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Positional information in the forelimb of the axolotl: properties of the posterior skin

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SUMMARY

Two series of experiments were carried out to investigate the properties of the positional information carried by posterior skin of the axolotl forelimb. The skin was assayed by grafting it to the anterior side of a normal limb and then amputating through the graft region. The formation of a double posterior regenerate indicates that the grafted skin carried the posterior coding.

In the first series, double posterior limbs were created by grafting posterior half limb rudiments to the flank of tailbud-stage embryos. The animals were reared for several months and then a half cuff of anterior skin, judged in relation to the whole body axes, was assayed by grafting to one of the host limbs. The results show that both sides of the double posterior limb carry the posterior coding and confirm our expectation that the visible anatomy is a good guide to the underlying codings.

In the second series animals were prepared by embryonic grafts so that they bore an extra normal limb on the flank. This extra limb was marked by pigmentation and in some cases by triploidy. When the limbs had developed, posterior skin from the extra limb was grafted to the anterior side of a host limb. The host limbs were amputated at intervals ranging from 2 weeks to 1 year after the skin graft. The results show that the posterior coding carried by the graft is stable even in an anterior environment.

Studies of the cellular composition of regenerates which had received triploid grafts showed that the graft epidermis was progressively replaced by that of the host. The dermis on the other hand retained triploid cells throughout.

INTRODUCTION

It has been known for several years that a graft of skin from one part of an amphibian limb to another can derange the pattern of a regenerate which is formed after amputation through the graft (Droin 1959, Rahmani 1960, Lheureux 1973). It is thought that the dermis rather than the epidermis is the active component (Carlson 1975) and it has recently been shown that the activity is destroyed by freezing and thawing of the graft, implying the need for viable cells (Tank 1981).

In a previous paper (Slack 1980*a*) I showed that a highly specific type of abnormal regenerate, the double posterior duplication, could be produced by such a procedure. Such regenerates are formed if posterior skin from the lower

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forelimb is grafted to the anterior side and the limb later amputated through the graft. The use of triploid cells as a marker showed that the duplications arose partly by metaplasia of those cells in the graft which contribute to internal tissues such as cartilage, and partly by the reprogramming of anterior host tissues to form posterior structures. By contrast, regenerates from lower forelimbs with double anterior skin were normal or slightly hypomorphic.

The present paper reports the results of two experiments designed to investigate further the nature of the 'posterior coding[1]' presumed to be carried by the posterior skin. The first asks whether double posterior limbs arising from operations on embryos carry the posterior coding on the anterior as well as the posterior side. The second asks how stable is the posterior coding in tissue which is grafted into an anterior environment. The results show that both sides of a double posterior duplication do indeed carry the posterior coding, and that the posterior coding is very stable, persisting in a skin graft for more than 12 months.

MATERIALS AND METHODS

These are essentially as described in Slack (1980*b*) for operations on embryos and Slack (1980*a*) for skin grafts. There were however three changes. Firstly, the skin grafts for the stability experiment were not sutured into position since the animals were rather too small at the time. The grafts were simply placed on the wound bed arising from removal of the host skin and the animals were kept anaesthetised on damp paper towels in a 10 °C incubator for a few hours before returning to water.

Secondly, a pressure shock instead of a temperature shock was used to induce triploidy in fertilized eggs. A large Aminco pressure cell was used in a French Press and a treatment of 6000 'high ratio units' (nominal 10 000 psi) for 6 min at 1 h after fertilization was found to be most satisfactory. This gives about 25 % survival with 90 % of survivors being triploid. In axolotls, triploid cells are easily identified since they have three nucleoli (Frankhauser & Humphrey 1943).

Thirdly, the Bismuth staining procedure was introduced during the course of the work (K. Muneoka, personal communication). It has some advantages over the Unna Pappenheim stain used previously: in particular the nucleoli of fibroblasts are more clearly visible, and it was used to analyse the final group of triploid–diploid combinations in the stability experiment.

In both experiments the original embryonic graft was marked by skin pigmentation, usually a black graft on white host. At the stage in question, black and white embryos are not distinguishable. However, the pigmentation is determined by a single genetic locus and black is dominant over white. So if eggs from

[1] The term 'coding' is preferred to 'positional value' for two reasons. Firstly it is obvious that in experimental situations there need not be a one-to-one relation between positional value and position. Secondly what we really want to know is the 'epigenetic code' which relates coding and final anatomy.

black parents are used as donors and eggs from white parents as hosts then either 75 % or 100 % of cases will be black-on-white.

RESULTS

Skin grafts from duplicated limbs

Double posterior limbs can be created by any operation on the embryo which sandwiches a piece of determined limb tissue in between two regions of flank tissue. The most reliable operation of this type is a graft of the posterior half of the limb rudiment (tissue ventral to somites 4 and 5) to a position on the flank, usually but not necessarily ventral to somite 8. The grafts were performed at stage 30 and 24 animals were prepared which bore double posterior limbs on the flank in addition to their four normal limbs. Most of the grafts were carried out between embryos from adult axolotls of different skin colour so that in the end there were 18 black limbs on white hosts, 3 white limbs on black hosts and 3 limbs on hosts of the same colour. The animals were reared for 4–6 months until they were about 10 cm in length and then skin from the anterior edge of the double posterior limb, judged by its relation to the principal body axes, was grafted to the anterior edge of the host forelimb on the same side of the body. The graft was a half cuff from the region between elbow and wrist and was sutured into position replacing an equivalent area of host skin. In six cases, skin was grafted from *both* sides of the duplication to the anterior of both host limbs, as shown in Fig. 1. The grafts were allowed to heal for two weeks and then the host limbs were amputated through the graft and allowed to regenerate. The donor limbs were also amputated at a level corresponding to the proximal limit of the skin which had been removed.

The structures of the resulting regenerates are shown in Table 1 with photographs in Fig. 2. It is clear that most of the regenerates arising from the graft of anterior skin are double posterior duplications (Table 1 – row 1). This

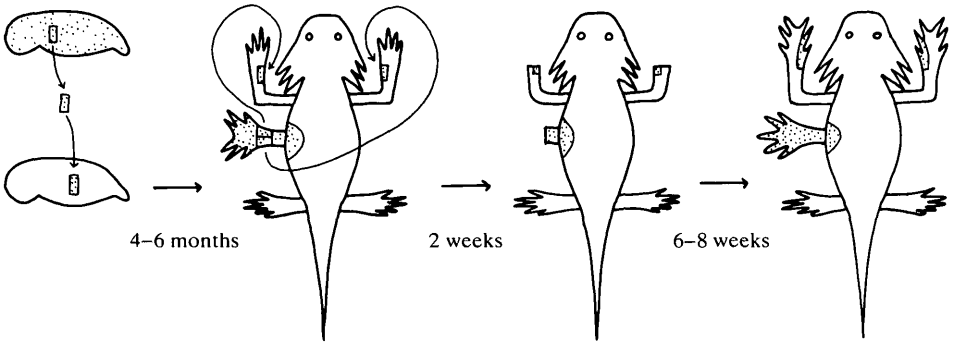


Fig. 1. Procedure used to assay the coding of the two sides (anterior and posterior) of embryonically produced double posterior limbs. In 6 cases anterior and posterior skin was grafted, as shown, and in a further 18 cases anterior skin only.

Table 1. *Equivalence of anterior and posterior skin from embryonically produced duplications*

Limb	Cases	Structure				Digit number of duplications	
		Duplication	Other hypermorph	Hypomorph	Normal	Mean	Range
Regenerated host limbs with graft of anterior skin from duplication	24	16	6	0	2	5·7	5–8
Regenerated host limbs with graft of posterior skin from duplication	6	5	0	0	1	5·8	5–6
Regenerated donor limbs	24	16	0	6	2	5·1	3–8
Original donor limbs	24	24	–	–	–	5·9	4–8

"Duplicated" are mirror-symmetrical double posterior limbs.
"Other hypermorphic" includes partial duplicates and duplicates with serial repetition of structures.

suggests that the anterior skin from an embryonically produced duplication has the same coding as posterior skin from normal limbs. As expected, the posterior skin from the donor limb also evokes the formation of duplications (Table 1 – row 2).

The amputated donor limbs also regenerate as duplications (Table 1 – row 3). However it should be noted that these regenerates display a slight pattern contraction compared both to the original donor limbs and to the regenerated host limbs. Typically a six-digit duplication of the type **4-3-2-2-3-4** regenerates as a five-digit duplication of the type **4-3-2-3-4**. This effect has been described before (Slack & Savage 1978). Embryonically produced duplications are also prone to regenerate hypomorphs, something which is never found following the skin graft + amputation procedure.

Most of the grafts were carried out with skin marked by pigmentation and so it was possible roughly to estimate the proportions of each duplicated regenerate derived from graft and host tissue. In this respect the case shown in Fig. 2 (C,D) is fairly typical since the regenerates usually showed donor pigmentation on only the two digits of most anterior position. Since most of the duplications had six digits this indicates that some of the regenerate anterior to the mirror plane is formed from host tissue.

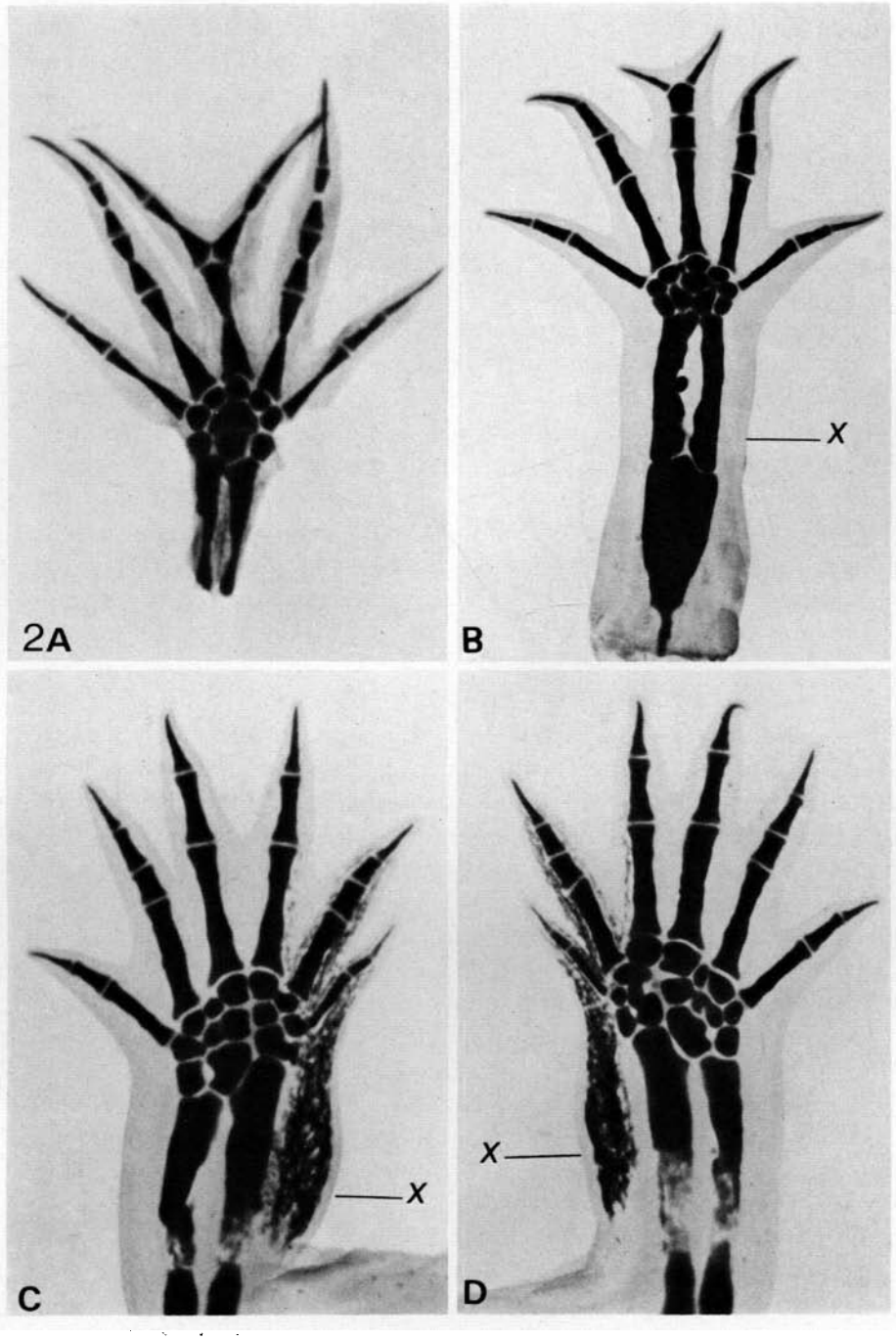
Stability of the posterior coding.

For this experiment 44 white animals were prepared which carried an extra normal black limb on the flank. These were made by grafting the whole limb rudiment and its surroundings (tissue ventral to somites 3,4 and 5) from donor embryos derived from black parents to host embryos derived from white parents. In addition some of the donor embryos had been pressure shocked after fertilization. After the operation these were kept alive and at about stage 40 were scored for triploidy by examining tail tip or liver squashes under Nomarski optics. In all 16 of the 44 black donor limbs were triploid.

When the animals reached 3 months of age (about 6 cm long) posterior skin was grafted from the black limb to the contralateral white limb. The grafts were smaller than the half cuffs used previously: about 90° of the circumference. In contrast to previous series, and presumably because of the lack of sutures, 8 of the 44 cases developed supernumerary digits at the distal graft–host junction. They were used for the experiment along with the others. At various times ranging from 2 weeks to 1 year after the skin graft the host limb was amputated through the graft and allowed to regenerate. Three animals were excluded because the colour of the skin graft had disappeared by amputation time.

The complete procedure is shown in Fig. 3, the results are listed in Table 2, and typical regenerates are shown in Fig. 4.

It is clear that the time delay between skin graft and amputation makes no difference to the structure of the regenerates, most of which are duplications or other hypermorphic structures. So it seems that the posterior coding of the graft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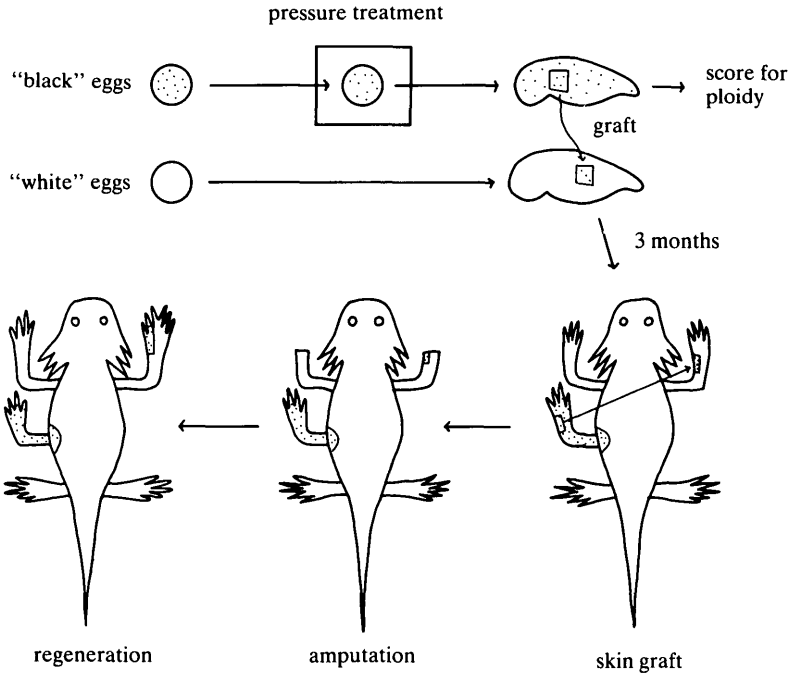

Fig. 3. Procedure used to assay the stability of the posterior coding of skin grafted to an anterior environment. The time interval between skin graft and amputation varied from 2 weeks to 1 year.

is quite stable even though it is surrounded by anterior tissues for over a year. Interestingly there is no significant change in the digit number of the duplications despite the fact that the limbs and therefore also their regeneration blastemas widen from about 2 mm to about 5 mm as the animals grow up. In analagous experiments on chick limb buds the number of elements is highly sensitive to the width of the bud (Smith & Wolpert 1981).

Owing to the vagaries of the staining procedures only 12 of the regenerates arising from triploid skin grafts could be examined histologically. These were drawn from the 2-week group (two cases), the 22½-week group (five cases) and the 54-week group (five cases). The donor limbs from the same animals were also examined.

The 2-week cases were essentially the same as cases described in the previous paper (Slack 1980*a*) where the delay between skin graft and amputation was also

Fig. 2. Four limbs from a single experimental case. (A) Shows the original donor limb. Both skin grafts (anterior and posterior) have been removed and the pigmentation has been bleached to reveal the skeleton. (B) Shows the regenerate of the donor limb (also bleached). (C) and (D) show respectively the regenerates from left and right host limbs. Both are duplicated in distal carpalia and digits. They are unbleached and the black pigmentation marks the donor derived regions. X – amputation level.

Table 2. *Stability of the posterior coding*

Time between skin graft and amputation (weeks)	Number of cases	Structure of regenerate			Digit number in duplications	
		Duplicate	Other hypermorphic	Normal	Mean	Range
2	6	4	2	0	6·4	5½–8*
5½	8	5	2	1	6·0	5 –7
8	5	4	0	1	5·7	5 –6
22½	6	3	2	1	6·0	6
28	8	5	2	1	6·8	6 –7
54	8	6	2	0	6·4	6 –7
Totals	41	27	10	4		

* branched digits are counted as 1½

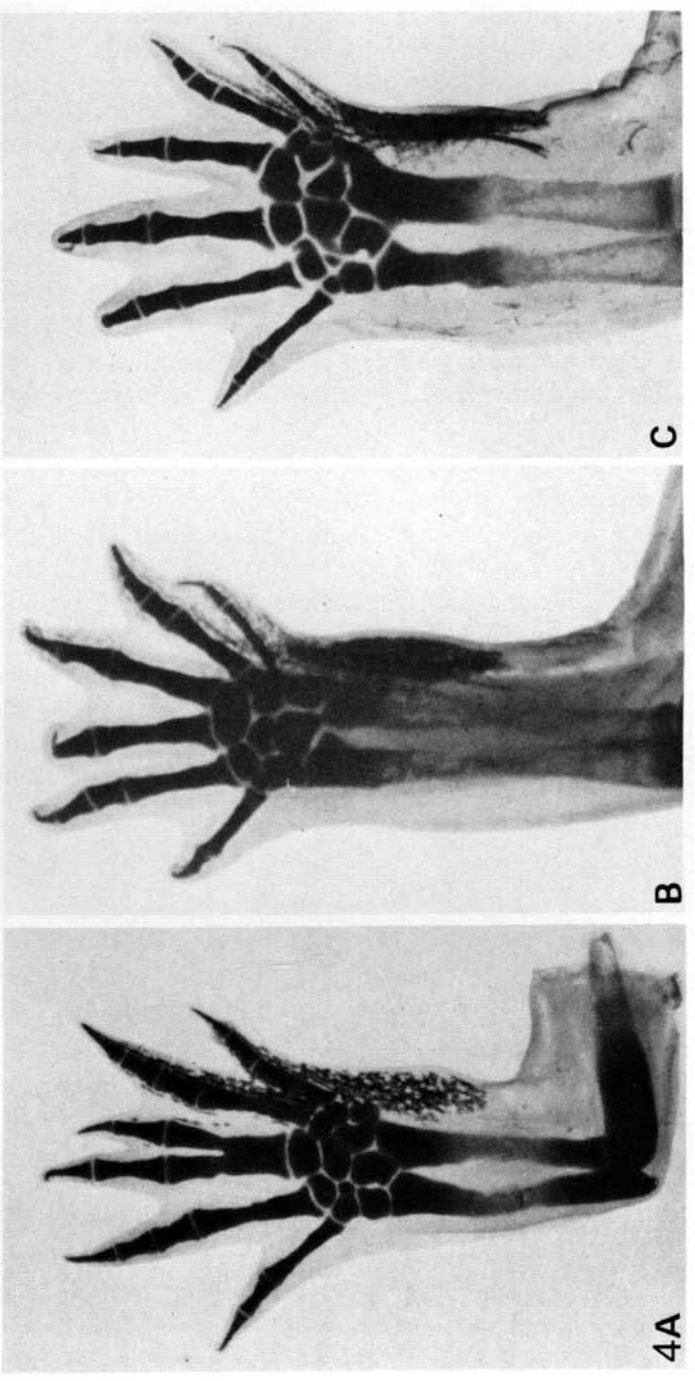

Fig. 4. Typical regenerates arising from the stability experiment after different intervals between skin graft and amputation. (A) 2 weeks (B) 22½ weeks (C) 54 weeks. The black pigment marks donor-derived tissue.

2 weeks. Abundant trinucleolar cells were found anterior to the mirror plane in epidermis, dermis and cartilage, and a few in the muscle. In the cases with the long time intervals the proportions of trinucleolar cells fell and there were significant differences between the tissues. In the epidermis it was possible to find patches of trinucleolar cells in two of the 22½-week cases but in none of the 54-week cases (although one case had a few 'doubtful' 3n cells). On the other hand it remained fairly easy to find trinucleolar cells in the dermis even in the 54-week group (Fig 5). They tended to be in the same regions as the melanocytes suggesting that the pigmentation pattern in black–white combinations is a reasonably good guide to the underlying cellular composition. Trinucleolar cells could also usually be found in the cartilage neighbouring these regions and, in three of the ten long-time-interval cases, there were a few trinucleolar muscle cells (Fig 5).

In the donor limbs all cases showed abundant trinucleolar cells in all tissues, regardless of the length of time since the embryo graft (3 months, 8 months and 15 months for the three groups) and in no case was there any sign of immunological rejection.

These results suggest that triploid epidermal cells in small grafts become overgrown by host cells while those in complete triploid limbs do not. This should not be surprising since the epidermis is a tissue which undergoes continuous renewal during adult life. This means that at any given time most of the cells will be descended from a small proportion of cells at a previous time, so overgrowth of small grafts is probable even if the graft cells are at no selective disadvantage.

DISCUSSION

Equivalence of anterior and posterior in duplications

Although there is disagreement about the arrangement of epigenetic codings on the tissues of the limb and on the rules for their interactions (Wallace 1981, Tank & Holder 1981), all contemporary workers seem to agree on three things: firstly that anterior and posterior dermis carry different codings, secondly that abnormally juxtaposed codings interact during regeneration, and thirdly that there is a one-to-one relationship between coding and final anatomy.

The last of these points implies that abnormal morphologies embody codings which are the same as the codings carried by the corresponding structures in the normal morphology. However this has not been tested directly because it requires an assay for the codings in the various regions of the abnormal morphology which is independent of the methods used to create the morphology in the first place. Such an opportunity is however, provided by the mirror duplicated limbs created by embryological operations since there are three independent criteria for tissues bearing the posterior coding:

(1) They look posterior.

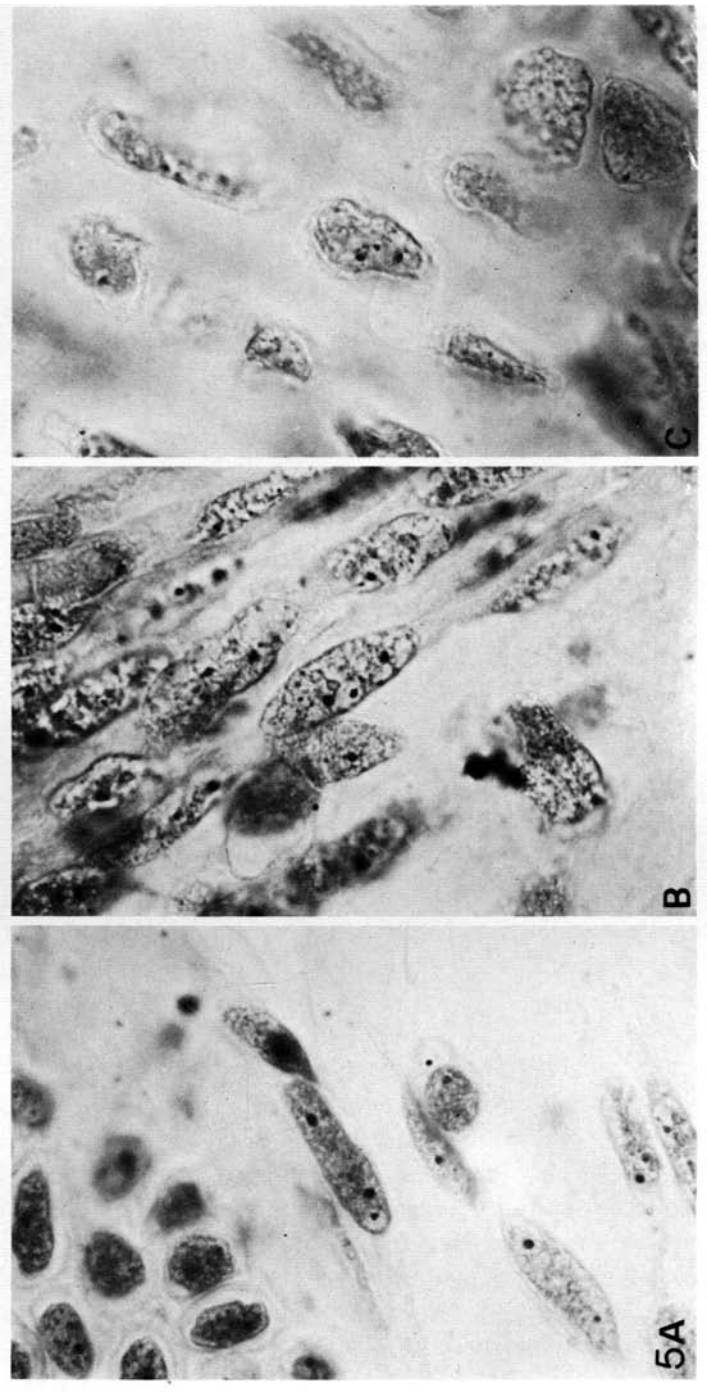

Fig. 5. Trinucleolar cells in regenerates from limbs with a 54-week delay between triploid skin graft and amputation. (A) dermal fibroblast, (B) Myotube nucleus, (C) Chondrocyte. Stained by the bismuth procedure. Final magnification ×755.

(2) On exposure at an amputation surface they regenerate posterior structures (Slack & Savage 1978, Stocum 1978, Holder, Tank & Bryant 1980).
(3) During regeneration they can recruit neighbouring anterior tissues into posterior structures (Slack 1980*a*).

The experiment described here shows that both sides of an embryonically produced duplication carry the posterior coding not only by virtue of (1) but also by virtue of (2) and (3), and therefore confirm our expectation of morphogenetic equivalence. Although the result is the expected one it is not trivial since a divergence between visible anatomy and the underlying pattern of codings has more than once been suggested to explain aberrant results.

Stability of the posterior coding

The stability of codings in differentiated tissues has been suspected since Carlson (1974) left animals with rotated skin cuffs for up to 2 years and still obtained abnormal regenerates after amputation. However, the present result represents the first demonstration of this using marked tissue combinations. It is shown that donor-derived cells persist for long periods in the dermis but not in the epidermis and this must reinforce the earlier conclusions of Carlson (1975) that the dermis is the tissue responsible for the pattern alteration, and of Tank (1981) that viable cells are required to carry the posterior coding.

In the present experiments, as in previous ones, much of the tissue anterior to the mirror plane in the duplications appears to be derived from the host, implying that the codings of these cells were respecified from anterior to posterior during regeneration. So it is most important to distinguish between the nature of the codings in the differentiated tissues and in the blastema. In differentiated tissues the codings are extremely stable and discontinuities persist indefinitely, while in the blastema the codings are labile and discontinuities become smoothed out as regeneration proceeds.

Metaplasia

The present results do not add anything to what is already known about the interconversions which are possible between different cell types during regeneration (Slack 1980*c*, Wallace 1981). Workers who have studied this question conclude that epidermis and mesodermal tissues are not interconvertible; that dermal fibroblasts and chondrocytes are interconvertible; and reserve judgement on whether muscle can interconvert with the other mesodermal tissues (Wallace, Maden & Wallace 1974, Namenwirth 1974, Dunis & Namenwirth 1977).

In several of the regenerates examined in the stability experiment a few trinucleolar muscle cells were found. However, as in the previous studies this does not necessarily prove the possibility of conversion from fibroblast to myotube since the labelled cells are few in number and not found in all cases. It is possible for example that they are derived from muscle satellite cells contaminating the skin grafts. It is also possible that they are artifacts, since with

some stains, including the bismuth stain, there are other granules in myotube nuclei which stain similarly to nucleoli. The metaplasia of fibroblasts to chondrocytes is much more clearcut since skin grafts cannot be contaminated with cartilage and because the labelled cartilage cells are found in large numbers and in most experimental cases.

To a large extent our expectation about metaplasia to and from muscle depends on whether the prospective muscle cells in the larval limb bud turn out to have a different origin from the other mesenchymal cells. It has of course been shown in recent years that prospective muscle cells in the chick limb bud are derived from the somites (Chevallier, Kieny, Mauger & Sengel 1977, Christ, Jacob & Jacob 1977). This question is now under investigation in several laboratories using triploid–diploid axolotl combinations and we await the results with interest.

Conflicts of results

The results presented here incidentally also show the repeatability of certain experiments of mine about which there has been some controversy and I now believe that all disagreements can be accounted for by differences in experimental technique.

There is no question that in my hands embryonically produced double posterior limbs always regenerate and that the regenerates are usually also duplicated. This result is disputed by Tank (1982) who obtains little or no regeneration, and it has also been known for some time that surgically produced double posterior limbs regenerate poorly unless amputation is carried out immediately after the operation (Bryant 1976, Tank & Holder 1978, Tank 1979, Holder & Tank 1979). In my view this is because the American workers habitually clip off the bone(s) which protrude from the amputation surface. This creates a flat stump and a favourable opportunity for skin to heal over the wound and inhibit regeneration. I have previously tried to emphasize the importance of the competition between regeneration and healing in cases where the amputation surface does not carry a 'complete circle' of codings (Slack 1980*d*). Where there is a complete circle healing cannot occur because internal codings will be intercalated at the discontinuities and recreate the original wound. However in a mirror-symmetrical amputation surface each territory can heal to the equivalent territory on the other side and this will inhibit regeneration as surely as if skin was sutured across the wound. Originally I left the protruding bone simply because it seemed more 'natural' to do so. But now I feel that it plays an important role by preventing equivalent cell populations from meeting. By the time the bone has regressed the blastema has developed far enough for regeneration to be irreversible. In the case of surgically produced double posterior limbs which are amputated immediately, it is likely that a midline barrier is created by clotted blood and dead tissue, and so in this case regeneration does occur.

A somewhat similar situation probably obtains concerning the formation of

supernumerary structures from the juxtaposition of differentiated tissues with non-neighbouring codings. Previously I have not observed these while the Americans have. I now think that they tend to arise when the grafts are not sutured since this was the case in the stability experiment described above (8/44 cases), while in all my previous experiments of this type sutures have been used and no supernumeraries observed. In a case where sutures have not been employed it is more likely that a non-congruent graft–host junction will remain open long enough to initiate regeneration.

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