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Genetic inhibition of mesenchymal cell death and the development of form and skeletal pattern in the limbs of *talpid*^{3} (*ta*^{3}) mutant chick embryos

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SUMMARY

Vital staining reveals that in homozygous (*ta*^{3}/*ta*^{3}) *talpid*^{3} embryos, the areas of mesenchymal cell death which occur regularly in normal limb development are absent or reduced. The necrotic locus in the central mesenchyme (the 'opaque patch') which in the normal chick limb reaches maximum development at stages 24 and 25 (4½–5 days) is absent or much reduced in *talpid*^{3} fore- and hindlimb-buds. Autoradiographic studies, following application of a 2 h pulse of 40 μCi of $^{35}SO_4$ to the vitelline circulation, show that normal tibia and fibula incorporate $^{35}SO_4$ into chondroitin sulphate at stage 24 and more strongly at stage 26 during the process of chondrogenesis. The mesenchyme in the opaque patch region of normal limbs ceases to incorporate $^{35}SO_4$ into chondroitin sulphate at stage 24. *Talpid*^{3} mesenchyme cells in the equivalent position at stages 24 and 26 continue to incorporate $^{35}SO_4$, remain viable and become chondrogenic. It is suggested that absence or reduction of this central necrotic locus in *talpid*^{3} is causally related to the fusion of radius/ulna and (in some cases) of tibia/fibula characteristic of the later stages (28–35) of *talpid*^{3} limb development. This evidence supports the hypothesis that cell death in the opaque patch plays a morphogenetic role in separation of radius/ulna and tibia/fibula.

The digital plate of stage 32 (7½ days) normal limbs is characterized by massive necrosis of the interdigital tissue. In *talpid*^{3} forelimbs of stages 30–35 interdigital necrosis is absent, and there is no regression of the tissue between the digits ('soft tissue syndactyly'). In *talpid*^{3} hindlimbs of stage 30–35 interdigital necrosis is either absent or much reduced, and there is little or no erosion of the soft tissue between the digits. This evidence supports the hypothesis that the morphogenetic role of interdigital cell death is in causing separation of the digits through shaping and remodelling the contours of the digital plate.

INTRODUCTION

Cell death in well-defined loci in the mesenchyme is a prominent feature of chick limb morphogenesis, and recent studies have shown that at least one of these regions, the posterior necrotic zone (PNZ), is determined by a hierarchy

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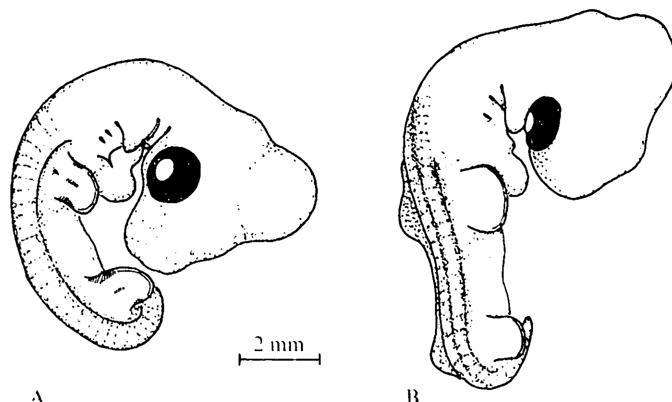

Fig. 1. (A) Normal and (B) *talpid*[3] embryos at $4\frac{3}{4}$ days (stage 25). Areas of cell death in the limb-buds are shaded.

of factors: genetic, spatial and temporal (Saunders, Gasseling & Saunders, 1962; Zwilling, 1964; Saunders, 1966). There are three major areas of cell death: the anterior and posterior necrotic zones (ANZ and PNZ), the 'opaque patch' of the central limb mesenchyme, and the interdigital areas (INZ). While the morphogenetic role of the first two of these areas of necrosis is still the subject of controversy, it has been suggested that the INZ plays a role in the shaping of the contours of the digits by removing the interdigital areas of mesenchyme (Saunders & Fallon, 1966).

Since both experimental and teratological studies of limb development (Menkes & Deleanu, 1964; Saunders, 1966) have failed to resolve this problem, genetic variants in which normal control of cell death is disturbed as a result of mutation may clarify the functional role of cell death. The effect of increasing a naturally occurring area of cell death is known in the classic study of Zwilling (1942) on *Rumplessness*, and more recently, in the *wingless* (*ws*) mutant, in which a precocious and enlarged ANZ removes wingbud mesenchyme (Hinchliffe & Ede, 1973).

The *talpid* mutant is an interesting example of genetic suppression of cell death in areas where it would normally take place. ANZ and PNZ are absent (Hinchliffe & Ede, 1967) while *talpid* cells *in vitro* are able to survive in conditions which normally lead to cell death (Ede & Flint, 1972, and unpublished work of Cairns, quoted in Ede, 1971). The present investigation concerned genetic suppression in *talpid*[3] embryos of cell death in two regions of the limb not previously examined, which correspond to the normal opaque patch and the INZ. In addition, absence of the opaque patch is examined in relation to the establishment of *talpid*[3] chondrogenic pattern, which was studied autoradiographically by examination of the pattern of $^{35}SO_4$ incorporation into chondroitin sulphate (Searls, 1965*b*).

MATERIALS AND METHODS

Talpid^3 and normal embryos were obtained from Light Sussex hens and the stage of embryonic development determined according to the Hamburger–Hamilton system (1951). The *talpid*^{3} (*ta*^{3}*ta*^{3}) embryos were obtained by crossing known *talpid*^{3} carriers (heterozygous for the *ta*^{3} gene). This mutant strain is descended from the original *talpid*^{3} stock described by Hunton (1960) and Ede & Kelly (1964).

Areas of cell death in normal and *talpid*^{3} embryos were mapped by vital staining. Nile blue sulphate or neutral red at a concentration of 1:40000 in Ringer's solution was applied *in ovo* for $\frac{1}{2}$–1 h to the vitelline circulation. Embryos were either photographed directly, or for higher magnification the limbs were dissected out, mounted in Ringer's solution in a cavity slide, and photographed.

Two types of histological procedure were employed. Whole limb-buds were fixed in Bouin's fluid, processed for conventional wax histology, sectioned at 8 μm and stained with haematoxylin and eosin or with alcian blue and Mayer's acid haemalum. Alternatively, limb tissue was fixed by an electron microscopy double fixation procedure (Trump & Bulger, 1966) and embedded in epoxy resin. Sections 1–2 μm thick were cut on a glass knife, mounted on glass slides and stained with 1% toluidine blue in borax.

The pattern of chondroitin sulphate synthesis in developing normal and *talpid*^{3} fore- and hindlimbs was analysed by autoradiography using radiosulphate as a labelled precursor (Searls, 1965*a*; Amprino, 1955); 0·02 ml of a sterile aqueous solution of ^{35}S-labelled sulphate (Amersham Radiochemical Centre) with a total activity of 40 μCi was applied through a window to the area vasculosa, and the sealed egg returned to the incubator for 2 h. The embryos were then fixed in Bouin's fluid, and the limbs dissected off, blocked in paraffin wax and sectioned at 8 μm. The sections were dewaxed, coated with stripping film (Kodak Autoradiographic Stripping Plates AR 10) and exposed for 12 days at 4 °C, after which they were developed in Kodak D 19 developer, mounted in 'Clearmount' and viewed by dark-field illumination.

RESULTS

Suppression of cell death in the talpid^3 *central limb mesenchyme*

Normal fore- and hindlimb-buds at stages 24 and 25 possess a characteristic and well-defined region (the 'opaque patch') of cell death in the central limb mesenchyme (Figs. 1 and 2). The 'opaque patch' region of necrosis is clearly defined by vital staining (Fig. 4) due to accumulation of the vital dye in the phagosomes of the macrophages. By contrast, in *talpid*^{3} fore- and hindlimb-buds of the same age, there is no comparable 'opaque patch' region of necrosis in the central *talpid*^{3} limb mesenchyme, which fails to accumulate vital dye

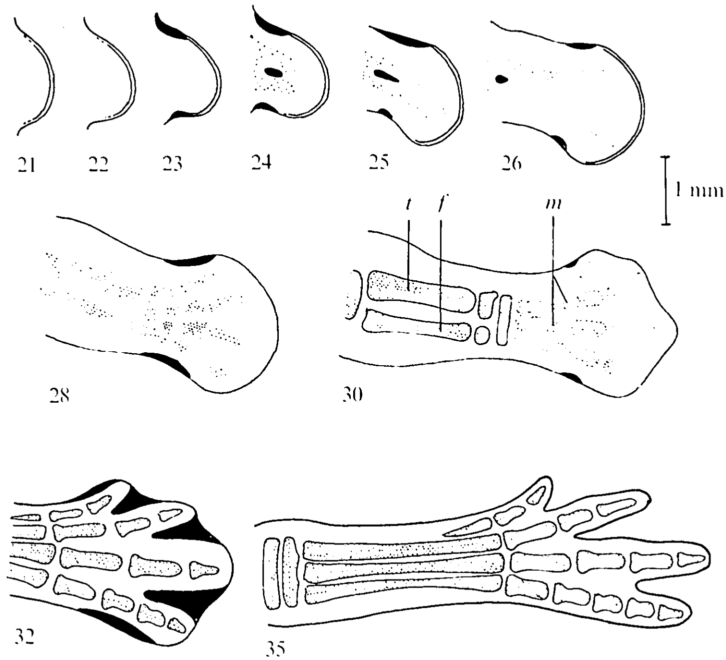

Fig. 2. Patterns of chondrogenesis and cell death in the normal hindlimb ($3\frac{1}{2}$–9 days, stages 21–35). Areas of cell death in solid black. Stippling represents precartilaginous condensations (areas of high $^{35}SO_4$ uptake). Cartilaginous elements outlined in black: *f*, fibula; *m*, metatarsals; *t*, tibia.

(Fig. 3). *Talpid*^{3} fore- and hindlimb-buds also show clearly at this stage the absence of ANZ and PNZ. In both normal and *talpid*^{3} limb-buds the apical ectodermal ridge (AER) is clearly defined by the vital dye-accumulating dead cells which are found along the distal margin of the ridge.

Histologically the normal mesenchyme from this area is distinguished by the presence of numerous densely staining, moribund and dead cells and of cellular debris (Fig. 5A). Often such material is present as phagocytic inclusions within the cytoplasm of large, distended, macrophagous cells (Dawd & Hinchliffe, 1971). This contrasts markedly with the mesenchyme from the equivalent area of the majority of *talpid*^{3} limbs of the same developmental stage (Fig. 5B). The central blood vessel serves as a useful marker to identify the equivalent central area of *talpid*^{3} limbs. No such cell necrosis is present and the mesenchyme cells present an apparently healthy and viable appearance. An exception to this general rule is shown by a minority of *talpid*^{3} limbs which possess regions of cell death corresponding to the opaque patch, but the number of dead cells is reduced and the dead cells are more widely scattered than normal.

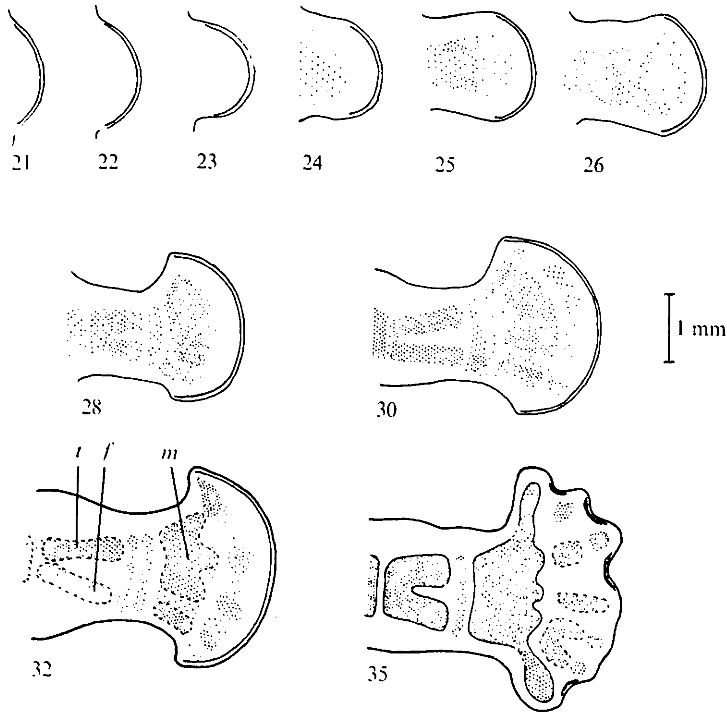

Fig. 3. Patterns of chondrogenesis and cell death in the *talpid*^{3} hindlimb ($3\frac{1}{2}$–9 days, stages 21–35). Note limited interdigital cell death at stage 35. The 'opaque patch' is absent. Conventions as for Fig. 2.

Autoradiographic ($^{35}SO_4$) *analysis of skeletal development in* talpid^3 *limbs*

The use of a radioisotope precursor of chondroitin sulphate provides information about the development of metabolic pattern in the limb. At stages 20 and 22, in both *talpid*^{3} and normal fore- and hindlimbs, there is uniform uptake of sulphate in both central and peripheral areas of the mesenchyme (Thorogood, 1972). At stage 23 the intensity of uptake of central Y mesenchyme is greater than that of peripheral mesenchyme.

At stage 24 the autoradiographic pattern of normal and *talpid*^{3} limbs differ for the first time. In the stage 24 normal limb, the axial area of increased incorporation (i.e. the chondrogenic area – Searls, 1965*a*) is divided distally into two arms (tibia and fibula) by a funnel-shaped area of low uptake (Fig. 6A) which corresponds with the 'opaque patch' of necrosis. This result is interpreted as reduced [^{35}S]sulphate metabolism at this locus, indicating a cessation of chondrogenic activity. The equivalent *talpid*^{3} limb likewise possesses a central core of mesenchyme which has high uptake of the isotope but the distal end of this region is ill-defined and no distal division by a low incorporation

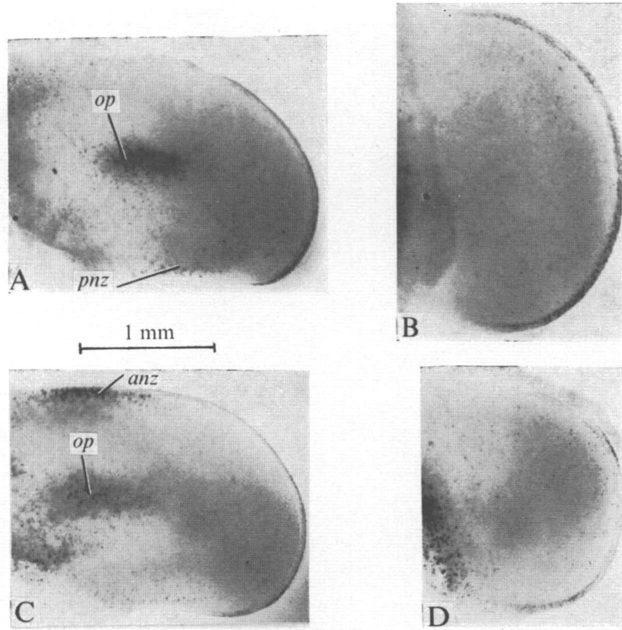

Fig. 4. Cell death at 4½ days (stage 24) in normal limb-buds and absence of cell death in *talpid*³ limb-buds. Vitally stained. (A) Normal forelimb, (B) *talpid*³ forelimb, (C) normal hindlimb, (D) *talpid*³ hindlimb. Note absence of opaque patch (*op*) and anterior and posterior necrotic zones (*anz* and *pnz*) in *talpid*³ limb-buds.

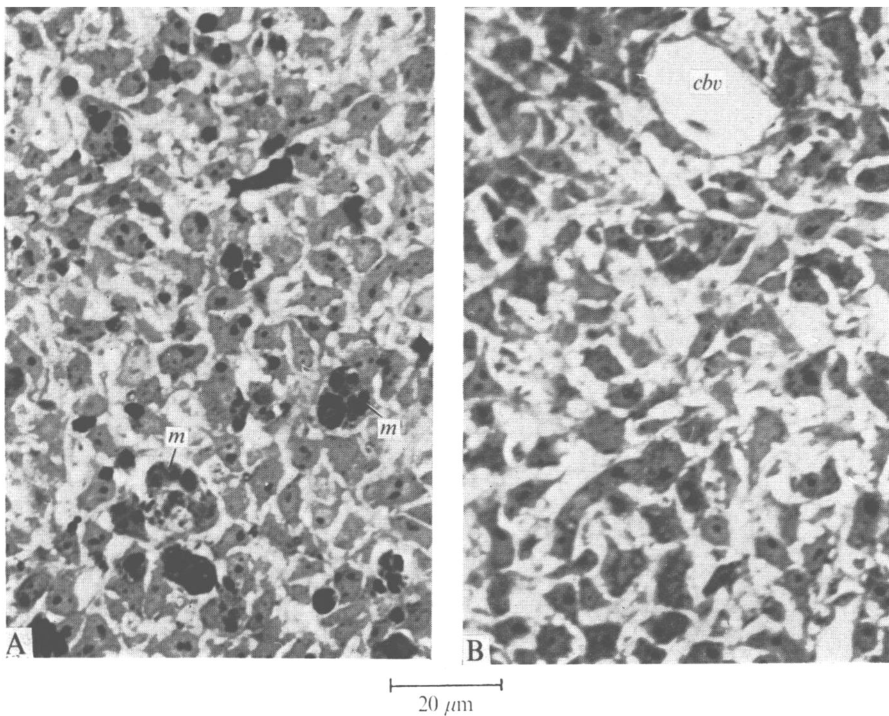

Fig. 5. Transverse sections (1 μm) stained with toluidine blue at the opaque patch level in stage 24 (4½ days) hindlimbs. (A) Normal (note macrophages *m*), (B) *talpid*³ (central blood vessel *cbv*).

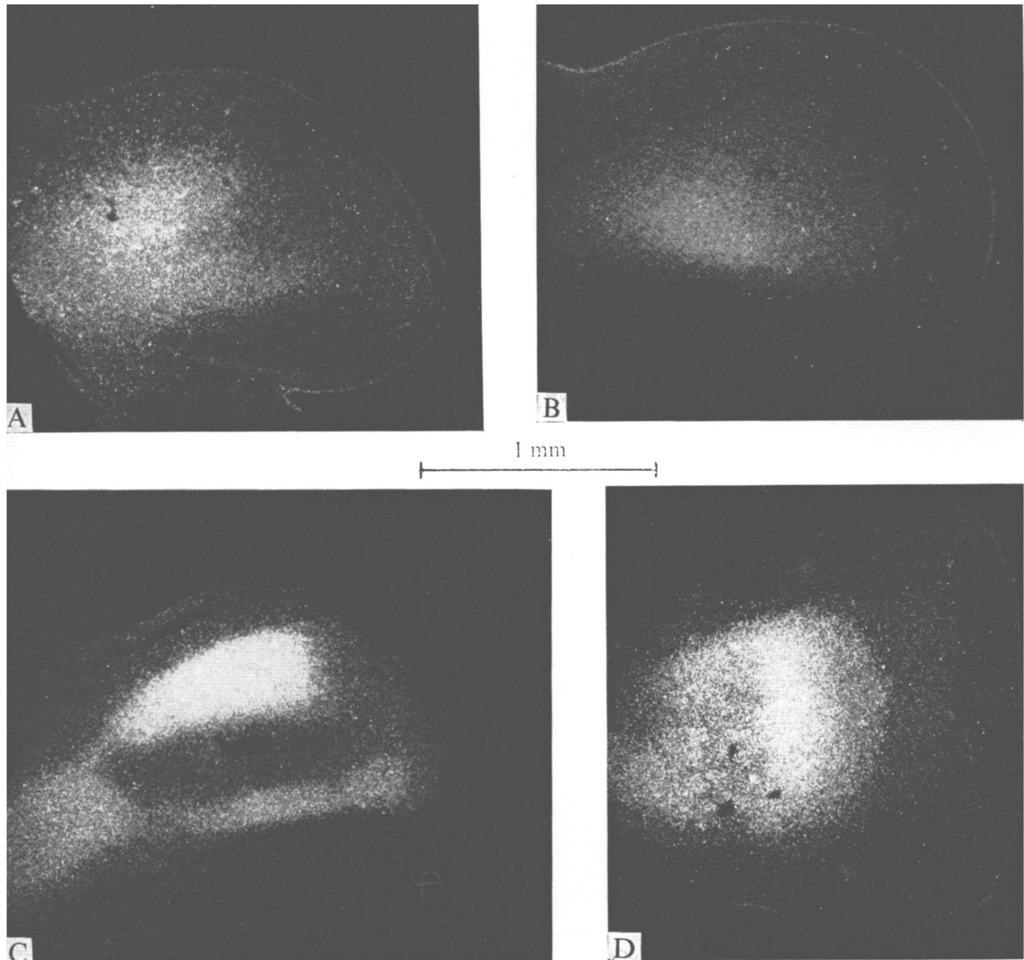

Fig. 6. Autoradiographs of $^{35}SO_4$ uptake into chondroitin sulphate in normal and *talpid*[3] hindlimbs, photographed under dark-field illumination. (A) Normal, stage 24 (note Y shape of the region of high uptake). (B) *talpid*[3], stage 24. (C) Normal, stage 26 (note well-defined tibia and smaller fibula). (D) *talpid*[3], stage 26 (note single central region of high uptake, representing fused tibia and fibula).

area can be identified (Fig. 6B). Thus the cells at this locus not only remain viable as judged by histological appearance but continue to exhibit an elevated incorporation of radioisotope and presumably remain chondrogenic.

In the normal limb at stage 26, the pattern of high uptake clearly outlines the chondrogenic pattern of the skeleton; high activity is confined to the cartilage primordia of the long bones (Fig. 6C). The peripheral soft tissue shows an almost total lack of label. The *talpid*[3] hindlimb shows a marked deviation from the normal (see Fig. 6D). Central regions of high uptake are present but their distribution bears little resemblance to that seen in the normal limb.

There is a diffuse area of increased incorporation within which are blocks of high activity, representing the long bone cartilaginous elements within a much enlarged and disturbed chondrogenic region. Separate tibia and fibula elements cannot be identified. The forelimb in *talpid*³ shows an even more disturbed pattern of incorporation which takes the form of a single central block of labelled chondroitin sulphate. In the *talpid*³ condition the border between high and low activity is indistinct and shows a blurred gradation of activity from high to low; in the normal limb the chondrogenic area has a sharp 'edge' and regional differences in uptake contrast sharply. It is also noteworthy that in *talpid*³ the areas of highest uptake seem to be marginally less intense than the equivalent areas in the normal limb.

These differences between the uptake pattern of normal and *talpid*³ limbs at stage 26 correlate well with the chondrogenic pattern which is now demonstrable by histochemical means, using alcian blue to stain the acid mucopolysaccharides characteristic of cartilage matrix (Fig. 3 and Hinchliffe & Ede, 1967). It should be borne in mind that the latter pattern represents accumulated mucopolysaccharides, whereas the autoradiographic technique reveals the actual synthesis of mucopolysaccharide over a 2 h period. Alcian blue staining of later stages (30–35) of *talpid*³ hindlimb development shows that tibia and fibula are sometimes fused proximally and sometimes separate (Fig. 3). Formation of separate elements may represent a later 'recovery' from an initial stage 26 single chondrogenic block; alternatively it may be that since few *talpid*³ embryos survive to this stage, these embryos have been heavily selected for normality and show less extreme hindlimb skeletal abnormalities. *Talpid*³ forelimbs in these later stages (30–35) always show fusion of radius and ulna.

Suppression of cell death in the talpid³ *interdigital regions*

Vital staining of normal embryos shows that well-defined areas of mesenchyme cell death are found between the digits of the foot and wing during stages 31–34 (7–8 days) (see Fig. 2). The process reaches a climax at about stage 32, and coincides with the transition from the rounded limbplate to the separated digits characteristic of the adult limb.

*Talpid*³ forelimbs during the period 7–10 days showed a complete absence of interdigital cell death. This absence correlates with the absence of any regression of the mesenchyme tissue between the digits, which remain linked to their

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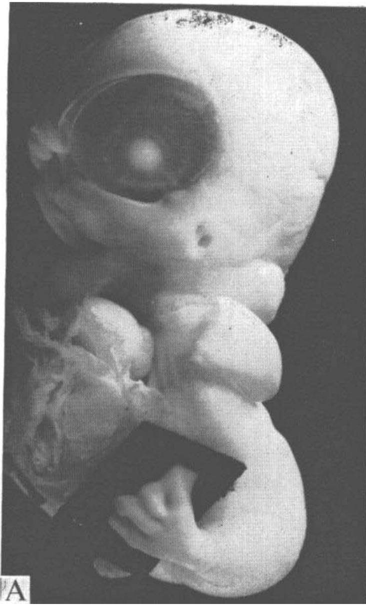
FIGURE 7

(A, B) Nine-day *talpid*³ embryo. Both fore- and hindlimbs show soft tissue syndactyly, but in the hindlimbs there is slight regression of the interdigital tissue. (C–G) Sections of fore- and hindlimbs of this embryo. (C–D) Forelimb stained with alcian blue. (D) Detail of (C) (arrowed). Interdigital cell death is absent. (E–G) Hindlimb, stained haemotoxylin and eosin. (F) Detail of (E) (arrowed), and (G) detail of (F) (arrowed), with arrows indicating dead cells and macrophages.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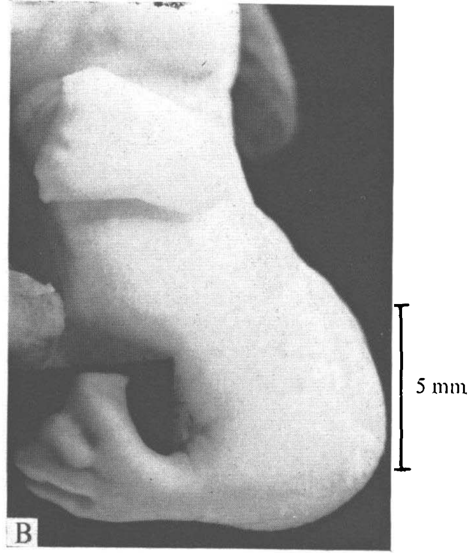
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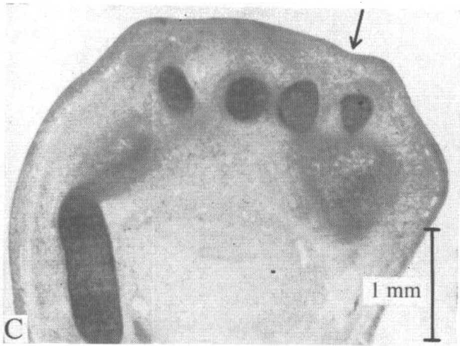
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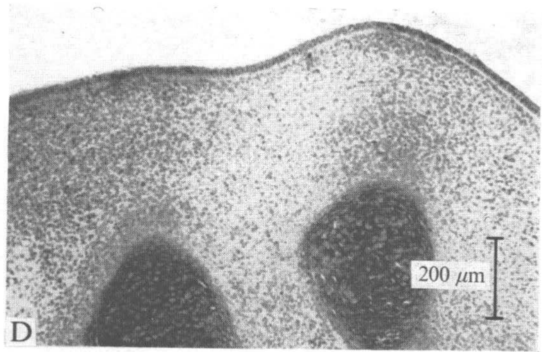
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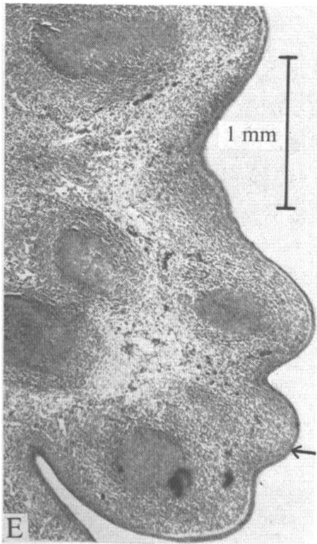
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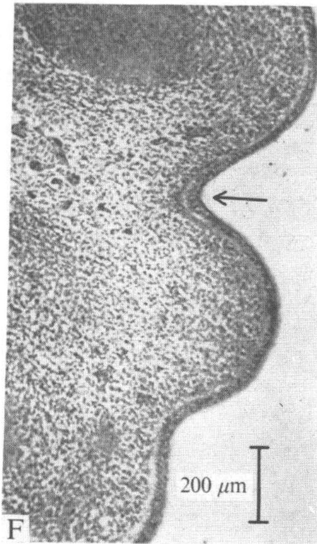
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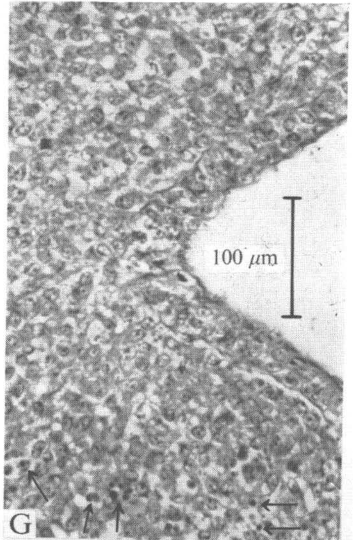
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neighbours by webs ('soft tissue syndactyly') (see Fig. 7A–D). In the case of many of the 7- to 10-day *talpid*^{3} hindlimbs a similar description applies: there is absence of interdigital cell death, correlated with survival of mesenchyme tissue between the digits. However, in some hindlimbs characterized by a small degree of interdigital regression (Fig. 7B), a limited amount of interdigital cell death is found, but this is very much reduced as compared with the normal (Fig. 7E, F, G). This finding is in line with other evidence indicating that abnormalities of the *talpid*^{3} hindlimb are less pronounced than those of the forelimb.

DISCUSSION

Genetic suppression of cell death in the talpid^3 *limb*

Talpid^3 limb development is characterized by the absence of mesenchymal cell death; the absence of ANZ and PNZ has been reported previously (Hinchliffe & Ede, 1967) and the present report clearly indicates absence or severe reduction of cell death in OP and INZ regions in both fore- and hindlimbs. *Talpid*^{3} limb mesenchyme cells in general are resistant to processes which normally cause cell death; Ede & Flint (1972) found comparatively few cell deaths in reaggregating trypsinized *talpid*^{3} limb mesenchyme cells and they quote unpublished work by Cairns who reports that *talpid*^{3} limb mesenchyme survives the stripping off of the AER for much longer than does normal limb mesenchyme. It appears, however, that this effect is confined to the limb mesenchyme, and that cell death is found in other sites. Cell death takes place in the distal edge of the apical ectodermal ridge of both *talpid*^{3} and normal limb-buds (Hinchliffe & Ede, 1967) and cell death is found in other regions of the mesoderm, for example, the limb base and the somites of *talpid*^{3} embryos.

Absence of the opaque patch and emergence of chondrogenic pattern

Fell & Canti (1934) originally described the opaque patch as occurring initially in prospective femur tissue, but this conclusion was based on the culture of isolated limb fragments in which regulation may well have occurred. A more accurate picture of the emergence of chondrogenic pattern in the intact limb is given by $^{35}SO_4$ autoradiography which indicates that the opaque patch separates tibia and fibula in the hindlimb and radius and ulna in the forelimb at the time of their first formation during stages 24 and 25. The emergence of a Y-shaped blastema, with tibia and fibula representing the two arms of the Y, is the first evidence of the development of a chondrogenic pattern in the limb.

Dawd & Hinchliffe (1971) have recently proposed a model to account for the initial Y-shaped chondrogenic pattern of the limb. The essential features of the model are a central process of condensation forming an axial rod which is divided distally into two by the process of cell death in the opaque patch. The two distal condensations subsequently increase in size as a result of inter-

stitial and appositional growth as peripheral cells are assimilated to the primary condensations, as suggested by Holtfreter (1968). The opaque patch in addition to containing dead cells and macrophages, contains cells which appear to be undergoing autophagy. This was tentatively interpreted as evidence of de-differentiation: that is, chondrogenic cells eliminating cytoplasm programmed for production of cartilage intercellular material. Autoradiographic results presented in this report demonstrate that radiosulphate uptake ceases in the opaque patch area at this time, and such a finding is compatible with the idea that chondrogenic activity is suppressed in the cells which form the non-chondrogenic tissue separating tibia and fibula. Thus the opaque patch cells fail to develop any chondrogenic potential: in the mild form this expresses itself as a dedifferentiation and in a more extreme form, they actually die.

From such a hypothesis it is reasonable to predict that in the absence of the opaque patch the distal portion of the primary chondrogenic blastema will fail to divide into the two distinct 'arms' of the Y. At the later stage of appositional growth the effect of this failure will be more pronounced and the normal separation and definition of the paired chondrogenic centres will be affected. This prediction is tested by the *talpid*^{3} mutant. The cell death at the opaque patch position is suppressed; no dying or autophagic cells or phagocytic macrophages are present, and in fact these cells maintain their chondrogenic ability as indicated by [^{35}S]sulphate incorporation. Thus the fate of these cells is not switched to a non-chondrogenic one. At a later stage radius and ulna and frequently the tibia and fibula remain partially joined proximally or they form a single central and ill-defined chondrogenic mass (Ede & Kelly, 1964; Hinchliffe & Ede, 1967). These findings reinforce the suggestion made by Dawd & Hinchliffe (1971) that the opaque patch makes a morphogenetic contribution to the shaping of the initial chondrogenic pattern.

In addition to suppression of cell death, the *talpid*^{3} gene also causes an increase in adhesiveness of the mesenchyme cell, which has been demonstrated by *in vitro* studies (Ede & Agerbak, 1968; Ede & Flint, 1972). This effect has been invoked as the major cause of the *talpid*^{3} abnormal limb outgrowth and also of the disturbed limb skeleton. To explain its effect on the skeleton, Ede has postulated that the increased adhesiveness and the reciprocal decreased cell motility inhibit the cell movement involved in condensation formation, thus resulting in 'fusion' of adjacent cartilage elements. Absence of the opaque patch and increased adhesiveness may both contribute to the fusion of the radius/ulna and tibia/fibula. It is the contention of the present authors that the increased cellular adhesiveness also affects the skeleton at a later stage. The poor definition indicated by the 'blurred edge' to the autoradiographs of individual *talpid*^{3} skeleton elements at stage 26 may be the result of inadequate appositional growth of existing ill-defined condensations. Appositional growth of the blastema presumably involves small-scale cell movement as surrounding cells are recruited and aggregate onto the blastema. If *talpid*^{3} cell movement is reduced,

presumably appositional growth will be inhibited and poorly defined cartilage elements will result.

The model which has just been outlined has features which parallel the results of a number of studies, both theoretical and experimental, of limb development and structure. Searls' (1965*a*) study of $^{35}SO_4$ incorporation led him to the conclusion that initially all stage 21 limb mesenchyme cells are capable of synthesizing chondroitin sulphate, but that beginning at stage 22 this activity is repressed in non-chondrogenic regions and enhanced in chondrogenic areas. In computer simulation of a biochemical model of the development of the cartilage pattern of the chick limb, a derivative of the Turing model using one morphogenetic substance and two cellular thresholds for synthesis and destruction, when combined with simulation of limb growth, generated a limb-like pattern in which the number of chondrogenic elements increased in a proximo-distal direction and the elements were arranged in transverse rows distally (Wilby & Ede, 1974). Simple bifurcating patterns are also found in the paddles of Crossopterygian lung fish such as *Eusthenopteron* (Andrews & Westoll, 1970) and *Sauripterus*, and also in the primitive tetrapod limbs of urodele amphibia such as the axolotl (Sewertzoff, 1908; Hinchliffe, 1974). In addition, dissociated chick and duck limb mesenchyme is known to form, following reconstitution, a simple bifurcating skeleton much simpler than that of the normal limb (Pautou, 1973). Valuable insight may well be obtained by considering the complex higher vertebrate chondrogenic pattern of the limb as being evolved through modification of a simpler bifurcating pattern.

Absence of the INZ and the survival of 'webbing' in talpid[3] *limbs*

Saunders' discovery of dying cells between the digits in the stage 32 ($7\frac{1}{2}$ day) chick limb led him to suggest that the INZ shaped the external limb form (Saunders *et al.* 1962). This conclusion was strengthened by analysis of the development of the duck leg in which the INZ is absent or reduced, and in which webbing survives between the digits of the foot (Saunders & Fallon, 1966).

Experimental modification of the normal pattern of the INZ has been attempted by Janus green treatment of chick embryos. This dye suppresses the INZ and the resulting limbs show soft tissue syndactyly (Menkes & Deleanu, 1964; Deleanu, 1965; Saunders & Fallon, 1966). However, this experiment does not provide unequivocal proof of a causal relationship between these two events. Janus green is known to have widespread teratogenic effects on embryonic development, and its use therefore introduces additional parameters into the system; suppression of the interdigital clefts may be a secondary effect of a disturbed pattern of limb growth caused by Janus green.

The *talpid*[3] mutant presents a limb system in which the INZ is absent without experimental manipulation. A poor definition or shaping of the individual *talpid*[3] digits is observed due to persistence of soft tissue between the digits.

The degree of 'shaping' is directly proportional to the amount of cell death. Thus in the hindlimb the INZ is reduced but not always totally absent and a partial separation of the digits may be observed, whereas in the forelimb the INZ never arises and complete soft tissue syndactyly occurs. In the normal embryo this tissue is removed by the interdigital necrosis. A similar situation is demonstrated in the limb development of the polysyndactylous mutant of the mouse, in which soft tissue syndactyly is associated with suppression of interdigital cell death in homozygous (*Ps/Ps*) embryos (Johnson, 1969). *Talpid*^{3} embryos thus provide important supporting evidence to the hypothesis that the morphogenetic role of the INZ is in shaping the digital contours.

Fallon (1972) has recently analysed the relationship between the apical ectodermal ridge and the INZ. The survival of the AER until 7½ days appears to be necessary if cell death is to appear in the subjacent INZ at 8½ days. Various treatments, including Janus green, which result in early flattening of the ridge, also inhibit the INZ. Fallon concludes that the presence of the AER until 7½ days is necessary if the INZ is to appear. In the *talpid*^{3} mutant, however, inhibition of the INZ is associated with survival of the AER, which persists beyond the time at which the AER normally regresses.

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