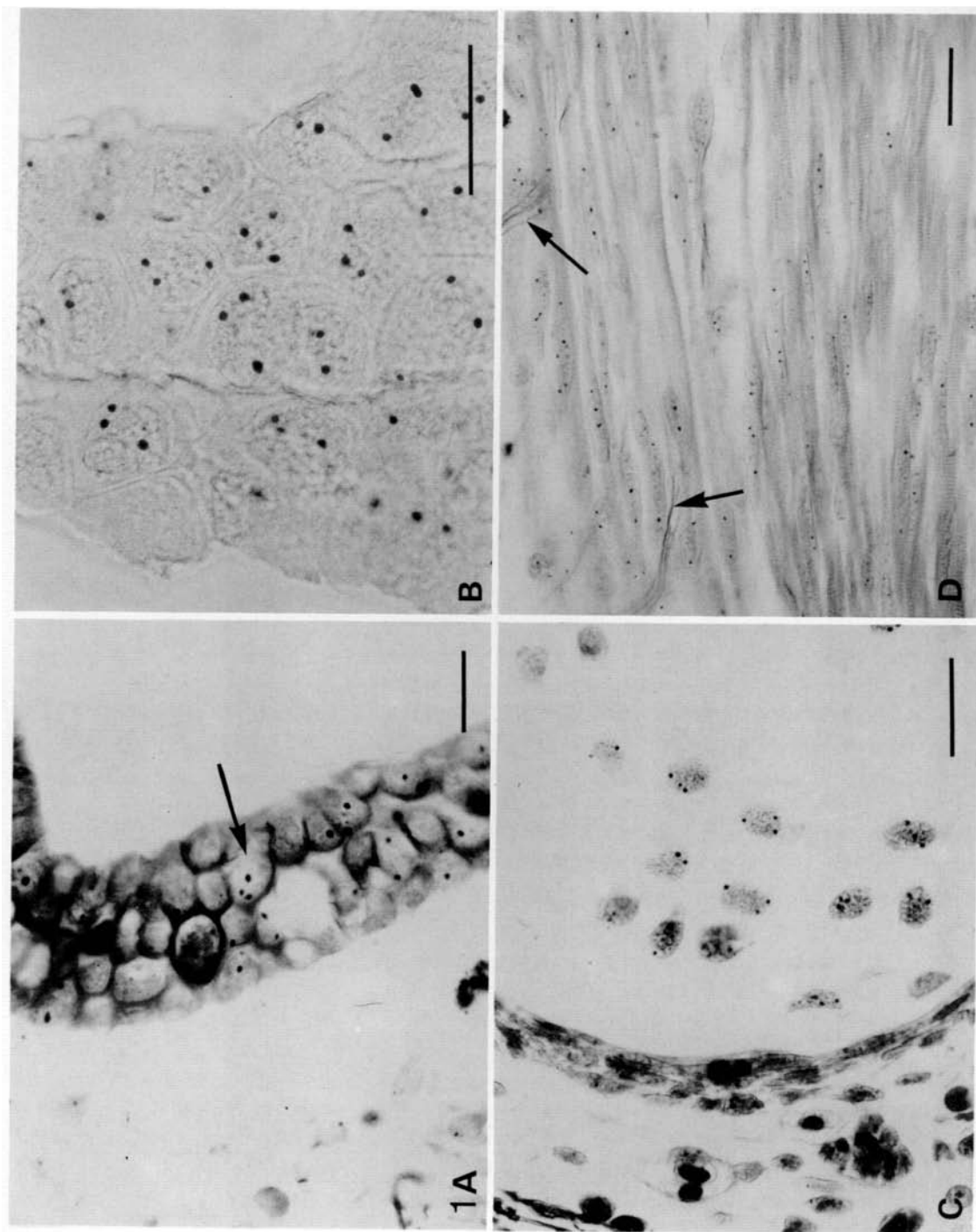
values of 37–49% for cartilage and 30–76% for dermis. These authors have concluded that the frequency of 3n cells varies considerably from animal to animal, perhaps this is the reason for the discrepancies.
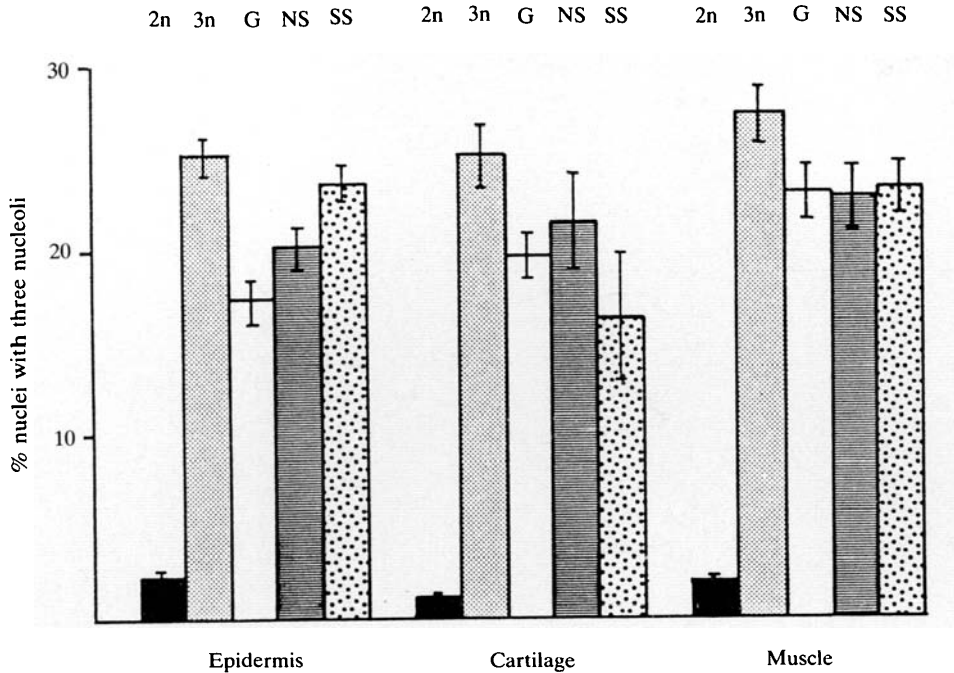

Fig. 2. The percentage of nuclei with three nucleoli in epidermis, cartilage and muscle of normal diploid (2n-solid columns), normal triploid (3n-dotted columns), grafted triploid limbs (G-open columns), a normal supernumerary limb generated by rotating a 2n blastema on a 3n stump (NS-cross hatched columns), and a symmetrical supernumerary limb generated by rotating a 2n blastema on a 3n stump (SS-large dots in columns). Each column represents a total count of 5000–7000 cells from one, two or three limbs. Bars represent standard errors.

Grafted limbs

The grafted blastemas of opposite polarity and colouration to the stump develop into complete limbs themselves as well as participate in the induction of supernumerary limbs (Fig. 3). We can therefore extract useful information from sections of these limbs with regard to any cellular invasions from the stump or alterations in the normal pattern. A total of 22 grafted limbs was analysed.

Is the epidermis of the graft replaced by the stump epidermis? In view of the recent report by Lheureux (1983) that there is a constant distal migration of epidermis down the limb, we might expect the epidermis of the graft blastema to be replaced by that of the stump. Of the 22 cases sectioned, 17 (77%) had

epidermis of graft origin, that is, no significant contribution from the stump. This was assessed by examining the sections for the presence or absence of 3n cells rather than detailed counts of each case. However, counts of the epidermis of two triploid grafts confirmed this conclusion (Fig. 2) although a minor contribution may have caused the slight increase from normal (17% *versus* 21–25%) recorded in the histogram (column 3). Three (14%) had areas of stump invasion and in two (9%) cases stump epidermis had completely replaced that of the graft. Since the grafted blastemas had been allowed to develop for 8 weeks it is likely that we had just begun to see the results of distal
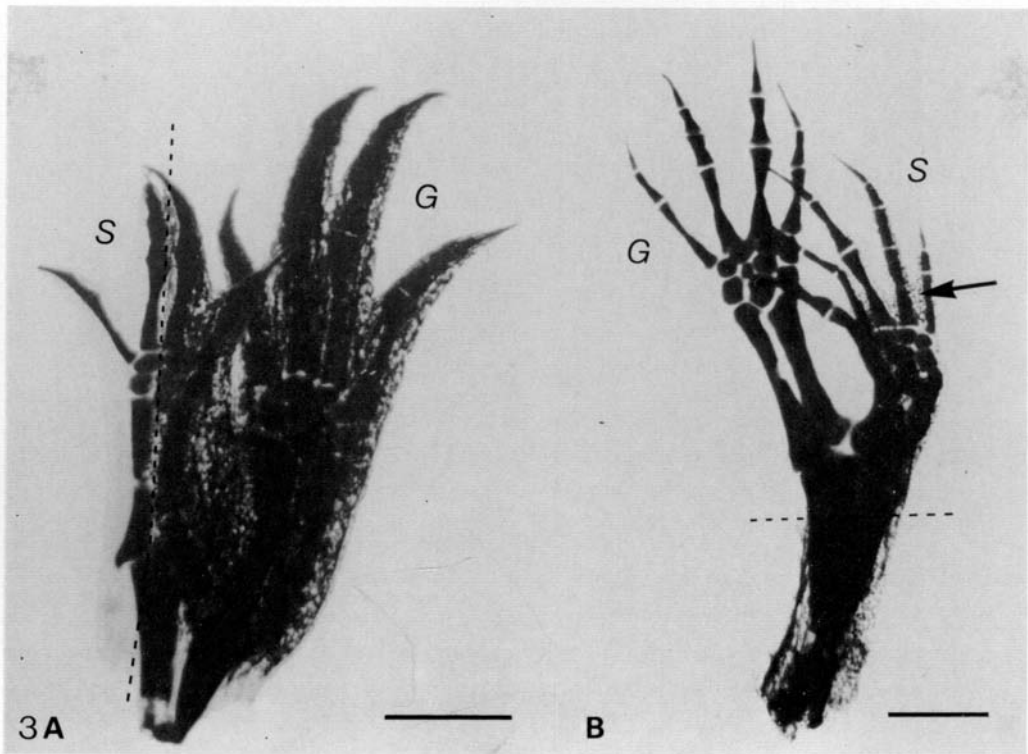

Fig. 3. Victoria-blue-stained whole mount of 180° supernumerary forelimbs to show the use of the black/white marker. (A) The result of a blastema from a black larva grafted onto a white stump. The grafted blastema develops into a normal, but upside down black limb *(G)*, packed with melanophores. A supernumerary limb *(S)* of normal cartilage structure developed consisting of both black and white tissue i.e. formed from both stump and graft cells. The boundary between black and white tissue runs almost exactly down the middle of the limb (dotted line). The white tissue on the left of the line has no melanophores in it. (B) The converse situation, a white graft blastema (G) grafted onto a black stump. The supernumerary limb is again composed of both black and white tissue. Melanophores (arrow) are present in the right (posterior), side of the supernumerary. The dotted line marks the blastema/stump boundary. Bar = 1mm.

migration of epidermis. Presumably if the limb had been fixed later a greater number of grafts would have been covered by stump epidermis.

Does the stump contribute mesodermal cells to the grafted limb? This was most easily analysed in diploid grafts onto triploid stumps of which there were ten cases. In all but one the presence of triploid cells was detected. They were reliably found comprising the walls of the blood vessels, as Schwann cells or as connective tissue sheath cells of the nerve bundles, areas in which stump cells might be expected. But they were also detected in other limb tissues such as cartilage at a frequency (approximately 1%) no greater than the number of trinucleolate cells in a normal diploid limb (Fig. 2). Thus we could not distinguish between a small, but real contribution and no contribution at all. Counts from the muscle and cartilage of triploid grafts onto diploid stumps produced similar values to normal triploid limbs (Fig. 2).

Do melanophores from the stump invade grafts of white blastemas? Eleven cases were of such a combination and ten of them did not have any melanophores. One limb had quite a large black patch within it demonstrating that the black/white marker is good but not totally reliable.

Is the muscle structure of the grafted limb always normal? We would expect the graft to develop into a normal, albeit upside down limb. All of the 22 limbs analysed did indeed have perfectly normal and inverted muscle patterns.
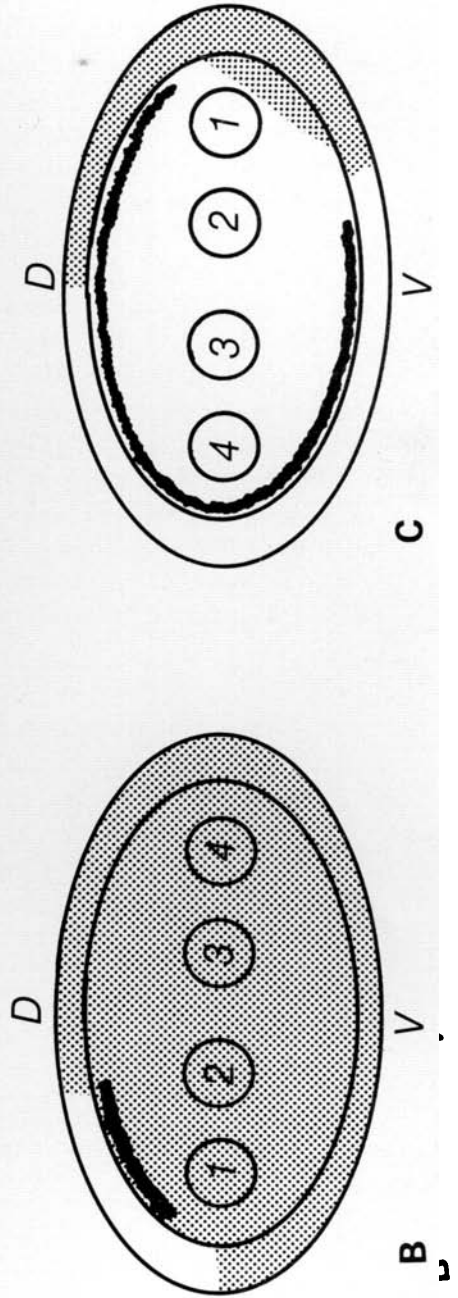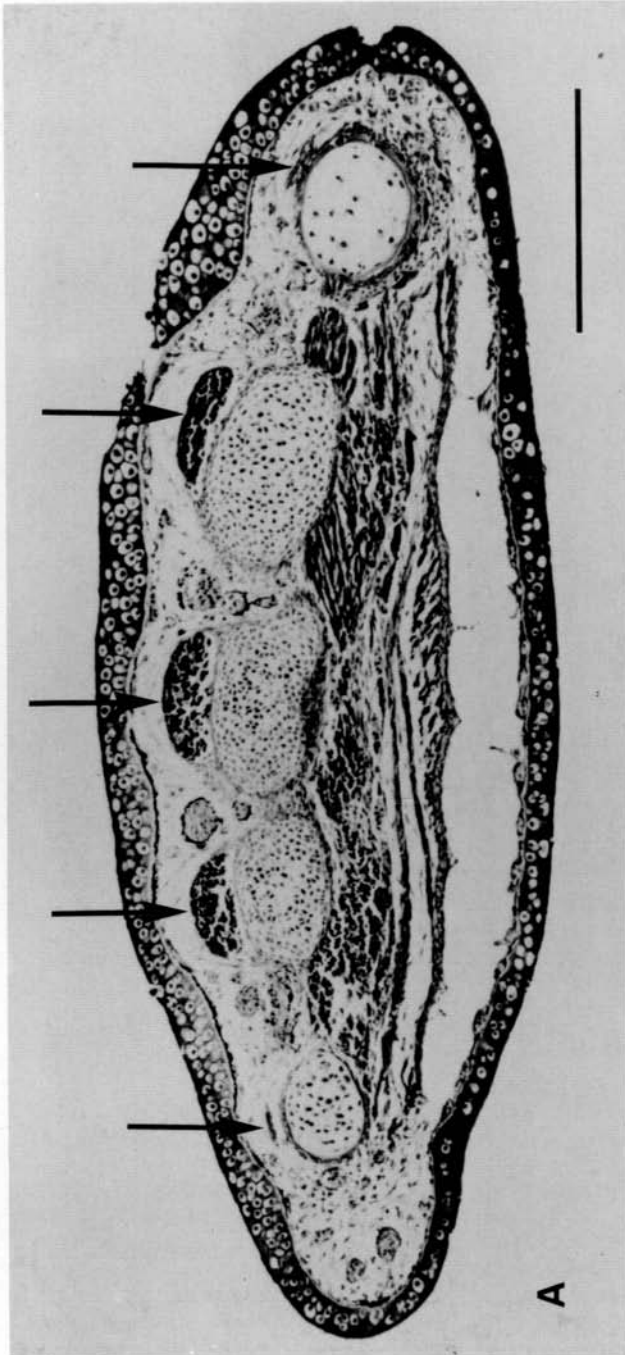
Supernumerary limbs

A total of 36 supernumerary limbs was analysed whose muscle patterns were of the four classes previously identified (Maden & Mustafa, 1983). These were 8 normals, 6 symmetrical, 17 part normal/part symmetrical and 5 part normal/part inverted. The details of the cellular contributions to each class will be described in turn.

Class 1 – normal supernumeraries.

There were eight supernumeraries of normal structure (Fig. 4A), arising either in the posterodorsal quadrant or the anteroventral quadrant. All eight of

---

Fig, 4. Normal supernumeraries – class 1. (A) Section through the metatarsal level of a normal hindlimb supernumerary showing the characteristic distribution of muscles (Rager staining). On the upper, dorsal surface of the limb the crescent shaped *extensores digitorum breves* (edb) sit on top of the cartilages (arrows). On the lower surface a large mass of 16 muscles stretches from side to side. Bar = 500μm. (B and C) Diagrammatic representations of the 2n: 3n contributions to two normal forelimb supernumeraries. Hatched areas = 3n, thick black lines represent melanophores in the dermis, outer elipse = epidermis, inner circles = cartilages. (B) A normal supernumerary from 2n blastema rotated a 3n stump. All the mesoderm plus most of the epidermis came from the stump, despite a small strip of melanophores in the dermis. (C) A normal supernumerary from a 3n blastema rotated on a 2n stump (the converse of B). All of the mesoderm except for a small patch on the anterior side is of 2n stump origin, a large segment of epidermis is of 3n blastemal origin.

stump handedness. The mesoderm of six cases was composed exclusively of stump cells, despite having a small patch of melanophores (or absence of melanophores as the case may be) on the side of the supernumerary adjacent to the blastema (eg. Fig. 4B). The other two had a small area of mesoderm of blastemal origin, also on the side adjacent to the blastema. This small patch, composed of connective tissue and dermal cells, was distinct but obviously not significant enough to cause any change in pattern.

The epidermis of seven of these supernumeraries contained a contribution from the blastema, again on the side adjacent to it. In one case (Fig. 4C) the contribution was quite extensive, approximately 30%. Thus the pattern of cell mixing in the epidermis bore no relation to the mesodermal contributions.

As mentioned above, each supernumerary also had an area of dermis with or without melanophores in it. The six cases composed exclusively of stump cells revealed that the melanophore pattern was not a reliable indicator of meso-dermal cell contributions. Instead it reflected cellular contribution patterns of the epidermis (Fig. 4B).

It was easy to establish that the 2n supernumeraries (eg. Fig. 4C) were of stump origin because of the absence of 3n cells. To establish the converse, one of the other cases (eg. Fig. 4B) was counted and the results (Fig. 2 NS columns) confirmed that the frequency of 3n cells in epidermis, cartilage and muscle was within the normal range of control triploid tissue.

Thus we can conclude that the mesodermal tissues of normal supernu-meraries are mostly or entirely composed of stump cells.

Class 2 – symmetrical supernumeraries.

Six supernumeraries were perfectly symmetrical, four double dorsal (Fig. 5A) and two double ventral (Fig. 5B). The mesodermal contributions to these supernumeraries were not consistent. The majority (four out of six) were

---

Fig. 5. Symmetrical supernumeraries – class 2. (A) Section through the metacar-pals of a double dorsal supernumerary showing the crescent-shaped edb on both sides of the limb (Rager staining). (B) Section through the metacarpals of a double ventral limb showing the large ventral muscles fusing in the mid line to form a continuous tube of muscle down the digits. No edb are present (Rager staining). Bar = 500μm. (C–E) Diagrammatic representations of 2n: 3n contributions to three examples of supernumeraries. Same key as Fig 4. (C) A double dorsal supernumerary from a 2n blastema rotated on a 3n stump. All the epidermis and most of the mesoderm except for a strip of dermis (with melanophores in) came from the stump. (D) A double ventral supernumerary from a 3n blastema rotated on a 2n stump. Most of the mesoderm except for an upper strip of dermis and an anterior muscle came from the blastema in contrast to (C). All of the epidermis came from the stump. Note the presence of melanophores in 3n tissue in the lower dermis of the limb. (E) A double dorsal supernumerary from a 3n blastema on a 2n stump. Here the mesodermal contributions are virtually 50:50 along the mid-line. All the epidermis came from the stump and the melanophore distribution reflects the epidermal contribution not the mesodermal one.
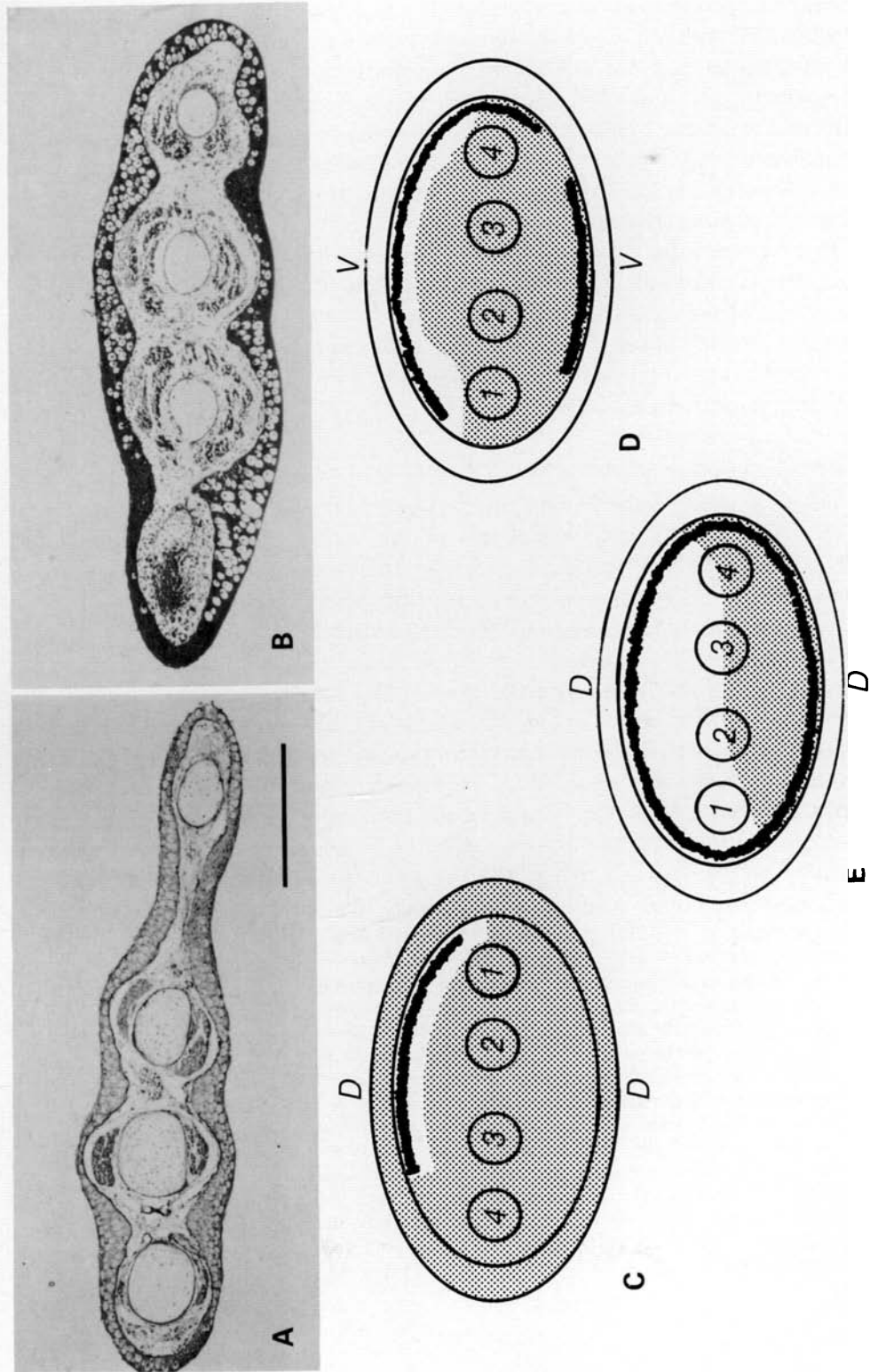
composed almost entirely of stump cells with the exception of a small strip of dermis of opposite ploidy running along the edge of the limb adjacent to the blastema (Fig. 5C). One of the remaining cases was composed almost entirely of blastemal cells, again with a strip of opposite ploidy, this time along the edge adjacent to the stump (Fig. 5D). The final case had an extensive mixture of cells and was nearly 50% stump/50% blastema cells with a boundary running approximately along the mid-line (Fig. 5E). One of the supernumeraries which was composed almost entirely of 3n cells was counted. The results (Fig. 2 SS columns) demonstrate that this is so.

Thus symmetrical supernumeraries do not seem to be formed in a particular way. They can be half stump/half blastema along the mid-line or mostly one or the other. However they do not seem capable of being formed exclusively by stump or blastemal cells, a strip of dermis of opposite ploidy at least needs to be present. This interesting observation was also seen in two cases of class 3 supernumeraries (see below).

Class 3 – part normal/part symmetrical supernumeraries.

There were 17 limbs in this category, 16 part normal/part double ventral (Fig. 6A) and one part normal/part double dorsal. Again, neither the cell contributions to the epidermis nor the melanophore distributions bore a consistent relation to the pattern formed by the mesoderm.

By examining the mesodermal cell contributions it was immediately obvious how these complex supernumeraries were formed. In 15 of the cases the normal part of the limb was entirely of stump origin and formed the largest component. For example in Fig. 6B, a limb which is in half normal/half double ventral, three quarters of the mesoderm is of stump origin and one quarter (the two aberrant ventral areas) is of blastemal origin. Whether this blastemal tissue included the relevant cartilage elements or not was variable. The same principle is exemplified in Fig. 6C, a limb which is three quarters normal/one quarter double ventral, that is the normal area is of stump origin and the aberrant ventral part is of blastemal origin. It is worth noting that the same

Fig. 6. Part normal/part symmetrical supernumeraries – class 3. (A) Rager-stained section through the metacarpal level of a supernumerary showing the two digits on the left as double ventral and the two digits on the right as normal. Arrows mark the edb muscles in the normal digits. This is therefore half normal/half 2V. Bar = 500µm. (B–D) Diagrammatic representation of the 2n:3n contributions to three examples of supernumeraries. Same key as Fig 4. (B) A half normal/half 2V supernumerary from a 3n blastema rotated on a 2n stump. The normal part is all of stump origin and the two aberrant ventral muscles of the 2V part are of blastemal origin. (C) Same graft as B which produced a three quarters normal/one quarter 2V supernumerary. Here only the single aberrant ventral muscle is of blastemal origin. (D) A three quarters/one quarter normal supernumerary produced from a 2n blastema rotated on a 3n stump. Here the converse principles apply, the stump produced the symmetrical part (as in class 2) and the blastema the normal part.
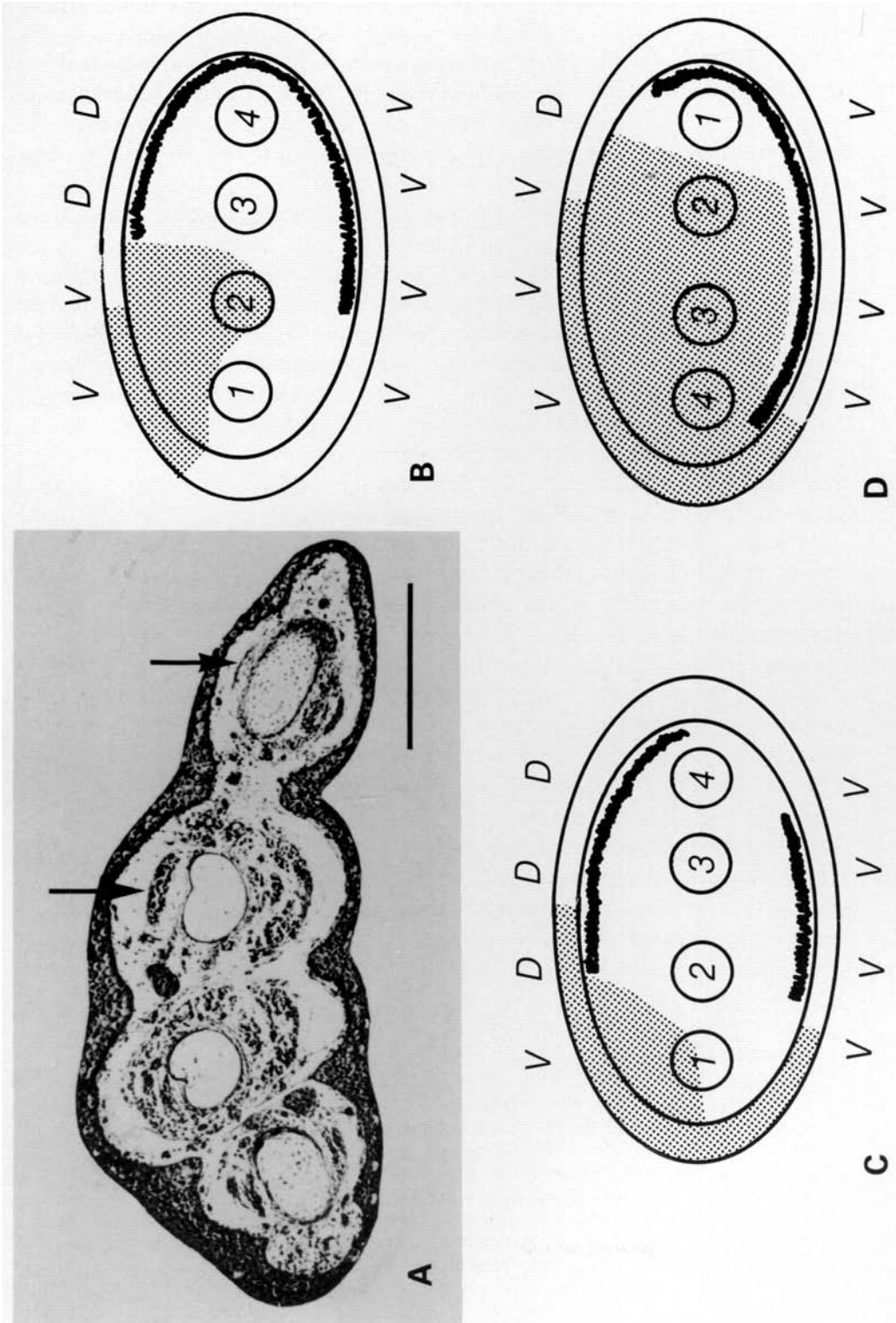
principles applied to hindlimb supernumeraries, but having five digits instead of four (see Fig. 4A) meant that the normal/symmetrical areas were assessed in fifths. Therefore the way in which these limbs are surely formed is that the stump begins to produce a supernumerary of its own accord (i.e. it would have been a normal supernumerary – class 1), but that during outgrowth the blastema contributes a quantity of tissue which changes the pattern of the limb in that area.

This conclusion was further substantiated by analysing the handedness of the normal part of the complex limbs. In every case this part was of stump handedness. In addition, in every case the normal part of the supernumerary was on the side adjacent to the stump and the symmetrical part was on the side adjacent to the blastema. This is exemplified in Fig. 7A & B with line drawings of two cases, showing the position of the supernumeraries and surrounding tissues.

The other two cases of part normal/part symmetrical supernumeraries followed opposite rules to those described above, but the same principles. Here, the symmetrical part of the limb was the largest component (i.e. they were one quarter normal/three quarters symmetrical) and the stump tissue formed the symmetrical part, not the normal part (Fig. 6D). Thus these were formed by the development of a fully symmetrical supernumerary of mostly stump origin (see class 2) which became altered by enough tissue from the blastema to cause a change in pattern. In both cases the normal part of the supernumerary was adjacent to the blastema and the symmetrical part was adjacent to the stump (Fig. 7C). The symmetrical part also contained a strip of dermis of opposite ploidy just as most of the fully symmetrical limbs did (see class 2). This observation tends to imply that the dermal strip plays some role in the generation of symmetrical supernumeraries.

Class 4 – part normal/part inverted supernumeraries.

There were five supernumeraries of this type, each being half normal/half inverted (Fig. 8A) and they behaved consistently. The epidermal cell contribution was not a reliable marker of mesodermal pattern and the black:white distribution tended to be more extensive than the ploidy patterns. That is,

Fig. 7. Diagrammatic representation of the origin and structure of three part normal/part symmetrical (class 3) supernumeraries. Outer large circle represents the stump, inner small circle (hatched) represents the rotated blastema and the elipse represents the supernumerary. (A) An anterodorsal supernumerary. The normal part of this limb was of stump origin, stump handedness (labelled right) and was adjacent to the stump. The aberrant ventral area (hatched) was of blastemal origin and adjacent to the blastema. (B) The same principles apply to this posterodorsal supernumerary. (C) This posterodorsal supernumerary followed opposite rules – the stump contributed the symmetrical part and the blastema the adjacent right-handed part. It is clear that the relative contributions determine the structure and handedness of the supernumeraries.
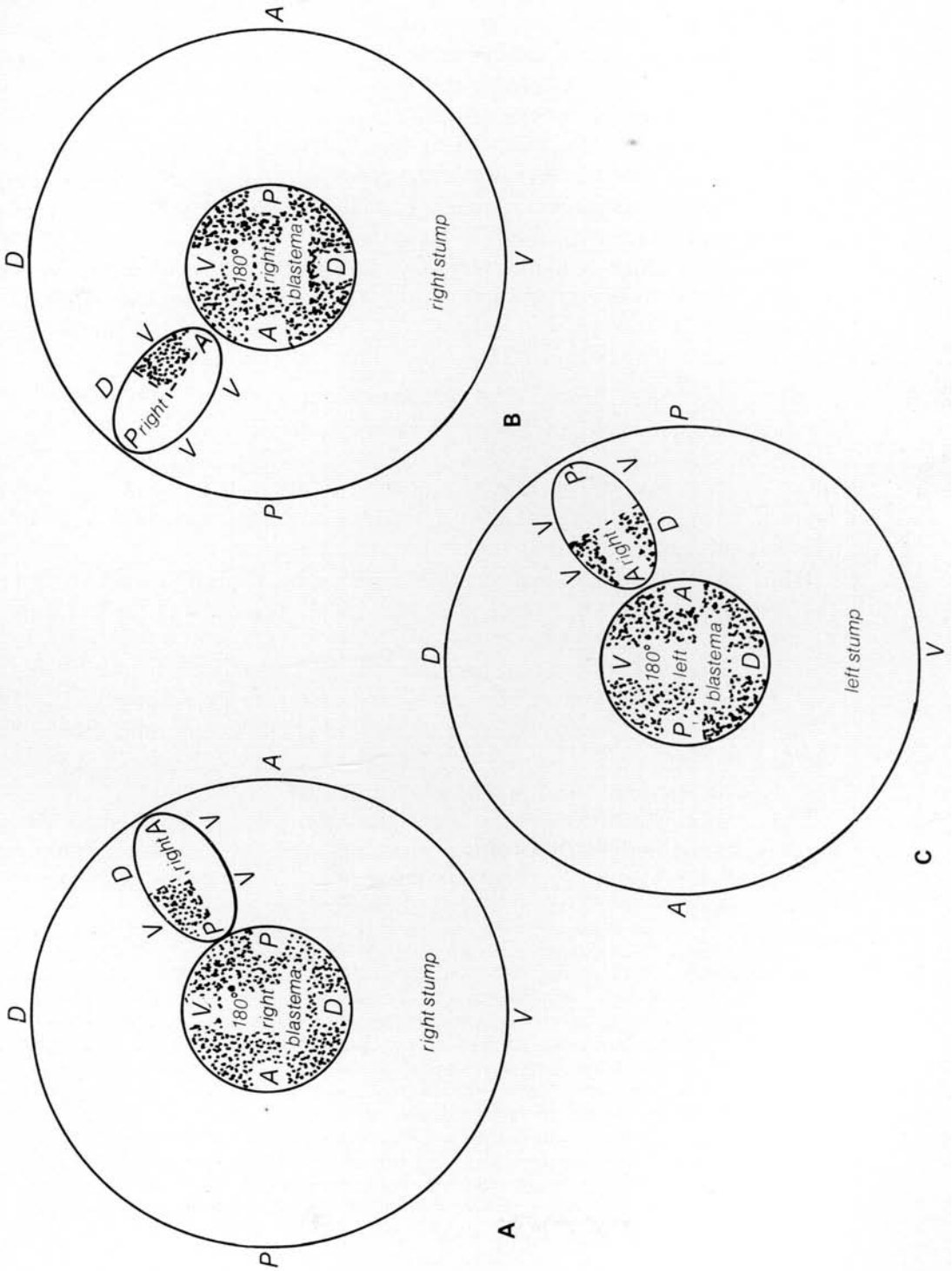
melanophores invaded 3n territory (Fig. 8B). The mesodermal contributions however were perfectly clear: the half of the limb adjacent to the stump (normal half) originated from the stump and the half nearest the blastema (inverted half) came from the blastema (Fig. 8B). The ploidy boundary was not exactly down the middle of the limb, but tended to be skewed. The handedness of each half of the supernumerary confirmed this analysis and an example is shown in Fig. 8C. The half of blastemal origin was of opposite handedness to the blastema (because the dorsoventral axes were the same, but the anteroposterior axes mirror imaged) and the half of stump origin was of the same handedness as the stump.

Thus we can conclude in part normal/part inverted supernumeraries the cell contribution boundary of the mesoderm coincides with the line of pattern discontinuity.

DISCUSSION

Once the characteristics of triploid staining and counting have been established it is a simple, if laborious matter to identify triploid and diploid areas of tissue within a limb. In this way cellular contributions to 180° supernumerary limbs have been determined after grafting rotated blastemas onto stumps of opposite ploidy.

Four types of supernumary structure have previously been identified (Maden & Mustafa, 1982) and were also found here. These are:– normal, symmetrical (double dorsal and double ventral), part normal/part symmetrical and part normal/part inverted. By using the triploid marker it was revealed that three of these four classes of supernumeraries have a consistent pattern of stump/ blastemal cell contribution. This leads to the following conclusion – the stucture of 180° supernumerary limbs is, in these cases, determined by the relative contributions of stump and blastemal cells.

Thus the mesoderm of all normal supernumeraries was found to be mostly or entirely of stump origin and of stump handedness. So the way these limbs were

Fig. 8. Part normal/part inverted supernumeraries – class 4. (A) Section through the metacarpal level (Rager staining). The digits on the left are inverted with small edb on the bottom (arrows) and strips of ventral on the top. The two digits on the right are in normal orientation with edb above (arrows) and ventral muscle below. Bar = 500μm. (B) Diagrammatic representation of the 2n:3n contributions to a supernumerary. Same key as Fig 4. A 3n blastema rotated on a 2n stump showing that the relative contributions to the mesoderm are virtually 50:50. The melanophores are slightly more extensive than would be expected and the epidermis, while being composed to both tissue bears no relation to the mesodermal pattern. (C) Diagrammatic representation of the origin and structure of the supernumerary. Same key as Fig 7. The part of the supernumerary adjacent to the blastema is of blastemal origin and of opposite handedness to the blastema and the part adjacent to the stump is of stump origin and of the same handedness as the stump.
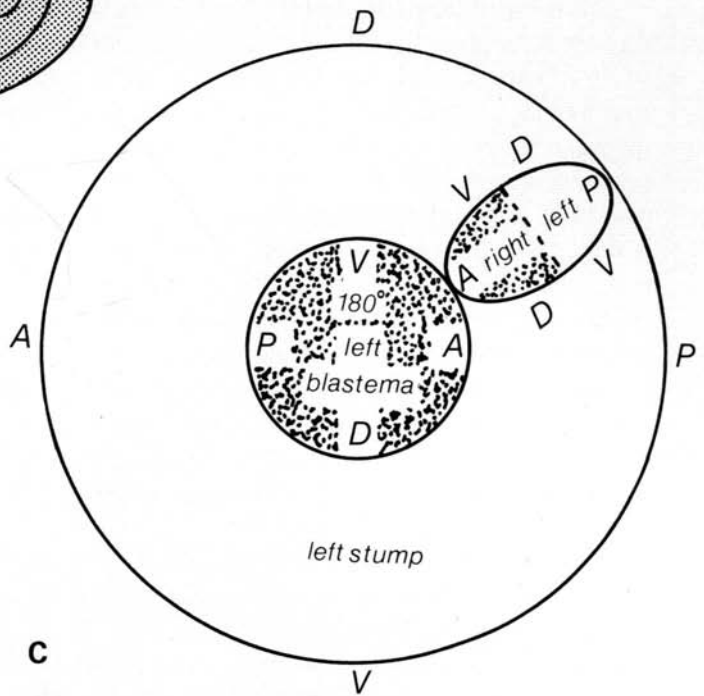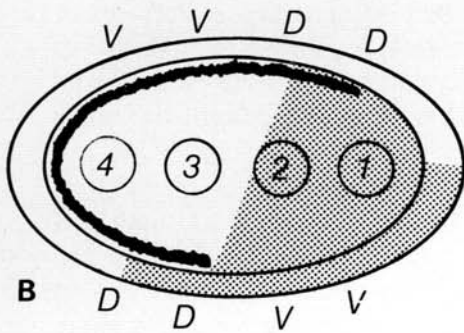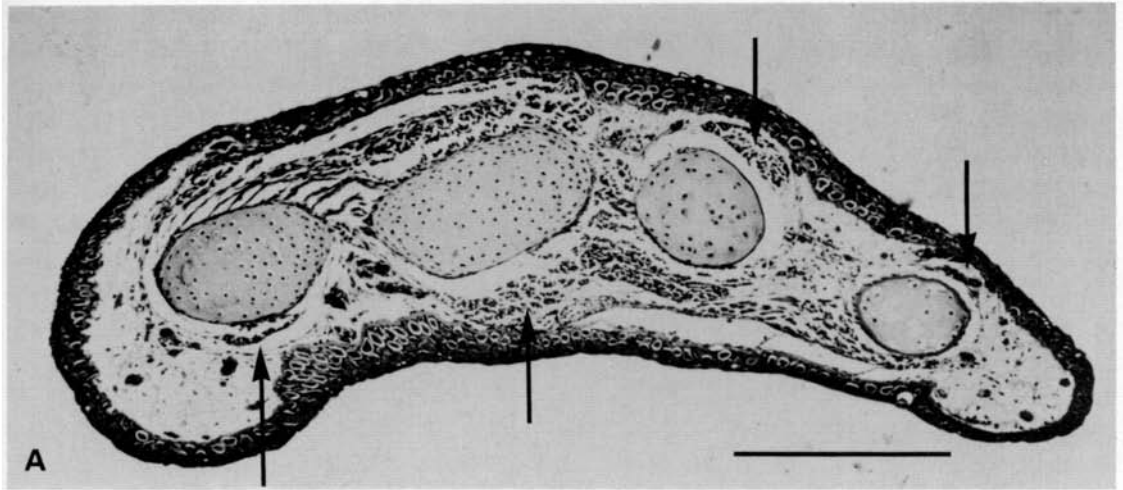
formed was presumably by the stump 'ignoring' the inverted blastema and producing a regenerate of its own. Most of the part normal/part symmetrical supernumeraries had their normal part of stump origin and handedness and the abnormal area generating the symmetrical muscles was of blastemal origin. So these must have been produced by the stump initiating a normal supernumerary, as above, but which during outgrowth incorporated enough blastemal tissue to change the pattern. If a larger amount of blastemal tissue is incorporated (half and half) then the supernumerary becomes part normal/part inverted, that is the fourth type of supernumerary structure. This was established because the line of pattern discontinuity coincided with the cell contribution boundary and the handedness of each part was consistent with its origin. Thus there is a gradient of cellular contributions from all stump (normal), through three quarter stump/one quarter blastema (part normal/part symmetrical), half stump/half blastema (part normal/part inverted) to all blastema (normal). As the relative contributions change, so the structure changes, therefore the contributions must determine the structure.

Examples of supernumeraries composed of all blastemal tissue were not, however, observed here. They must occur, albeit infrequently, since cases of two supernumeraries both of normal structure have been observed (Maden & Mustafa, 1982 – Table 2). They are always of opposite handedness so only one could have come from the stump (the one of stump handedness), the other must have come from the blastema.

The composition and mode of formation of the remaining class of supernumerary structure, the symmetrical double dorsal or double ventral limb was more enigmatic. One of the cases was 50% stump/50% blastema along the mid-line as might be expected, but the others were almost entirely of one origin or the other except for a strip of dermis of opposite ploidy running along the upper surface of the supernumerary. This type of arrangement was also observed in the two of the part normal/part symmetrical supernumeraries in which the largest area was symmetrical rather than normal. The dermal strip may, therefore, play some role in the generation of symmetrical limbs – perhaps the dermis can, during development of the supernumerary, instruct myoblasts that should have been dorsal (in the case of double ventrals) or ventral (in double dorsals) to become the opposite. Thus connective tissue could be responsible for pattern generation (Bryant, 1978) with myoblasts acting as submissive packing material. This certainly seems to be the case in chick limb development (Chevalier and Kieny, 1982) and clearly needs further investigation in amphibians.

In the above discussion, emphasis has been laid on the pattern of mesodermal cell contributions because it was apparent early on in this study that the epidermal cell contributions did not reflect the mesodermal ones. Thus, as Thoms & Fallon (1980) concluded, the epidermis behaves differently during regeneration and supernumerary limb formation. The most likely reason for

this is because of the constant distal migration of epidermis down the limb (Lheureux, 1983). This phenomenon was also observed here and it means that both supernumerary and grafted limb will ultimately become covered entirely by stump epidermis as the distal migration replaces grafted epidermis.

The black/white marker was used in addition to the ploidy marker in the above experiments. It was concluded that the melanophore pattern reflected the cellular contributions to the epidermis rather than to the mesoderm. Keller, Lofberg & Spieth (1982) have shown that the migration of pigment cells in the axolotl embryo is controlled by the ectoderm and the same seems to be happening in the limb. Thus because of the discrepancy in epidermal and mesodermal contribution patterns the colour difference is not a reliable marker of mesodermal structure as Muneoka & Bryant (1984) have concluded. Furthermore, because of the distal migration of epidermis down the limb the colour patterns will change with time.

It is interesting to compare these results with others using similar cell marking techniques in development and regeneration. Muneoka & Bryant (1984) examined the stump/blastema contributions to supernumeraries generated by AP reversal of axolotl regeneration blastemas using the diploid/ triploid marker. They concluded that both stump and blastema contributed to AP supernumeraries in approximately equal proportions. The same conclusion was reached for AP supernumeraries generated by contralateral grafting of limb buds. Thus with axolotls the results from limb development and regeneration are consistent. In addition, complex supernumeraries like those described above have also been found after 180° rotation of *Rana* limb buds (Maden, Gribbin & Summerbell, 1983) suggesting that these too are formed from both stump and limb bud tissue.

In chick limbs, however, the situation seems to be different. Using the chick/quail marking system, Gribbin (unpublished results) has found that both AP and 180° supernumeraries, rather than being mixed, are always composed of either one tissue or the other, depending on their position of origin. Anterior ones are of stump composition and posterior ones are of grafted tip composition. It seems, therefore, that amphibians and birds behave differently in their mode of formation of supernumerary limbs.

What significance do these results have for models of pattern formation? Firstly, it is apparent that there are important differences in behaviour between the anteroposterior and the dorsoventral axes of the limb. The dorsoventral axis seems to be mosaic and is not easily altered because gross discontinuities in this axis appear within the limb (eg. part normal/part inverted limbs). This is in contrast to the behaviour of the anteroposterior axis which is always smoothly consistent in digit sequence. Thus supernumeraries which form mirror images with the rotated limb will have the digital formula of **43211234, 4321234, 432234**, etc. . . ., but never **43214321**. Secondly, as has been pointed out before, supernumeraries with internal discontinuities such as

part normal/part inverted or part normal/part symmetrical contravene the principle of continuity which is the cornerstone of concepts invoking local intercalation of positional discrepancies, such as the polar coordinate model (French, Bryant & Bryant, 1976). These models cannot accommodate either the development or the regeneration (Maden, 1982) of limbs with gross internal discontinuities. But then we are faced with the question of what is special about the discontinuities between the blastema and the stump which stimulates the production of the supernumerary limbs in the first place? We could conclude that no pattern formation rules are needed at all – the stump ignores the rotated blastema, initiates a regenerate of its own and varying blastemal contributions to this outgrowth from the stump generate the structural diversity of supernumeraries as described above.

There are several reasons why it cannot be as simple as this. Firstly, if supernumeraries are induced simply by the stump ignoring blastema after grafting, why does this not happen in control grafts? Secondly, cell/cell interactions clearly do take place because 180°-rotated blastemas can derotate. Thirdly, there is a relationship between the angle of rotation and frequency of supernumerary limb formation (Turner, 1981). Fourthly, AP supernumeraries would be expected to contain the same variation in cellular contributions as 180° supernumeraries, but instead they are always approximately half and half (Muneoka & Bryant, 1984).

Some rules must be needed therefore, and one model which can cope with this complexity is the boundary model of Meinhardt (1983). This proposes that a limb is generated whenever a dorsal–ventral boundary is present in anterior competent tissue flanked by a posterior organizer. The predictions of this model concerning the handedness of individual parts of complex supernumeraries have been tested before (Maden, 1983) and those generated here also conform perfectly. It can explain much of the diversity of supernumerary limb structure, but has problems with symmetrical ones since there is no dorsal–ventral boundary present. Perhaps the dermal strip of opposite ploidy observed in these cases can provide a solution for the moment. However, further experiments at the cellular level will surely produce more information for the refining of such models.

REFERENCES

BRYANT, S. V. (1978). Pattern regulation and cell commitment in amphibian limbs. In *The Clonal Basis of Development*. pp 63–82 New York Academic Press.

BRYANT, S. V. & ITEN, L. E. (1976). Supernumerary limbs in amphibians: experimental production in *Notophthalmus viridescens* and a new interpretation of their formation. *Devl Biol.* **50**, 212–234.

CHAVALLIER, A. & KIENY, M. (1982). On the role of the connective tissue in the pattern-

ing of the chick limb musculature. *Wilhelm Roux Arch. devl Biol.* **191**, 277–280.

DUNIS, D. A. & NAMENWIRTH, M. (1977). On the role of grafted skin in the regeneration of X-irradiated axolotl limbs. *Devl Biol.* **56**, 97–109.

FRENCH, V. BRYANT, P. J. & BRYANT, S. V. (1976). Pattern formation in epimorphic fields. *Science* **193**, 969–981.

ITEN, L. E. & BRYANT, S. V. (1975). The interaction between the blastema and stump in the establishment of the anterior-posterior and proximo-distal organisation of the limb regenerate. *Devl Biol.* **44**, 119–147.

KELLER, R. E., LOFBERG, J. & SPIETH J. (1982). Neural crest cell behaviour in white and dark embryos of *Ambystoma mexicanum:* epidermal inhibition of pigment cell migration in the white axolotl. *Devl Biol.* **89**, 179–195.

LHEUREUX, E. (1983). Replacement of irradiated epidermis by migration of non-irradiated epidermis in the newt limb: the necessity of healthy epidermis for regeneration. *J. Embryol. exp. Morph.* **76**, 217–234.

MADEN, M. (1980). The structure of supernumery limbs. *Nature,* **287**, 803–805.

MADEN, M. (1982). Supernumerary limbs in amphibians. *Am. Zool.* **22**, 131–142.

MADEN, M. (1983). A test of the predictions of the boundary model regarding supernumerary limb structure. *J. Embryol exp. Morph.* **76**, 147–155.

MADEN, M. & MUSTAFA, K. (1982). The structure of 180° supernumerary limbs and a hypothesis of their formation. *Devl Biol.* **93**, 257–266.

MADEN, M. & TURNER, R. N. (1978). Supernumerary limbs in the axolotl. *Nature,* **273**, 232–235.

MADEN, M., GRIBBIN, M. C. & SUMMERBELL, D. (1983). Axial organisation in developing and regenerating vertebrate limbs. In *Development and Evolution* (B. C. Goodwin, N. Holder & C. G. Wylie eds) pp 381–397 Camb. Univ. Press.

MEINHARDT, H. (1983). A boundary model for pattern formation in vertebrate limbs. *J. Embryol. exp. Morph.* **76**, 115–137.

MUNEOKA, K. & BRYANT, S. V. (1984). Cellular contribution to supernumerary limbs in the axolotl, *Ambystoma mexicanum.* (Submitted to *Devl Biol.*).

MUNEOKA, K., WISE, L. D., FOX, W. F. & BRYANT, S. V. (1984). Improved techniques for use of the triploid cell marker in the axolotl, *Ambystoma mexicanum* (Submitted to *Devl Biol.*).

NAMENWIRTH, M. (1974). The inheritance of cell differentiation during limb regeneration in the axolotl. *Devl Biol.* **41**, 42–56.

PAPAGEORGIOU, S.. & HOLDER, N. (1983). The structure of supernumerary limbs formed after 180° blastemal rotation in the newt *Triturus viridescens. J. Embryol exp. Morph.* **74**, 143–158.

PESCITELLI, M. J. & STOCUM, D. L. (1980). The origin of skeletal structures during intercalary regeneration of larval Ambystoma limbs. *Devl Biol.* **79**, 255–275.

RAGER, G. LAUSMANN, S. & GALLYAS, F. (1979). An improved silver stain for developing nervous tissue. *Stain Tech.* **54**, 193–200.

SLACK, J. M. W. (1983). Positional information in the forelimb of the axolotl: properties of the posterior skin. *J. Embryol. exp. Morph.* **73**, 233–234.

TANK, P. W. (1978). The occurrence of supernumerary limbs following blastemal transplantation in the regenerating forelimb of the axolotl, *Ambystoma mexicanum. Devl Biol.* **62**, 143–161.

TANK, P. W. (1981). Pattern formation following 180° rotation of regeneration blastemas in the axolotl, *Ambystoma mexicanum. J. exp. Zool.* **217**, 377–387.

THOMS, S. D. & FALLON, J. F. (1980). Pattern regulation and the origin of extra parts following axial misalignments in the Urodele limb bud. *J. Embryol. exp. Morph.* **60**, 33–55.

TURNER, R. N. (1981). Probability aspects of supernumerary production in the regenerating limbs of the axolotl, *Ambystoma mexicanum. J. Embryol. exp. Morph.* **65**, 119–126.

WALLACE, H. & WATSON, A. (1979). Duplicated axolotl regenerates. *J. Embryol. exp. Morph.* **49**, 243–258.