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Genetic control of skeletal development in the chick embryo

By D. A. EDE, *Department of Zoology, University of Glasgow, Developmental Biology Building, 124 Observatory Road, Glasgow G12 9LU*

Autonomous growth programmes in skeletal development

The mature skeleton of the fowl is remarkable for its complexity, brought about partly by differential growth and partly by the development of secondary fusions. An example is seen in the development of the wing which in its final form is a quite uniquely avian structure supporting the wing feathers, but in which the first embryonic rudiments of the digits are distinct and not greatly different from each other or from the basic pentadactyl limb pattern.

The rudiments of the skeletal elements are formed first as cartilage. This makes possible the rapid growth and remodelling of the embryonic skeleton, which would be impossible for bone where growth is by apposition and where remodelling goes on at the surface: exceptions are most of the bones of the head and also the clavicle, which are formed directly from mesenchyme.

Skeletal growth control in the first half of embryonic development is therefore concerned with the control of cartilage growth. Any factors, intrinsic or extrinsic, that affect the proliferation of cartilage may be considered to control growth. These factors may also affect the pattern, i.e. the shape and distribution of cartilage rudiments. It was established by Murray & Selby (1930), who cultured chick limb rudiments on the chorioallantoic membrane, that the primary factors regulating skeletal development were instrinsic, i.e. genetic. It appears that from an early stage in the formation of the skeletal rudiment, i.e. in the stage when the mesenchyme is becoming closely packed in particular regions to form prechrondrogenic condensations, each rudiment has an autonomous programmed growth pattern. This has been demonstrated by examining the growth of isolated rudiments in organ culture (Fell & Canti, 1935). Once programmed, skeletal elements will grow at different rates and produce differently shaped structures in an autonomous manner. Thus, at a very early stage in the formation of the rudiments of the fibula and the tibia in birds (up to stage 22–24) the prospective areas are labile and any rearrangement will be compensated by regulation, so that a normal limb skeleton is formed. Thereafter, their autonomous programmes will produce structural differences of size and shape. At stage 27 the chick tibia is almost equivalent to the fibula in size but by 8 d of incubation the tibia is twice the length of the fibula and much broader. Isolation of the rudiments in culture leads to development of these same differences, even though the environmental and cultural conditions are equivalent. Unpublished in vitro studies by Hicks (reported in Hinchliffe & Johnson, 1983) suggest that tibia cells start smaller than fibula cells, but during hypertrophy become larger. Neither cell proliferation nor matrix synthesis appear to contribute to the differential growth of the tibia and fibula.

Mutants affecting cartilage skeletal growth

One approach to the analysis of the genetic control of the formation and development of the cartilage skeletal pattern is through the analysis of mutants whose phenotypic effects can be identified at an early stage and followed through to their ultimate manifestation as skeletal deformities. Three examples will be described which illustrate the manner in which genetic control is manifested at specific stages in chondrogenesis.

Development of cartilage rudiments occurs in embryonic mesenchyme where the cells are rather loosely packed together, with much intervening intercellular matrix. Rudiments are formed by mesenchyme cells becoming more closely packed together in particular regions. Following this the cells in these chondrogenic condensations begin to secrete cartilage matrix. The matrix gives character to the tissue: collagen fibres give it tensile strength and proteoglycans give it resilience and resistance to compression. The proteoglycans are enormous molecules consisting of a central core of hyaluronic acid, to which are attached branches of core protein. From these proteins branch off glycosaminoglycans, some of which are characteristic of cartilage, notably keratin sulphate and chondroitin sulphate.

Nanomelia

This mutant was discovered by Landauer (1965) and has been investigated by Goetinck & Pennypacker (1977). The mutation is inherited as a single recessive lethal autosomal gene, and homozygous embryos are characterized by having extremely shortened limbs and parrot-like beaks. The chondrocytes themselves are not visibly affected. Its effect is on the matrix secreted by the chondrogenic cells and the difference in molecular structure between normal and *nanomelic* matrix is clearly visible in electron micrographs. Normal matrix in scanning and transmission micrographs shows collagen fibres and electron-dense matrix granules which represent condensed proteoglycan molecules; in the mutant, the collagen fibres are present but proteoglycan molecules are almost absent. The effect of this on the cartilage is that chondrocytes in the mutant are much more closely packed together than in the normal cartilage. Observations on synthesis of proteoglycans in the mutant indicate that the mutation affects the availability of the proteoglycan core protein.

Micromelia

This mutation, again, is a single autosomal recessive gene and in the homozygous dose the length of the long bones of the limbs is reduced (though not nearly so much as in *nanomelia*) and there is a parrot-like beak. Once again, it is the matrix which is affected but in this case there is also an effect on the chondrocytes. Normal chondrocytes are heavily vacuolated and have scalloped edges at the cell margin. The vacuoles must be related to matrix synthesis and distribution of the scalloping may be a manifestation of this process. In *micromelia* there are very few vacuoles and no scalloping at the cell margins. The extracellular matrix granules are in this case not completely absent but they are greatly reduced

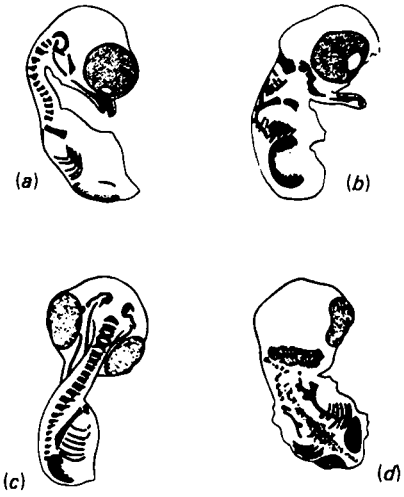

Fig. 1. Cartilage skeleton in 11 d chick embryos, limbs removed. (*a*), normal, lateral view; (*b*), normal, dorsal view; (*c*), *talpid*^{3} mutant, lateral view; (*d*), *talpid*^{3} mutant, dorsal view. Drawings made from alcian-blue-stained preparations.

in number. Biochemical analysis by Goetinck *et al.* (1981) has shown that this is due to reduction but not absence of available proleoglycan core protein.

Talpid^3

In *nanomelia* and *micromelia* it is the cartilage matrix which is affected. The pattern of skeletal development is relatively normal; only the dimensions of the long bones are affected. In the *talpid*^{3} mutant the effects of the gene, which is autosomal, recessive and lethal, are much more widespread. Cartilage is affected in all parts of the body and one effect is the inhibition of its transformation to bone; in alizarin preparations of late embryos, only the membrane bones of the head and the clavicle are stained, although cartilage rudiments of all parts of the skeleton are present. Furthermore, the pattern formed by the rudiments is altered, particularly in the occurrence of fused elements (e.g. in the neck vertebrae which in the normal chick embryo are separate entities (Fig. 1)). These abnormalities are clearly formed at a much earlier stage than the two matrix defects described previously, at the time in fact of chondrogenic mesenchyme cell condensation.

The effect on both growth and pattern can be seen most clearly in the development of the cartilage rudiment of the limb skeleton (Fig. 2). In normal wings and legs at about 10 d of incubation, the cartilages are distinctly separated, but in the homozygous *talpid*^{3} phenotype many of the rudiments are fused. Further, there are more than the normal number of digits (up to eight) which are more readily visible in the leg and which thus appears polydactylous.

The whole shape of the outgrowing limb bud is abnormal. In the normal wing bud (Fig. 3) the bud appears first as a simple swelling, then elongates, and then broadens at its distal end into a flattened paddle within which the digits develop.

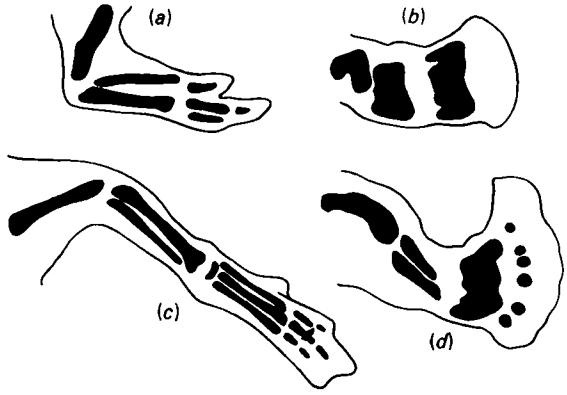

Fig. 2. Cartilage skeleton in limbs of 8 d chick embryos. (*a*), normal wing; (*b*), *talpid*^{3} mutant wing; (*c*), normal leg; (*d*), *talpid*^{3} mutant leg. Drawings made from alcian-blue-stained preparations.

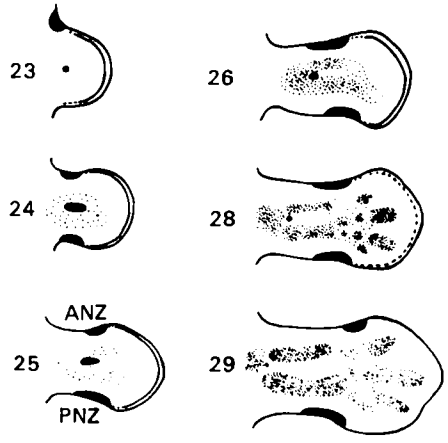

Fig. 3. Normal chick limb bud, stages 23–26, 28 and 29, showing areas of cartilage development (▨). ANZ, anterior necrotic zone; PNZ, posterior necrotic zone.

The whole of the limb bud, apart from its ectodermal covering, is composed of an apparently-homogeneous mesenchyme; within this mesenchyme the prechondrogenic condensations appear, first at the base of the bud, then progressively more distally. In *talpid*^{3} the initial swelling is broader and as it grows out the bud becomes fan-shaped, so that the developing limb is shorter and broader than normal. Within the mesenchyme, the chondrogenic condensations are much-less-well defined than normal, and tend to merge with each other. This characteristic is not dependent on the integrity of the limb bud. If limb mesenchyme cells from an early bud are dissociated and allowed to grow in culture, discrete condensations which stain for cartilage matrix are usually formed. In cultures of *talpid*^{3} cells a reticulated meshwork of connected condensations is formed (Ede, 1982).

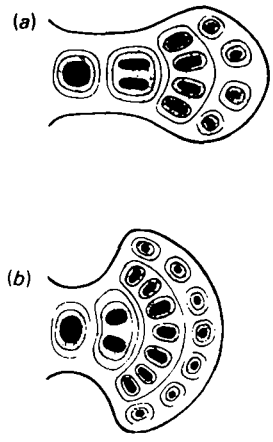

Fig. 4. Diagram of outgrowing shape of (*a*) normal and (*b*) *talpid*^{3} mutant limb buds, with prechondrogenic condensation fields. For detailed explanation, see p. 16.

The formation of the prechondrogenic condensations appears not to involve increase of packing density by cell proliferation but some sort of centripetal orientated movement of cells towards the condensation centre (Ede & Wilby, 1981). In *talpid*^{3} this orientation and movement appears to be impeded. Ede & Flint (1975) showed that cultured *talpid*^{3} limb mesenchyme cells were more adhesive to each other than normal cells, and moved more slowly on a plastic culture surface. These cell-behaviour characteristics would explain the failure of *talpid*^{3} prechondrogenic condensations to separate from each other clearly, producing the cartilage fusions that are observed in the limbs and elsewhere.

The cytological basis for these cell-behavioural defects has been investigated by Ede *et al.* (1974), who showed in transmission- and scanning-electron-microscopy studies that the cells were not polarized as normal mesenchyme cells are, but produced rather short filopodia around the cells (the filopodia of normal cells are chiefly at either end and of great length). No differences were found in the cytoskeletal elements (microtubules and microfilaments) so it is likely that the genetic defect is primarily on the cell surface.

Whereas in the case of *nanomelia* and *micromelia* the mutations affected only cartilage, and that chiefly in the long rudiments of the limbs, the cellular abnormalities of *talpid*^{3} are not limited to a single cell type. This may account for the fan-shaped outgrowth of the *talpid*^{3} limb buds, since some outward cell movement is required to produce the paddle-shaped elongation in the normal buds. If mesenchymal cell movement is restricted, the outgrowth will be fan-shaped and the limb bud will consequently be much broader at its apical end. If, as seems likely, there is some periodic basis to the spacing of the skeletal rudiments this will mean that progressively more rudiments will be formed at each successive level along the proximo-distal axis of the bud, culminating in an excessive number of digit rudiments at the apex. The consequent effects of the *talpid*^{3} gene on the wing skeleton is shown in Fig. 4. In each proximo-distal band the centres of

prechondrogrenic condensation are established by the periodic mechanism (the same number as normal in the humerus and radius/ulna bands) but an excess in the metacarpal and phalangeal bands. Movement of prechondrogenic mesenchyme cells towards each centre is indicated by the stippled fields around the centres. In normal embryos the cells will move through all of these fields to condense in the centre, but in *talpid*[3] embryos not all cells will move in from the peripheral fields. The result is the cartilage fusion that is observed, except in the case of the digits, which are generally distinct. Here we have a gene which affects the earliest stage of cartilage growth, the manifestations of which, however, extend to a much later stage, including (as mentioned previously) the inhibition of bone replacement.

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