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Original Article

Tissue patterning in the developing mouse limb

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ABSTRACT The developing mouse fore- and hindlimbs begin as bumps on the flank of the embryo and grow out to form miniature models of the adult limb during a five day period from E9.5 to E14.5. In this paper I show a series of embryos taken at half-day intervals during limb development and outline the timetable of patterning for each of the component tissues of a limb: epidermis, connective tissues, muscle, nerves and blood vessels. Scanning electron micrographs, supplemented by histological sections, are presented to define a set of standard stages for the description of mouse limb development. I discuss my observations of the mouse limb in the light of current theories of vertebrate limb development, which are based on classic manipulation experiments in the chick as well as more recent molecular data from the mouse system. The limb skeletal pattern in a mouse is laid down in a proximodistal direction, as it is in a bird: the E11.5 forelimb reveals the first signs of a humerus and by E14.5 even the most distal phalanges of the hand are formed. At this late stage ossification sleeves are seen around the proximal limb elements as the cartilage template begins to be converted to a bony skeleton. Myogenic cells stainable with the MF20 antibody against early muscle myosin heavy chain are first seen in the mouse forelimb at E11.5, which is also when the first nerve fascicles begin to enter the limb. From E11.5 to E14.5 both muscle and nerve patterns mature to give distinct muscles at all proximodistal levels of the limb, each muscle with its own nerve branch, and a cutaneous nerve plexus that extends to the fingertips. The developing skin of the mouse limb matures from a bi-layered epidermis overlying an avascular, but otherwise nondescript, prospective dermal layer of mesenchyme at E9.5, to a 4- or 5-layered epidermis with early hair placodes and the first signs of a distinct dermal layer at E14.5. Notable differences between mouse and chick limb development lie in the relatively late formation of the apical ectodermal ridge in the mouse and its unexpectedly close relations with blood vessels, in the absence of anterior and posterior necrotic zones and, possibly, in a late migration of myogenic cells into the mouse limb bud.

KEY WORDS: *mouse, limb, development, patterning*

Introduction

The vertebrate limb has long been an important model system for examining the mechanism of tissue patterning during development. Until recently almost all work on embryonic limb development has been carried out on birds (usually the chick) because their limb buds are easily accessible for manipulative experiments. It has been assumed, if only tentatively, that most of the data could be directly extrapolated to the rather more inaccessible developing mammalian limb. Tissue ablation and recombination experiments on the avian limb have yielded a mass of information about how component tissues interact to give the mature limb pattern (reviewed in Hinchliffe and Johnson, 1980; Martin and Lewis, in preparation). The next steps must be to determine the molecular nature of these tissue interactions, but the chick is a poor system for addressing molecular issues. The mouse has important advantages over birds in this respect, since far more mouse genes have been cloned and characterised, and a much wider range of cDNA probes and other molecular tools are available. Furthermore, improvements in techniques of embryo culture and surgery on the embryo *in utero* now make it possible to carry out manipulative experiments on the developing mouse limb (Beddington and Martin, 1989; Wanek *et al.*, 1989a). For these reasons it is becoming clear that it might be

Abbreviations used in this paper: AER, apical ectodermal ridge; ANZ and PNZ, anterior and posterior necrotic zones; GAGs, glycosaminoglycans; HA, hyaluronic acid; NGF, nerve growth factor; PZ, progress zone; RA, retinoic acid; SEM, scanning electron microscopy; ZPA, zone of polarising activity.

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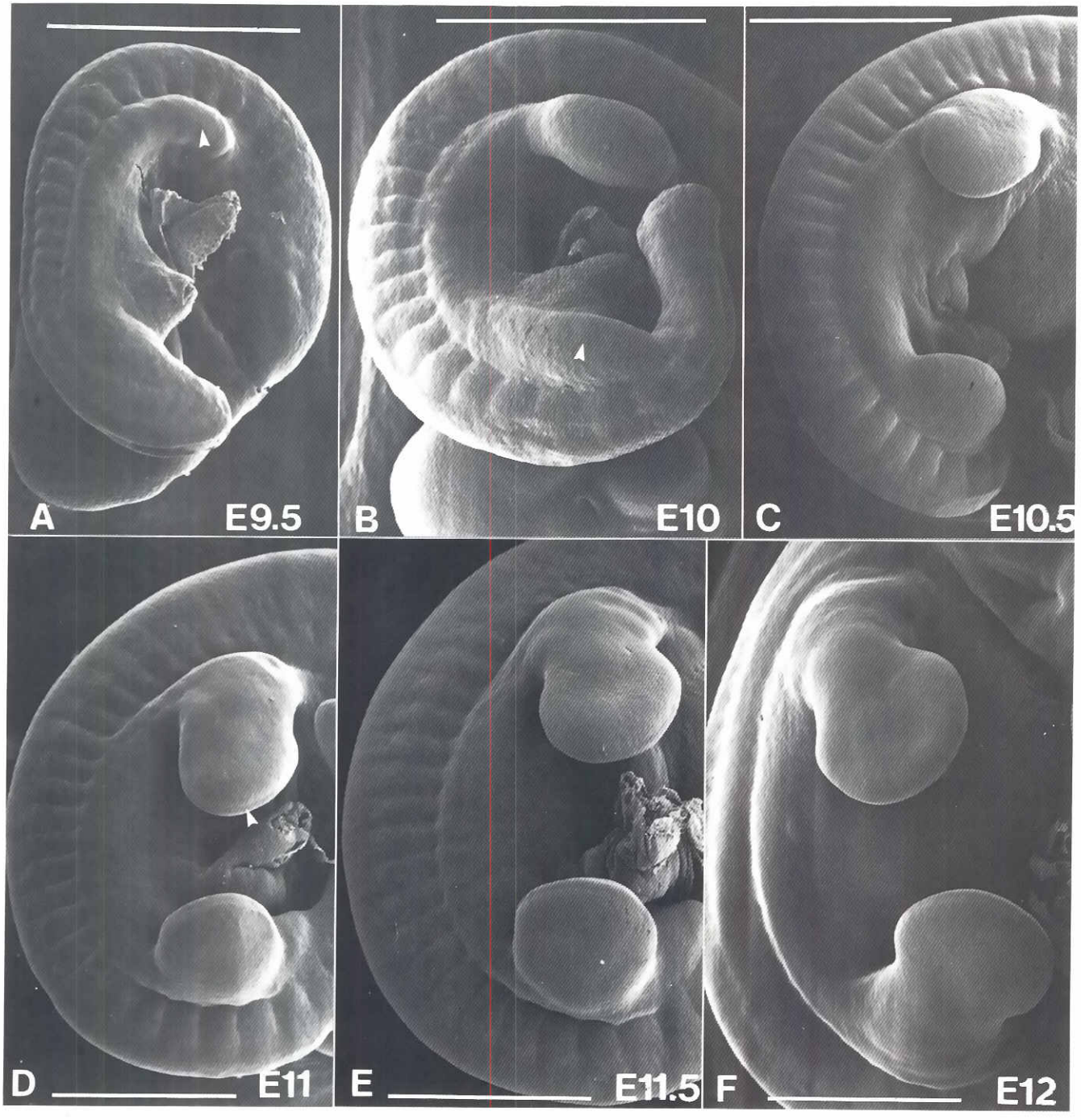
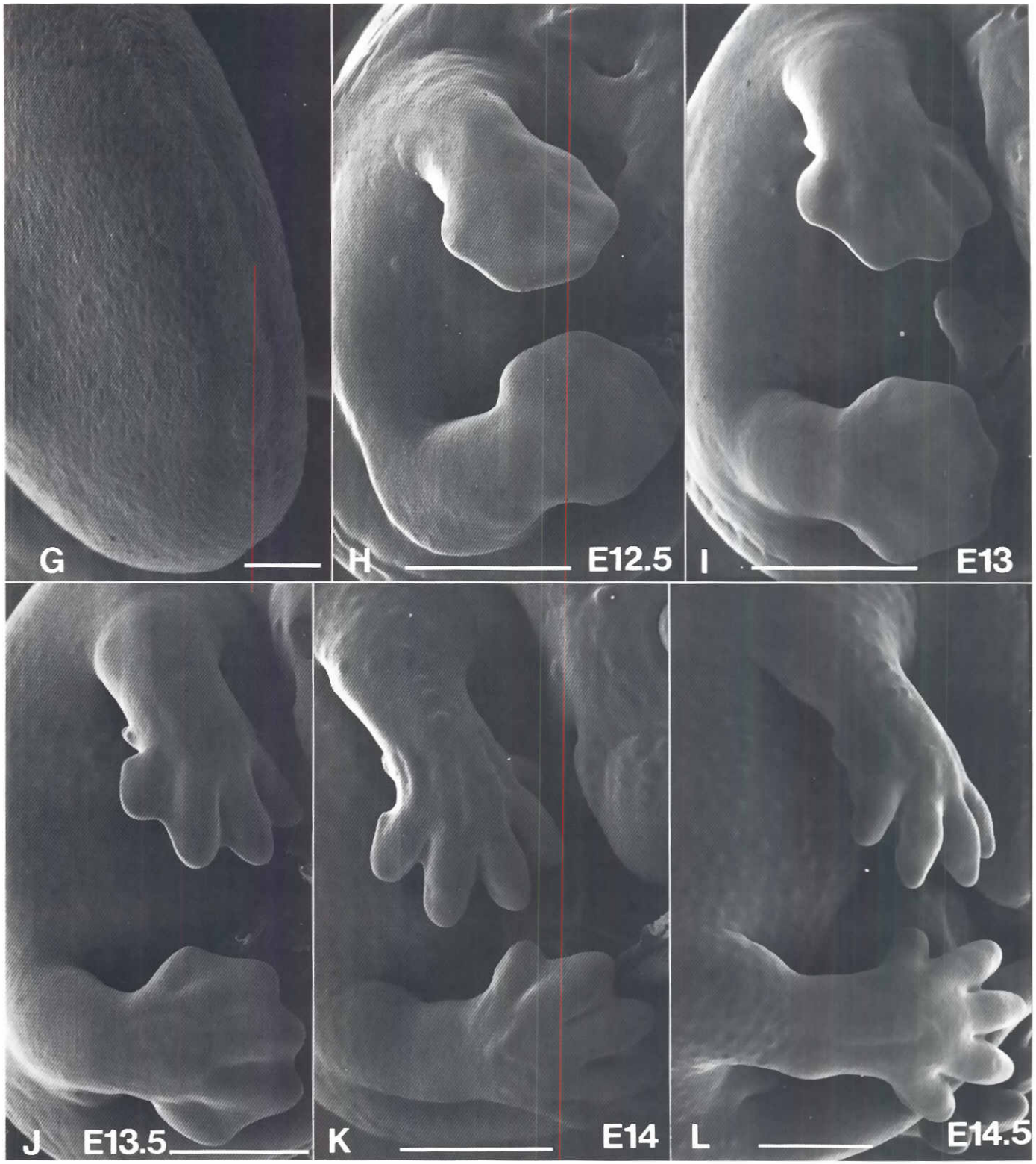


Fig. 1. A scanning electron micrograph series of mouse embryos taken at half-day intervals during the period when the fore- and hindlimbs are developing. *Each micrograph is oriented so that the forelimb is at the top.* **(A)** *E9.5 (21-29 somites) (Theiler, 1989: stage 15).The forelimb (arrow) is just beginning to protrude from the embryonic flank.* **(B)** *E10 (30-34 somites) (stage 16). Half a day later than the forelimb, the hindlimb (arrow) begins to develop.* **(C)** *E10.5 (35-39 somites) (stage 17).* **(D)** *(E11) (stage 18). The more advanced forelimb shows a distinct apical ectodermal ridge (AER) at the limb tip (arrows).* **(E)** *(E11.5) (stage 19). A handplate is beginning to form. The hindlimb also reveals a distinct AER.* **(F)** *(E12) (stage 20). A footplate has formed and the more advanced handplate is showing early signs of angulation.* **(G)** *(E12) High power view of a hindlimb apical ectodermal ridge.* **(H)** *(E12.5) (stage 20+). The handplate appears corrugated with the ridges correspoding to where the digits will form.* **(I)** *(E13) (stage 21). The handplate shows early signs of interdigital concavities.* **(J)** *(E13.5) (stage 21+). First signs of hair placodes on the forelimb. Interdigital concavities in the handplate have now become clefts.* **(K)** *(E14) (stage 22). Very little interdigital tissue now exists to separate each finger of the handplate.* **(L)** *(E14.5) (stage 22+).Toes as well as fingers are now clearly separated. Scale bars represent: (A-F and K-L) - 1mm; (G) - 200μm.*



useful to begin looking directly at development of the mammalian limb. A description of mouse limb development has been published by Wanek *et al.* (1989b), which concentrates on patterning of the skeletal elements but does not discuss muscle, nerve and blood vessel patterning within the developing limb. In this paper I look in turn at the timetable of patterning of all the main tissue components

TABLE 1

OUTLINE OF MOUSE LIMB STAGES AND TIMETABLE OF TISSUE PATTERNING

E stages	E9.5	E10	E10.5	E11	E11.5	E12	E12.5	E13	E13.5	E14	E14.5
Theiler (1989) stages	15	16	17	18	19	20	20.5	21	21.5	22	22.5
Wanek *et al.*, 1989b (forelimb)	1	1+	2+	4	5	6+	8	9	10	11	12
(hindlimb)	0	1	1+	2+	4	5+	7	8	9	10	11
Approximate chick stages (Hamburger and Hamilton, 1951) – forelimbs compared	18	20	22	23/24	25	27	29	30	32	34	35
Skeletal elements					Hint of humerus		Digits appearing			Proximal elements ossifying	Most distal phalanges forming
Muscle			Myogenic cells at base of forelimb		Faint MF20 staining in forelimb (and myogenin, MyoD1, Sassoon *et al.*, 1989)		Clear muscle blocks seen with MF20		Muscles first revealed by birefringency		Muscles in metacarpals
Nerves					First nerve fascicles enter forelimb		Nerve branches to muscle and skin				Dense cutaneous plexus extending to fingertips
Skin			AER visible in sections				Bi-layered epidermis begins to stratify				Epidermis consists 4/5 layers. Dermis beginning to form

of the limb: epidermis, connective tissues (including the skeletal elements), muscles, nerves and blood vessels and compare observations in the mouse with the extensive data from chick.

Mouse fore- and hindlimbs develop in almost identical fashion but slightly out of synchrony with one another, the hindlimb lagging behind by about half a day throughout the developmental process. By looking at a series of specimens spaced at half day intervals (for scanning electron microscopy) or one day intervals (for histological analysis) between E9.5 and E14.5 we get a good picture of limb development beginning with the onset of limb outgrowth and culminating with a limb which is essentially a miniature model of its adult counterpart.

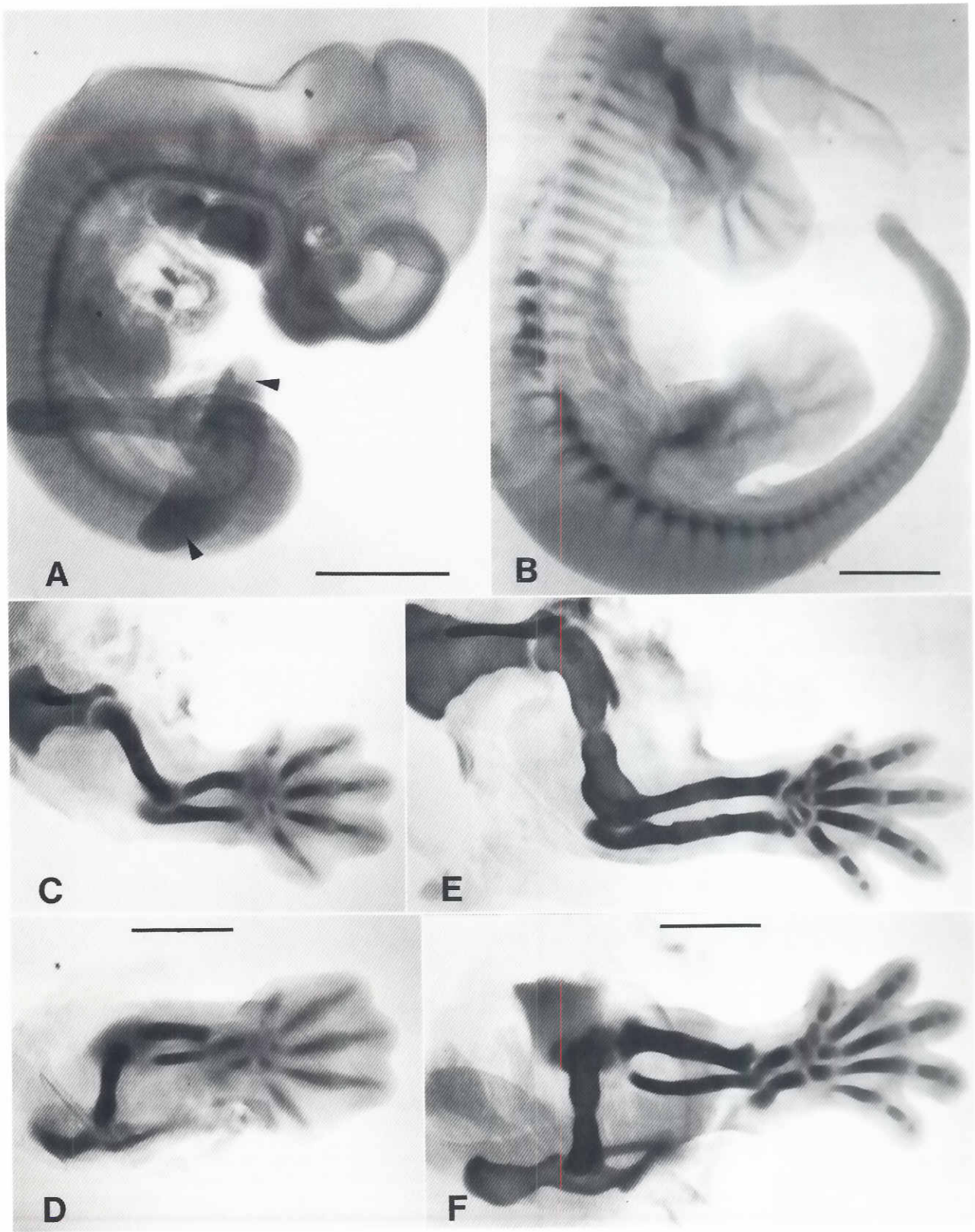
Results

The fore- and hindlimb buds of the mouse embryo first appear as bumps on the flank adjacent to somites 7-13 and 27-31 (Milaire and Mulnard, 1984), during the 10th and early 11th days of gestation (E9.5 and E10) respectively. A good overview of limb development is revealed by scanning electron microscopy of embryos from E9.5 - E14.5 (Fig. 1). The earliest appearance and subsequent development of the forelimb precedes that of the hindlimb by about half a day during early stages but the sequence of development for each tissue is similar for both limbs. In the chick both wing and hindlimb buds have been used for manipulative studies and in the mouse it is clear that particular experiments will favor using either the fore- or hindlimb depending on the developmental stage required; experiments involving whole-embryo culture of mouse embryos are best done on early stages (cultures commencing at E8.5 -12.5) and so favor manipulations of the forelimb because of its temporal advantage (Beddington and Martin, 1989) while surgery on the fetus *in utero* is best on later stages (E11.5 onwards), favoring manipulation of the later developing hindlimb (Wanek *et al.*, 1989a). For these reasons I show figures and briefly describe the timetables for both limbs, but where the details for both are very similar I concentrate here on the forelimb. A numbering system for the standard stages of mouse limb development, adopted from Theiler (1989), is outlined in Table 1 and Fig. 1, and correlated with chick limb developmental stages.

The cartilaginous skeletal elements are laid down sequentially in a proximodistal direction

At its earliest stages of development the limb bud consists of a jacket of epidermis, overlying a vascularized, apparently homogeneous, connective-tissue. In the chick the connective-tissue skeletal pattern is laid down in a proximodistal direction as development proceeds (Summerbell, 1976). This is also true for the mouse: by E11.5, whole-mounts stained with Alcian green reveal a hint of cartilage glycosaminoglycans (GAGs) in the proximal core of the forelimb, in the region destined to become the humerus, while at the

Fig. 2. A series of Alcian green stained and cleared wholemount specimens taken at one day intervals. (A) *E11.5. Cartilage staining is visible in the proximal core of the forelimb (*).The hindlimbs (arrows) are as yet devoid of such localized staining.* **(B)** *E12.5. Humerus, radius and ulna and metacarpals of digits 2, 3 and 4 are revealed in the forelimb. The corresponding digit elements are just becoming apparent in the hindlimb.* **(C and D)** *E13.5 Fore- and hindlimb respectively.* **(E and F)** *E14.5 The distalmost phalangeal elements of each finger have now formed. The corresponding distal elements of the toes are not yet apparent. Scale bars represent: (A-F) - 1mm. (C and D and E and F share a scale bar.)*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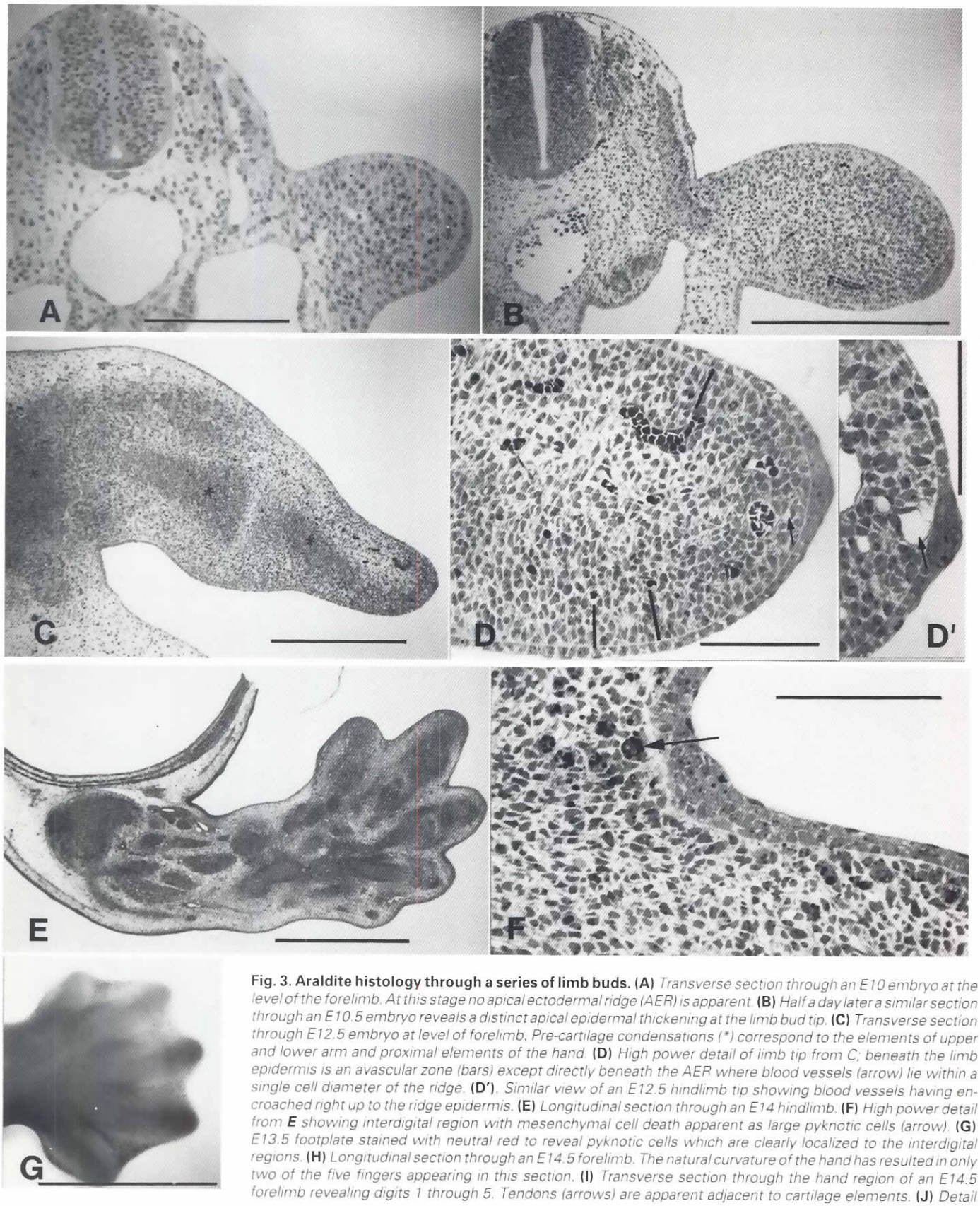
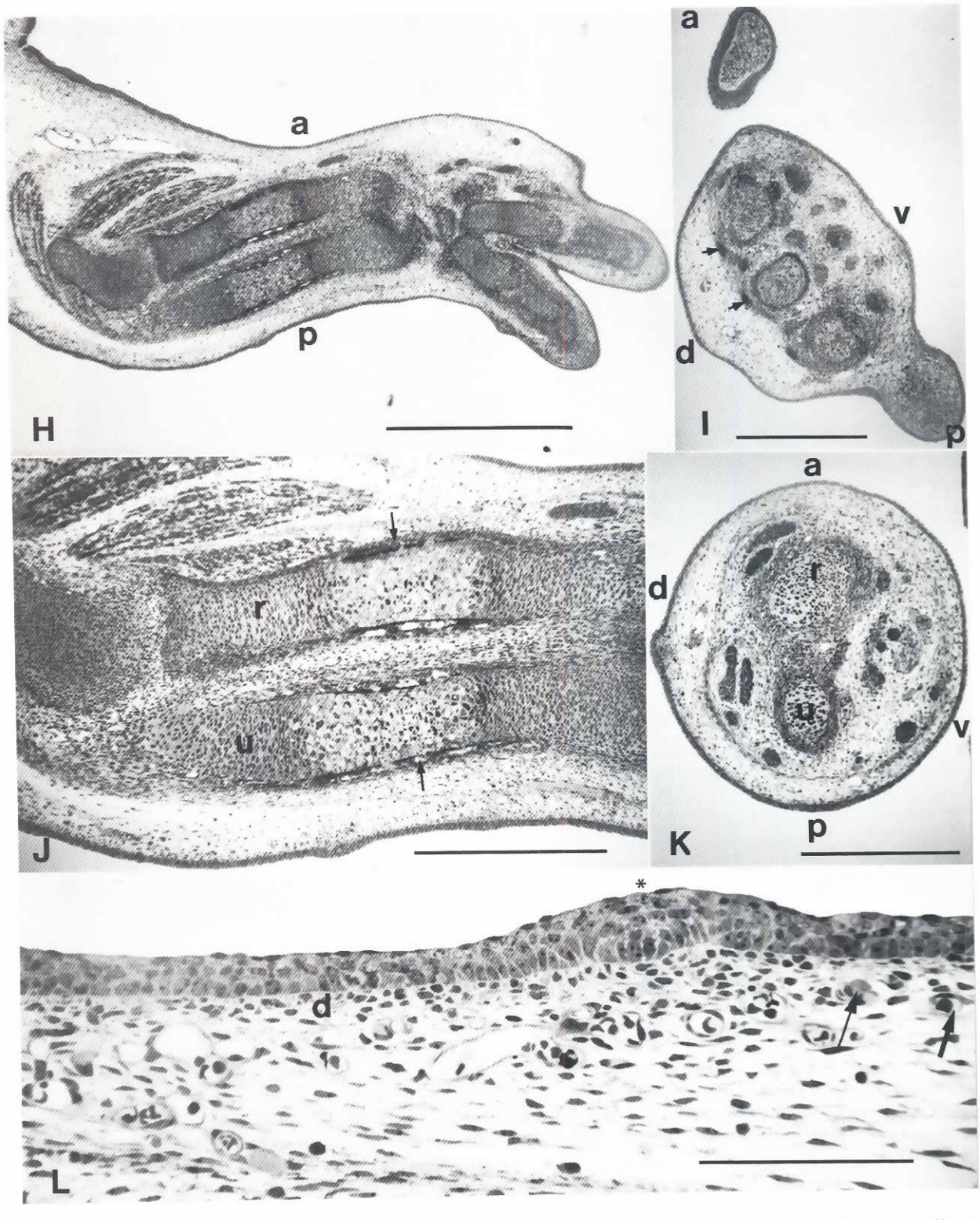


Fig. 3. Araldite histology through a series of limb buds. (A) *Transverse section through an E10 embryo at the level of the forelimb. At this stage no apical ectodermal ridge (AER) is apparent.* **(B)** *Half a day later a similar section through an E10.5 embryo reveals a distinct apical epidermal thickening at the limb bud tip.* **(C)** *Transverse section through E12.5 embryo at level of forelimb. Pre-cartilage condensations (*) correspond to the elements of upper and lower arm and proximal elements of the hand.* **(D)** *High power detail of limb tip from C; beneath the limb epidermis is an avascular zone (bars) except directly beneath the AER where blood vessels (arrow) lie within a single cell diameter of the ridge.* **(D')**. *Similar view of an E12.5 hindlimb tip showing blood vessels having encroached right up to the ridge epidermis.* **(E)** *Longitudinal section through an E14 hindlimb.* **(F)** *High power detail from* ***E*** *showing interdigital region with mesenchymal cell death apparent as large pyknotic cells (arrow).* **(G)** *E13.5 footplate stained with neutral red to reveal pyknotic cells which are clearly localized to the interdigital regions.* **(H)** *Longitudinal section through an E14.5 forelimb. The natural curvature of the hand has resulted in only two of the five fingers appearing in this section.* **(I)** *Transverse section through the hand region of an E14.5 forelimb revealing digits 1 through 5. Tendons (arrows) are apparent adjacent to cartilage elements.* **(J)** *Detail*



*from **H** to show chondrogenesis and ossification within the radius (r) and ulna (u) elements. A periosteal sleeve (arrows) overlies regions of hypertrophying cartilage in both elements.* **(K)** *Transverse section through an E14.5 forearm, cutting radius (r) and ulna (u) more distally than the regions of cartilage hypertrophy.* **(L)** *High power detail of posterior skin from **H**. The epidermis now consists of 4-5 cell layers, considerably thickened where a hair placode (*) is forming. Nerves (thin arrow) and blood vessels (thick arrow) approach within a few cell diameters of the epidermis. The first signs of a distinct dermis (d) are becoming apparent. Scale bars represent: (A) - 200 μm; (B, C) - 500μm; (D, D', F) - 100μm; (E, G) - 1mm.; (H) - 1mm; (I-K) - 500μm; (L) - 100μm. Throughout this figure, dorsal, ventral, anterior and posterior are indicated d, v, a and p.*

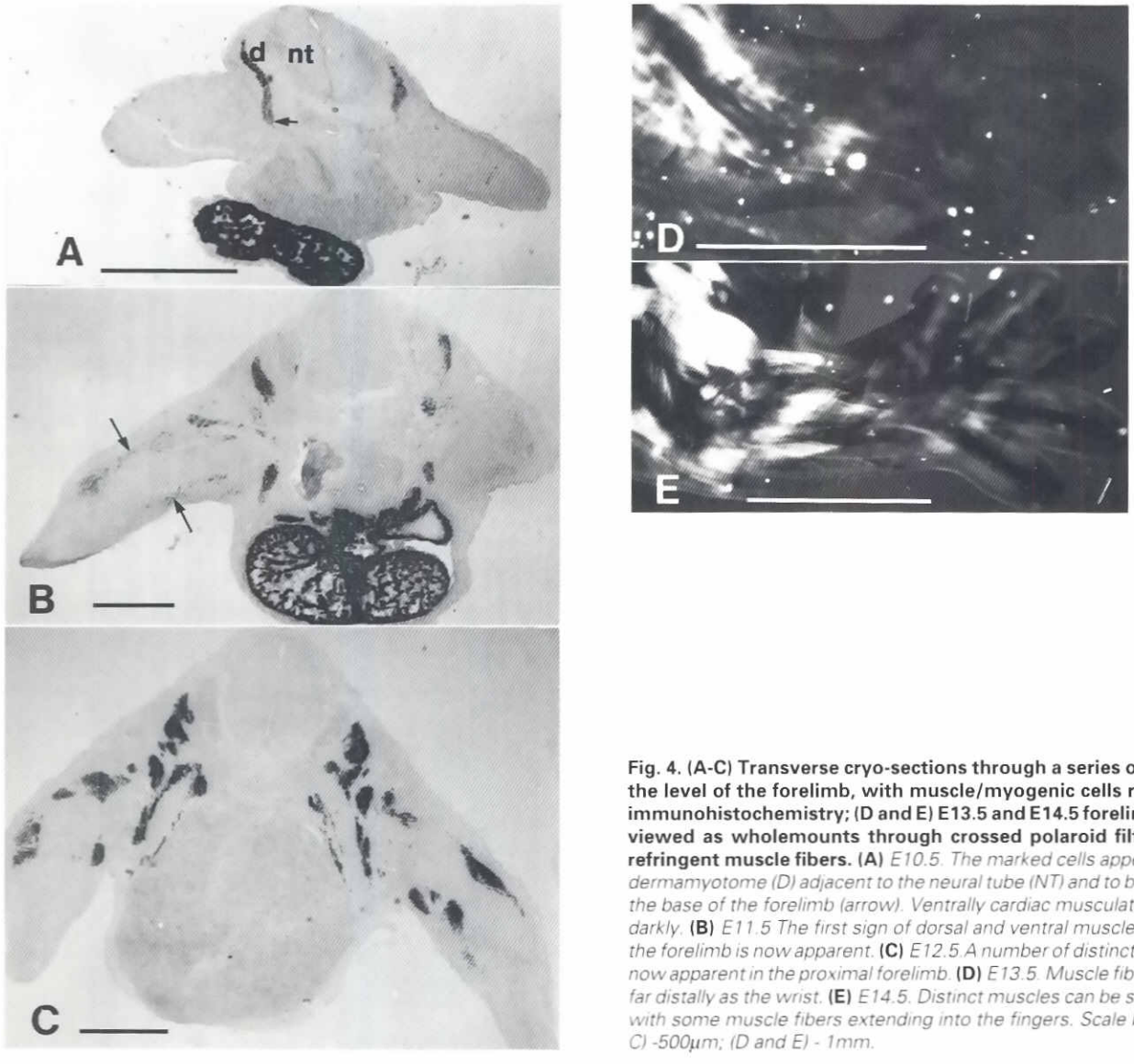


Fig. 4. (A-C) Transverse cryo-sections through a series of embryos, cut at the level of the forelimb, with muscle/myogenic cells revealed by MF20 immunohistochemistry; (D and E) E13.5 and E14.5 forelimbs respectively, viewed as wholemounts through crossed polaroid filters to reveal birefringent muscle fibers. (A) *E10.5. The marked cells appear to have left the dermamyotome (D) adjacent to the neural tube (NT) and to be heading towards the base of the forelimb (arrow). Ventrally cardiac musculature has stained up darkly.* **(B)** *E11.5 The first sign of dorsal and ventral muscle blocks (arrows) in the forelimb is now apparent.* **(C)** *E12.5.A number of distinct muscle blocks are now apparent in the proximal forelimb.* **(D)** *E13.5. Muscle fibers are revealed as far distally as the wrist.* **(E)** *E14.5. Distinct muscles can be seen in the forearm with some muscle fibers extending into the fingers. Scale bars represent: (A-C) -500μm; (D and E) - 1mm.*

same stage no such staining is visible in the hindlimb (Fig. 2A). A day later, at E12.5 the most proximal elements of the hand and foot are also beginning to be revealed by Alcian green staining, with middle digits appearing earlier than digits 1 and 5 (Fig. 2B). Sections through such a forelimb show precartilage condensations in the presumptive upper arm (humerus), lower arm (radius and ulna) and handplate (metacarpals) (Fig. 3C). By E14.5 all except the most distal phalangeal elements of the toes are apparent in Alcian green-stained wholemounts (Fig. 2F), while in the more advanced forelimb even the distalmost phalangeal elements of the fingers are visible (Fig. 2E). Transverse sections through the E14.5 lower arm reveal well-defined tendons adjacent to the radius and ulna cartilage elements (Fig. 3K), and the tendons are clear also in sections through the handplate, adjacent to the cartilage of the digits (Fig 3I). Ossification of the limb skeleton begins, much like the earlier process of chondrification, in the most proximal elements. Periosteal sleeves form around the humerus, radius and ulna at around E14. These sleeves are most obvious in the limb nerve wholemounts where the ossifying regions stain black in the silver stain reaction (Fig. 5D and E). Longitudinal sections through the E14.5 forearm reveal the familiar pattern of cartilage differentiation and ossification within each element (Bloom and Fawcett, 1975) with proliferating zones of rounded cartilage cells at both ends of the radius and ulna, and larger, more flattened cells towards the central diaphysis, with the cells eventually becoming hypertrophic and then degenerating in the region overlain by a periosteal bony sleeve (Fig. 3H and J).

An apical ectodermal ridge is not apparent until some time after the mouse limb has begun growing out

The epidermis overlying most regions of the developing mouse limb consists of two components, the basal ectodermal layer and

the superficial squamous, pavement-like layer of periderm. At the earliest stages of limb development (up to about E12.5) these are both single-cell layers (Fig 3D), but from about E12.5 the basal ectodermal layer begins to stratify and at E14.5 the forelimb epidermis consists of four to five layers (Fig. 3L), the single peridermal layer (now called *stratum superficiale*) overlying a stratum basale that is three to four layers thick (nomenclature of layers from Sengel, 1976). The first few limb hair buds are visible in SEM views of the E13.5 forelimb (Fig. 1J). A day later (E14.5) they are more pronounced and far more numerous (Fig. 1L) and in section are seen to consist of a thickened placode of epidermis overlying a small condensation of connective-tissue cells (Fig. 3L).

From the very earliest stages of bird limb development there is a specialized ridge of epidermis running along the distal margin of the limb bud (Saunders, 1948; Todt and Fallon, 1984) – the apical ectodermal ridge (AER) – which has been shown to play an essential role in the proximodistal outgrowth of the bird limb (Saunders, 1948; Summerbell, 1974). Curiously such a ridge of epidermis is not present during the earliest stages of mouse limb development (Wanek *et al.*, 1989b). Histological sections through the E10 forelimb reveal no sign of a distal epidermal specialization, although ventro-distal ectoderm is noticeably thicker than that on the dorsal surface (Fig 3A). By E10.5 a thickening of the distal tip epidermis is histologically distinct but no prominence in the outline of the outer epidermal surface has occurred. Such a prominence, making the ridge visible by SEM, becomes apparent about half a day later – E11 for the forelimb or E11.5 for hindlimb (Fig. 1D and E). By E14, at which stage almost all of the cartilage pattern of both fore- and hindlimb has been laid down, the forelimb AER has fully regressed but a slight ridge is still apparent in transverse histological sections through the footplate of the less mature hindlimb (data not shown).

An avascular zone exists beneath the early limb epidermis

The connective tissue just beneath the limb epidermis is notable at early stages for its lack of blood vessels. In the chick this avascular zone is well documented and extends to a depth of up to about 100 μm beneath the wing epidermis (Caplan and Koutroupas, 1973; Feinberg *et al.*, 1983; Martin *et al.*, 1989). Immediately beneath the avascular zone in the bird a cutaneous vascular (and at later stages neural) plexus forms (Feinberg *et al.*, 1983; Martin *et al.*, 1989). In the early mouse forelimb the avascular zone is significantly shallower than that of the bird, being only about 35-45 μm deep to the epidermis at stages up to E12.5 (see Figs. 3A-D), with an accompanying shallow cutaneous vascular plexus. Curiously, the one region in a mouse limb of this stage that noticeably lacks an avascular zone is directly beneath the AER, where capillaries encroach right up to the ridge epidermis (Fig. 3D and D'). (In the bird, by contrast, the avascular zone beneath the AER is if anything more pronounced than elsewhere). In the handplate region of a E14.5 mouse forelimb, much as at earlier stages, a 35-45μm avascular, aneural zone persists, but more proximally both blood vessels and nerves have entered the previous 'no-go' area to lie within one or two cell diameters of the rapidly maturing epidermis (Fig 3L). As late as E14 there is still no obvious dermal layer beneath the forelimb epidermis as there would be in a comparably staged chick wing.The first indication of dermis in the limb is not until E14.5 when a layer of rounded cells, 2 or 3 cells deep, lying just beneath the epidermis becomes distinguishable from the looser meshwork of deep mesenchyme in proximal regions of the limb (Fig. 3L).

Interdigital cell death aids in separation of the digits

Although cartilage formation is separate for each digit, fingers and toes are initially connected by soft tissue as part of a hand or foot plate in both chick and mouse embryos. Separation of the digits is in progress in the E14 foot and almost complete in the E14 hand (Fig. 1K). The process involves interdigital mesenchymal cell death, clearly visible in sections as a wedge of pyknotic cells (Fig. 3F), and retraction of interdigital epithelium into the dying mesenchymal cleft. Further remodeling of the digits is apparent as more mesenchymal cell death beneath the epidermis of the separated digit. Half a day later (E14.5) when the toes are fully separated there is no longer any sign of cell death (Fig. 3H and I).

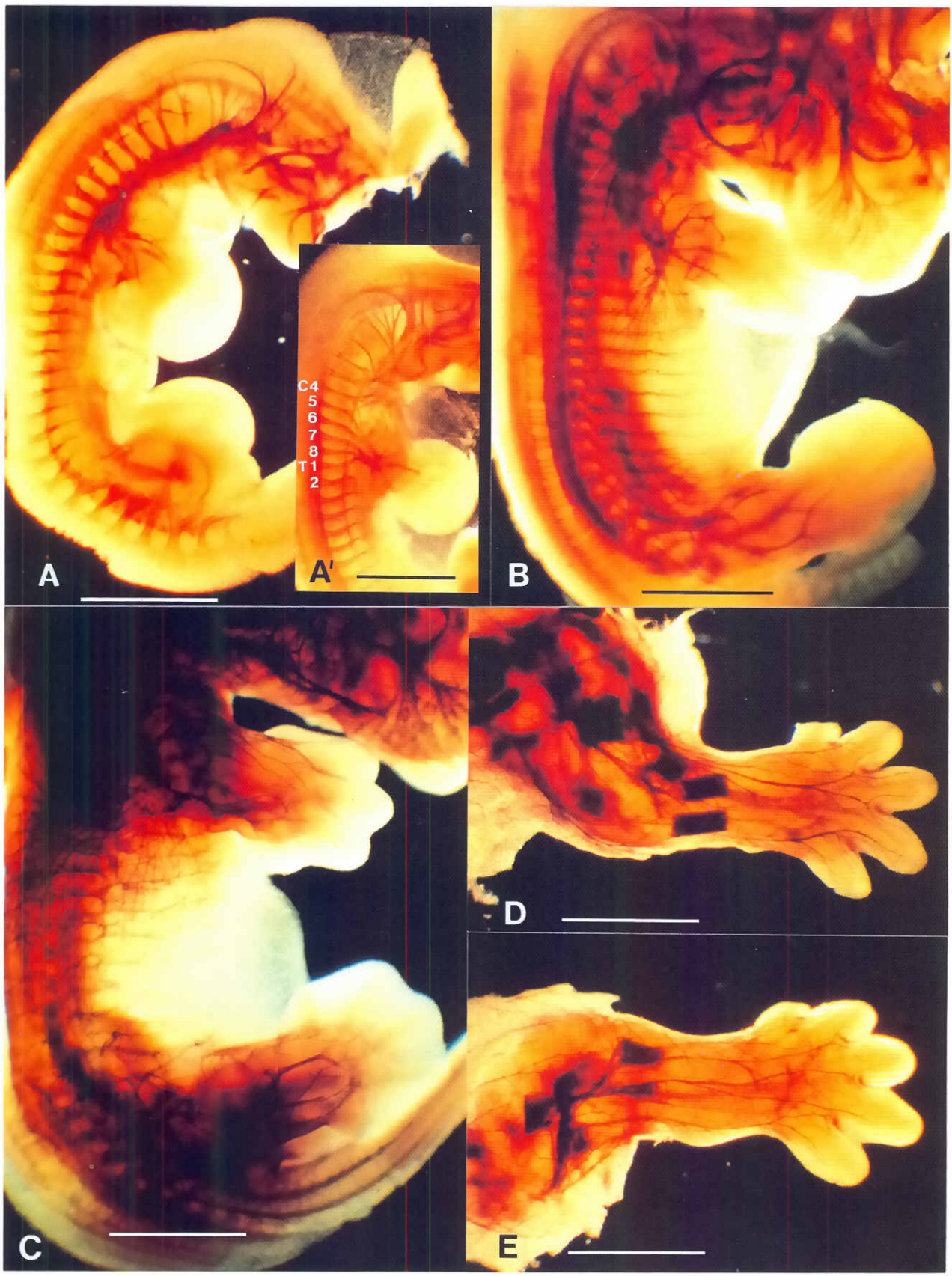
In the chick three other main regions of cell death have been noted during limb development, the anterior and posterior necrotic zones (ANZ and PNZ) and a region of mesenchyme between the radius and ulna (in the forelimb) or tibia and fibula (in the hindlimb), called the the opaque patch (reviewed in Hinchliffe and Johnson, 1980). Staining with neutral red (a vital dye concentrated in pyknotic cells) confirms the histological observation of interdigital cell death in the mouse E14 footplate (Fig. 3G) but does not reveal any sign of major zones of programmed cell death analogous to those seen in the early chick limb bud (data not shown).

The first myogenic cells are not seen in the forelimb by antibody staining until E11.5

Viewing cleared limbs with crossed polarizing filters, which highlight birefringent muscle fibers, I saw no indication of musculature at E12.5 in either fore- or hindlimb buds; but one day later (E13.5) birefringent muscles were apparent in both limbs, extending as far distally as the wrist in the developing forelimb (Fig. 4D). At E14.5, with digits now separate from one another, the muscles in upper and lower arm were now quite sharply defined and birefringent muscle fibers were apparent at the proximal ends of the metacarpals (Fig. 4E). The individual limb muscles visible by their birefringency in the E13.5 and E14.5 embryos are derived by repeated splittings from single dorsal and ventral muscle masses. These latter early rudiments are not visible by birefringency, but can be immunohistochemically revealed as early as about E11.5 (Fig. 4B) in the forelimb or E12.5 in the hindlimb (data not shown), using the antibody MF20 (gift from Dr. D. Fischman), raised against the light meromyosin fragment of early muscle myosin heavy chain (Bader *et al.*, 1982). Muscles are composite structures and in the bird embryo it has been shown that their contractile cell components are derived from myogenic cells that emigrate from the myotomes of somites adjacent to the limb (Chevallier *et al.*, 1977; Christ *et al.*, 1977). At E10.5 in the mouse, the MF20 antibody shows a band of myogenic cells that have left the dermamyotome and are lying along a pathway that appears to lead towards the base of the forelimb bud (Fig. 4A). A similar band of myogenic cells leading toward the base of the hindlimb is not apparent until E11.5 (data not shown).

The timetable of limb innervation is clearly revealed in silver-stained wholemounts

In the mouse, the forelimb receives innervation via the brachial plexus, from spinal nerves C4-8 and T1 together with a small contribution from T2 (Fig. 5A'), while the hindlimb is innervated by spinal nerves L1-6 via the lumbar plexus. At E11.5 the first nerve axons are seen to be encroaching upon the base of the hindlimb bud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while the forelimb bud has already been invaded by a number of thick nerve fascicles (Fig. 5A). At E12.5 both fore- and hindlimb buds reveal the rudiments of the main mixed nerve trunks that will supply the limbs (Fig. 5B). Side branches to muscles and skin begin to form in a general proximodistal progression; side branches that will serve proximal muscles are already apparent in the E12.5 forelimb and by E13.5, similar branches to muscle and skin extend well into the handplate and are beginning to enter the footplate (Fig. 5C). Ramification, particularly of the cutaneous nerve branches, is extensive during the next 24 hours, so that in the E14.5 fore- and hindlimb a dense network of innervation is revealed, extending to the tips of fingers and toes (Figs. 4D and E). As described in the chick (Swanson and Lewis, 1982), the pattern of nerve trunks and of nerve branches to muscle and skin is remarkably consistent from limb to limb while the ramifications thereafter within target muscles and skin appear random.

Discussion

In this study I have described the timetable of patterning for the various component tissues that make up a mouse limb during the critical 4- or 5-day initial period of its development. Forelimb development in the mouse commences at about E9.5 with the hindlimb lagging behind by about half a day. A miniature model of the adult limb has been formed 5 days later (E14.5 and E15 for fore- and hindlimb respectively). Later development is mainly just a matter of growth and maturation of the component tissues converting, for instance, the miniature embryonic cartilage template into the bony skeletal elements of the adult limb.

Proximodistal limb pattern

The pattern of cartilaginous skeletal elements as revealed by Alcian green staining of wholemounts develops in a proximodistal direction. This observation together with the fate-map studies of Muneoka *et al.* (1989) suggests that limb pattern in the mouse, as in the chick, is laid down in a proximal-distal sequence. In the chick, removal of the AER stops this process in its tracks resulting in truncated limbs, with the truncations progressively more distal the later the AER is removed (Saunders, 1948; Summerbell, 1974). Curiously in mouse an AER is not apparent until sometime after the onset of limb development and the chick experiment of removing early distal tip epidermis surgically has not yet been repeated for mouse. However, many mutations affecting normal development of the limb have been described in the mouse (Gruneberg, 1963). One such mutation ld^{Hd} (an allele of the gene *limb deformity*), which disrupts normal AER differentiation and subsequently causes proximodistal truncations of both fore- and hindlimb (Zeller *et al.*, 1989), suggests that a similar relationship between the AER (or at least distal tip epidermis) and underlying progress zone mesenchyme drives proximodistal patterning in mice just as it does in birds. Molecular analysis of genes like *limb deformity* should contribute to our understanding of the molecular mechanisms underlying the special epithelial-mesenchymal interaction occurring at the distal tip of the developing limb.

Anteroposterior limb pattern

In the avian wing it has been shown that anteroposterior pattern is modulated by a gradient of morphogen emanating from a group of mesenchymal cells at the posterior margin of the limb bud, collectively known as the zone of polarizing activity (ZPA). A graft of ZPA tissue to the anterior margin of the limb causes mirror-image duplication of limb pattern in the anteroposterior axis (Tickle *et al.*, 1975). The anteroposterior duplications resulting from ZPA grafts can be mimicked by implanting a bead soaked in retinoic acid (RA) beneath anterior epidermis (Tickle *et al.*, 1982) and together with the observation that a natural gradient of RA (running posterior to anterior) exists in the chick limb (Thaller and Eichele, 1987), this suggests that the anteroposterior morphogen in birds is RA. Similar experiments have not been reported for the mouse but it is known that posterior limb mesenchyme from a mouse limb will trigger chick limb duplications just as well as normal bird ZPA grafts (Tickle *et al.*, 1976). Moreover, it has recently been reported that two of the three mouse retinoic acid receptors (α and γ) are expressed in the limb bud at the critical time for anteroposterior patterning (Dolle *et al.*, 1989b). This important patterning mechanism is almost certainly conserved across amniote species (Fallon and Crosby, 1977).

Positional information in the developing limb connective-tissue might be marked by homeobox gene expression

The mouse limb is far ahead of its avian counterpart with regard to mapping of domains of homeobox gene expression. The recent report that a family of homeobox genes (the Hox 5 cluster on chromosome 2) is expressed in a temporal sequence (in the same order as they are spatially arrayed along the chromosome) as the limb grows out, is the most dramatic suggestion to date that homeobox gene expression might be the mechanism by which positional information is imparted to and 'remembered' by the limb connective-tissue cells (Dolle *et al.*, 1989a; and see Lewis and Martin, 1989). Other intriguing expression patterns for homeobox genes in the developing mouse limb include that of Hox-7, expressed by the progress zone mesenchyme at the limb tip and possibly by the overlying AER (Hill *et al.*, 1989; Robert *et al.*, 1989) and so perhaps involved in the AER/PZ interaction; and, less easily slotted into current limb patterning theories, an anteroposterior gradient of XIHbox1 protein in the mouse forelimb (Oliver *et al.*, 1988), which runs counter to the retinoic acid gradient seen in the chick limb.

Minor differences in tissue patterning exist between chick and mouse

It seems likely, though not yet proven, that the major mechanisms of vertebrate limb patterning will be largely conserved among the tetrapods, with minor modifications to allow, for example, for

Fig. 5. A series of silver-stained and cleared wholemount embryos, revealing patterns of limb innervation. (A) *E11.5. Nerves have yet to invade the hindlimb, while the first nerve fascicles are already present in the proximal forelimb bud.* **(A')** *Detail from a specimen of the same stage as* A, *revealing precisely the spinal nerves that contribute to forelimb innervation (C4-T2).* **(B)** *E12.5. Both fore- and hindlimbs contain the rudiments of the main mixed nerve trunks as well as some branches to muscle and skin.* **(C)** *E13.5. Nerve branches extend into both hand and foot plates.* **(D** and **E)** *E14.5 fore- and hindlimbs respectively, revealing nerve branches extending into the toes and almost to the tips of the fingers. Periosteal sleeves appear as dark jackets around the central diaphyseal regions of proximal cartilage elements. Scale bars represent: (A-E and A') -1mm.*

differing numbers of digits. However, there appear to be some finer details that have not been conserved across the bird/mammal evolutionary divide.

In the chick, much is made of the zones of programmed mesenchymal cell death seen in the developing limb and their probable role in the shaping of limb pattern; bird limb mutants often show reduced or enlarged zones of cell death associated with perturbed skeletal pattern and it was suggested that two of these cell death zones (the anterior and posterior necrotic zones- ANZ and PNZ) served the function of deleting mesenchyme that would otherwise give rise to digits 1 and 5 (reviewed, Hinchliffe and Johnson, 1980). This suggestion is encouraged by the finding that in the mouse limb there appear to be no major early zones of cell death equivalent to the ANZ, PNZ or the opaque patch of the bird, although Milaire and Rooze (1983) report observation of a small necrotic zone anterior to prospective digit 1. It appears that mesenchymal cell death is used by the mouse as a major morphogenetic tool only later in development, to separate digits in the hand and footplates.

Tissues associated with the embryonic skin develop to rather different timetables in chick and mouse. Condensation of the mesenchyme beneath the epidermis to form dermis is first apparent in the chick limb at a stage when the epidermis is still an immature bilayer of ectoderm and periderm. The bird dermis is coincident in many regions with the embryonic avascular/aneural zone beneath the epidermis (Martin and Lewis, 1989), but in the mouse the early, shallow avascular zone becomes invaded by blood vessels and nerves sometime before the appearance of a distinct dermis, by which time the overlying epidermis is already a fairly mature structure, consisting of 4 or 5 cell layers.

A further curious difference between chick and mouse limb development concerns the presence or absence of an avascular zone beneath the AER. In the chick the avascular zone beneath the ridge is deeper than beneath other regions of limb epidermis, but in the mouse it is non-existent, with blood vessels directly abutting the ridge epidermis. In the bird limb it has been suggested that high levels of hyaluronic acid (HA) synthesized beneath the AER might be responsible for the AER's ability to inhibit differentiation of sub-ridge mesenchymal cells in the so-called progress zone (Kosher and Savage, 1981), but since HA is known to inhibit vasculogenesis (Feinberg and Beebe, 1983) it would seem unlikely, at least in the mouse limb, that the AER exerts its influence over sub-ridge mesenchyme by synthesis of HA.

When do myogenic cells first enter the mouse limb?

In the bird, the full complement of myogenic cells has invaded the limb from dermamyotomes of adjacent somites at a stage resembling in shape that of an E10.5 mouse forelimb (Chevallier, 1978; Schramm and Solursh, 1988) but there is some controversy over the timetable of myogenic migration into mouse limbs. A recent study in which genetically marked somites from a transgenic mouse were grafted adjacent to the wild-type mouse forelimb bud suggests that myogenic migration into the limb is relatively late in the mouse, perhaps not even starting until E10.5 (Beddington and Martin, 1989). In this paper I show that the MF20 monoclonal antibody against early muscle myosin heavy chain, reveals at E10.5 a stream of muscle or myogenic cells that appears to be heading down from the dermamyotome towards the forelimb, but with no muscle cells detectable in the limb itself. In an E11.5 forelimb, the MF20 antibody faintly picks up myogenic cells as they are beginning to establish the earliest muscle blocks in the limb. MyoD1 and myogenin, both considered very early markers of myogenic cells, are also not expressed in the forelimb until E11.5 and then only in proximal regions (Sassoon *et al.*, 1989). However, if very early forelimb buds (24 somites or Sassoon *et al.*'s E9.25) are dissected from the embryo and grown in organ culture for 3-4 days, they too appear to express MyoD1 and myogenin proximally, suggesting that at least some myogenic cells may enter or already be present in the limb bud prior to its being explanted at the 24-somite stage (Sassoon *et al.*, 1989). Because of the difficulty of operating on very early mammalian embryos, the experiment of replacing the whole limb file of somites with marked somites from a donor embryo has not yet been done in the mouse and so the possibility still exists that not all muscle cells in a mouse limb are somite derived. In this regard it is worth noting that mouse somites transplanted to a chick host fail to contribute to the host limb musculature (Kieny *et al.*, 1987) and that chick somites grafted to a quail host give rise to limb musculature of mixed origin (Chevallier *et al.*, 1977).

Innervation of the mouse limb

The process of mouse limb innervation can be traced in silver-stained wholemount specimens from the stage when the first nerve fascicles enter the limb bud until the stage when a dense cutaneous neural plexus has formed. The fore- and hindlimbs are consistently supplied by spinal nerves C4-T2 and L1-6, respectively, and, as in chick (Lewis *et al.*, 1983), they show a very reproducible pattern of main mixed nerve trunks and branches to muscle and skin while terminal ramifications within these target tissues appear random. Nerve branches to muscles sprout off shortly after the time muscle blocks can first be revealed by immunohistochemistry (see above). No experiments have yet been reported addressing the mechanisms of nerve patterning in the developing mouse limb, but in the chick a wealth of manipulative experiments have defined a multiplicity of cues guiding nerves to their targets, operating at different points along the pathway (see for example, Lewis *et al.*, 1981; Landmesser, 1984; Tosney and Landmesser, 1985; Martin *et al.*, 1989). Even given this body of work on the chick limb, very little is known about the molecular nature of any of these neural cues, with the possible exceptions of NCAM, which appears to play a role in nerve branching within muscles (Landmesser *et al.*, 1988), and nerve growth factor (NGF) which may act as the trophic factor for cutaneous nerves once they have reached their target skin tissues (Davies *et al.*, 1987; Rohrer *et al.*, 1988).

Conclusion

The manipulation-friendly chick limb has supplied developmental biologists with the scaffold of an account of the tissue interactions that control patterning in the vertebrate limb. For the most part, the indications are that bird and mammalian limbs are patterned by similar mechanisms. Clearly, the next step is to fill in the molecular details, which will require directing attention more towards the mouse system with its wealth of molecular tools. As I have described earlier, the localization of mRNAs for retinoic acid receptors has already been reported in the mouse limb (Dolle *et al.*, 1989b), as have the temporal and spatial domains of mRNAs of homeobox genes, which are believed to play a part in defining positional values (Dolle *et al.*, 1989a). This paper supplies a series

of staged mouse embryos covering the critical period of limb development and a timetable of patterning for each of the component tissues, thus providing a foundation for interpretation of current and future molecular limb data.

Materials and Methods

Recovery and staging of embryos

For these studies of mouse limb development I have used an outbred strain of albino mice, strain PO (Pathology Oxford). Mice were kept on a light regime of 14h light and 10h dark with the midpoint of the dark period (presumed time of mating) at midnight. I have designated the morning on which a copulation plug was apparent as the first day post-coitum (or E0.5). Pregnant mice were killed by cervical dislocation at around midday (and also midnight for the SEM series) on days 10, 11, 12, 13, 14 and 15 post-coitum (p.c.) – that is, at gestational ages E9.5 – E14.5. Their embryos were dissected from the uterus into phosphate-buffered-saline (PBS) before processing in a variety of different ways to reveal the different tissue patterns within the limb.

It has been common practice, especially in studies of gene expression, to specify the stage of development of mouse embryos simply by gestational age. However, because of variations between species and even within litters, gestational age is not very precise as a means of specifying stage of development. Therefore, to accompany these studies I provide a series of standard SEM specimens (typical embryos from at least 3 litters for each stage) fixed at half day intervals and staged, according to Theiler (1989), by somite number for the earliest stages (up to E10.5) and by limb shape for the later stages. I have further calibrated these stages against the recently reported *in vivo* limb staging system (Wanek *et al.*, 1989b) and suggested comparative stages in chick limb development (see Table 1).

SEM and resin histology

For both scanning electron microscopy (SEM) and for resin histology, whole embryos were first fixed overnight in half-strength Karnovsky's fixative (Karnovsky, 1965), rinsed in 0.1M cacodylate buffer at pH 7.4 and then post-fixed for one hour in 1% osmium tetroxide, prior to dehydration through graded alcohols. Embryos were then either critical-point dried in the standard way for SEM or embedded in Araldite for routine thick-section (5mm) histology. Limbs from the older embryos were dissected free of the trunk before embedding in resin.

Identification of specific tissue types

Another batch of embryos were eviscerated in PBS and fixed in Bodian's fixative (ethanol 75%, water 15%, formalin 5%, glacial acetic acid 5%) for at least 24 hours. These fixed embryos were then processed in one of three ways: to visualize cartilage elements, embryos were stained in 0.1% Alcian green 2GX (BDH) in acid alcohol for three hours, differentiated overnight in acid alcohol, dehydrated and cleared in methyl salicylate before viewing under a dissecting microscope with bright field illumination. To visualize muscles in wholemount, embryos were not stained but simply dehydrated, cleared and viewed between crossed polarizing filters under a dissecting microscope with bright-field illumination – the specimen was rotated until the birefringent muscles were in optimal orientation, showing up bright on a dark background. To visualize nerves, embryos were processed by an adapted Bodian's silver method (Lewis *et al.*, 1981) prior to dehydration, clearing and viewing under dark-field illumination.

To visualize myogenic tissue by immunohistochemistry, 10μm cryosections were cut at the level of the fore- and hindlimb regions of unfixed whole mouse embryos embedded in gelatin. These sections were treated with a mouse monoclonal antibody (diluted 1:10 in PBS) to early muscle myosin heavy chain, MF20 (gift from Dr. D. Fischman) and positive cells were revealed with an HRP-labeled secondary antibody (Miles). At least 4 embryos of each stage were examined by each of the methods described above and the timetable of patterning for each of the tissues is summarized in Table 1.

To avoid clumsy terminology, I refer to the parts of the forelimb as arm, hand and fingers, and parts of the hindlimb as leg, foot and toes.

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