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Stage-specific expression patterns of alkaline phosphatase during development of the first arch skeleton in inbred C57BL/6 mouse embryos

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ABSTRACT

Timing and pattern of expression of alkaline phosphatase was examined during early differentiation of the 1st arch skeleton in inbred C57BL/6 mice. Embryos were recovered between 10 and 18 d of gestation and staged using a detailed staging table of craniofacial development prior to histochemical examination. Expression of alkaline phosphatase is initiated at stage 20.2 in the plasma membrane of mesenchymal cells in the distal region of the first arch. Expression is strongest in osteoid (unmineralised bone matrix) and presumptive periosteum at stage 21.32. Mineralisation begins at stage E23. Expression is present in the mineralised bone matrix. Secondary cartilages form in the condylar and angular processes by stage M24. The cartilaginous cells and surrounding cells in the processes are all alkaline phosphatase-positive and surrounded by the common periosteum, suggesting that progenitor cells of the processes, dentary ramus and secondary cartilages all originate from a common pool. Nonhypertrophied chondrocytes of Meckel's cartilage express alkaline phosphatase at stage M23. Expression in these chondrocytes is preceded by the expression in their adjacent perichondrium. This is true of chondrocytes in all other cranial cartilages examined. 3-D reconstruction of expression in Meckel's cartilage also revealed that the chondrocytes of Meckel's cartilage which express alkaline phosphatase and the matrix of which undergoes mineralisation are those surrounded by the alkaline phosphatase-positive dentary ramus. By stage 25, coincident with mineralisation in the distal section of Meckel's cartilage, most chondrocytes are strongly positive. The perichondria of malleus and incus cartilages express alkaline phosphatase at stage M24. Nonhypertrophied chondrocytes along these perichondria also express alkaline phosphatase. Superficial and deep cells in the dental laminae of incisor and 1st molar teeth become alkaline phosphatase-positive at the bud stage, stages 21.16 and 21.32, respectively. Dental papillae are negative until stage M24 when alkaline phosphatase expression begins in the dental papillae and follicles of the incisor teeth and the dental follicles of the 1st molar teeth. The dental papillae of the 1st molar teeth express alkaline phosphatase at stage 25. Expression in the dental papillae and follicles appears to coincide with cellular differentiation of follicle from papilla. The presumptive squamosal, ectotympanic and gonial membrane bones, lingual oral epithelial cells connected to the dental laminae of the incisor teeth, hair follicle papillae and sheath and surrounding dermis all express alkaline phosphatase in a stage-specific manner.

Key words: Dentary ramus; articulating processes; Meckel's cartilage; secondary cartilages; hypertrophy; incisor and molar teeth; mineralisation; hair follicles.

INTRODUCTION

Alkaline phosphatase, one of the earliest markers for osteogenesis, is expressed in a wide range of other tissues, e.g. teeth, germ cells, kidney, placenta, intestine, liver and brain (Milaire, 1974; Kwong & Tam, 1984; Wuthier & Register, 1985; Bronckers et al. 1987). Since alkaline phosphatase appears to have multiple functions including a role in mineralisation (Wuthier & Register, 1985) and because of its

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ubiquitous expression in a wide range of tissues, biochemical functions related to cellular events have remained unclear. Recent efforts to characterise different isoforms of alkaline phosphatase and their expression, however, have revealed that each isoform is expressed in a specific tissue at a specific time (Hahnel et al. 1990; Terao et al. 1990; Bossi et al. 1993; MacGregor et al. 1995). Protein domains of isoforms also play specific roles in cellular development (Bossi et al. 1993). One isoform known as a tissue nonspecific alkaline phosphatase (TNAP) is expressed in early mouse embryos from at least the 2-cell stage to 9.5 d, in primordial germ cells, placenta, testis, thymus, kidney, bone, liver and brain (Hahnel et al. 1990; Terao et al. 1990; Narisawa et al. 1994). Zernik et al. (1990) showed that mRNA and protein of the tissue nonspecific alkaline phosphatase in rats are expressed in the presumptive dentary ramus of the embryonic 1st arch.

That the mandibular bone consists of a large bone with incisor and molar teeth in the mouse 1st arch gives the impression that the 1st arch is composed of simple skeletal components. A developmental history of this skeleton, however, indicates diverse cellular origins and complex epigenetic interactions for differentiation and morphogenesis during both embryonic and postnatal periods (Atchley & Hall, 1991; Atchley, 1993). The skeletal components of the 1st arch—the dentary ramus, 3 articulating processes, Meckel's cartilage, 2 ear cartilages and teeth with alveolar bone—all undergo their own epigenetic interactions required for differentiation and morphogenesis (Atchley & Hall, 1991). Epithelial–mesenchymal interactions are required to initiate differentiation of ectomesenchymal cells into skeletogenic cells of cartilages, bones and teeth in the 1st arch (Thesleff et al. 1991; Hall, 1994). The condensation of progenitor cells for these skeletons marks a pivotal stage of differentiation and morphogenesis following the epithelial–mesenchymal interactions (Hall & Miyake, 1992, 1995). Other epigenetic factors such as mechanical loading (Herring, 1993*a*, *b*), the maternal uterine environment and postnatal care (Atchley et al. 1991; Atchley, 1993) also influence and modulate differentiation and morphogenesis.

Miyake et al. (1996*a*) developed a detailed staging table of craniofacial development of inbred C57BL/6 mouse embryos between Theiler's (1972) stages 18 and 21. By using this table, Miyake et al. (1996*b*) showed stage-specific onset of cellular condensation and matrix formation of Meckel's, malleus and incus cartilages in inbred C57BL/6 mouse embryos. In the present study we examined stage-specific expression of alkaline phosphatase during embryonic development of the 1st arch skeleton in inbred C57BL/6 mice using the staging table established by Miyake et al. (1996*a*). We employed BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium on histological sections to localise alkaline phosphatase expression in the developing skeleton. Our results show that alkaline phosphatase is expressed not only in developing bones, cartilages and teeth but also in hair follicles, surrounding dermis and lingual oral epithelial cells in a stage-specific manner.

MATERIALS AND METHODS

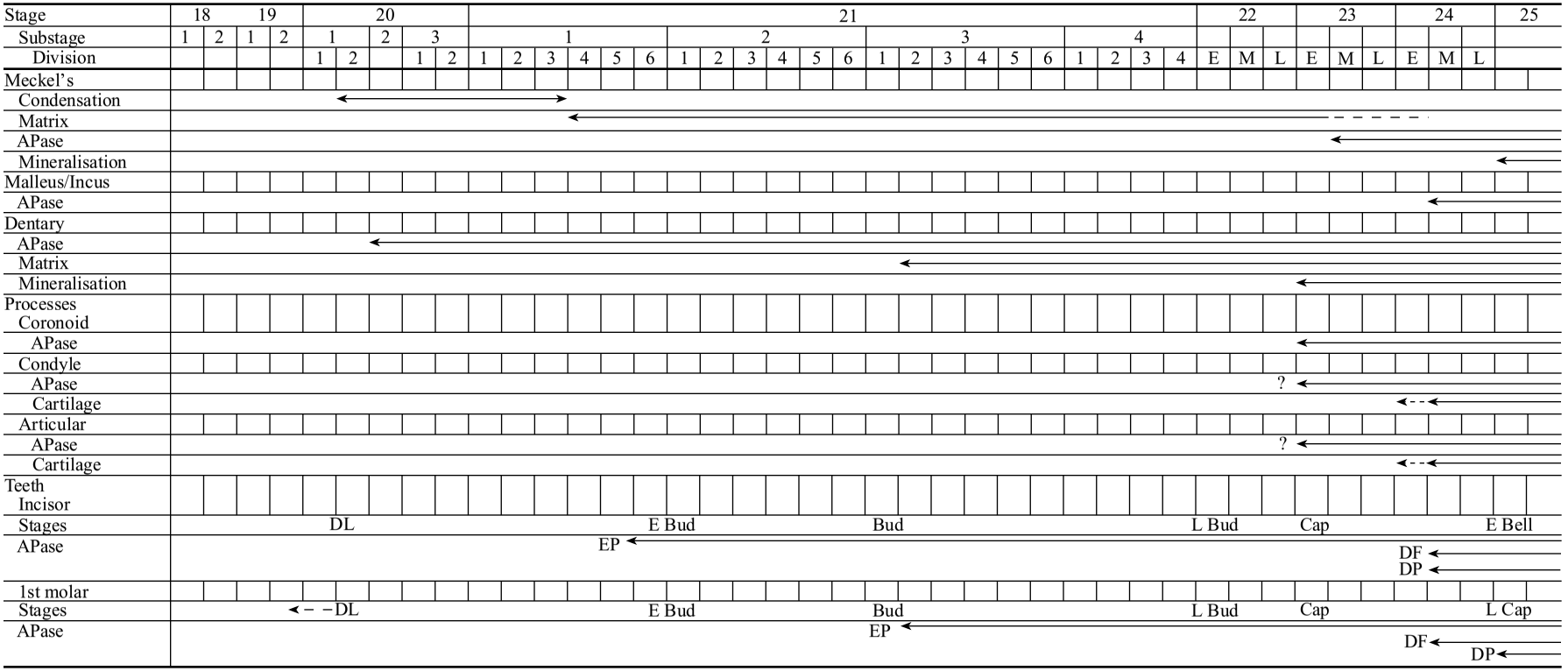
Breeding of mice and recovery and staging of embryos

Inbred C57BL/6NCr1BR mice (Charles River Canada, St-Constant, Quebec, Canada) were maintained and mated, and embryos were recovered and staged as described in Miyake et al. (1996*a*). Theiler's (1972) stages 18–21 were divided into substages and divisions based on craniofacial development (Miyake et al. 1996*a*). Stages 22–26 were divided into early (E), mid (M) and late (L) stages, since there is less intra- and inter-individual variation of stages of embryos at these stages than before stage 21.

Histochemistry of alkaline phosphatase

Embryos were fixed in 80% ethanol at 4 °C overnight. After serial dehydration in ethanol, embryos were embedded in low temperature paraplast. Some embryos were dehydrated in a series of ethanol and cleared in Histoclear at −20 °C to allow sections to be cut more easily from mineralised tissues. Alkaline phosphatase was visualised using a BCIP/NBT method modified from Blake et al. (1984). After deparaffinisation, sections were incubated in enzyme substrate medium at room temperature for 25 min in the dark. The medium consisted of 0.01 g 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma), 0.02 g nitroblue tetrazolium (NBT) (Sigma) and 400 ml 2 M $MgCl_2 6H_2O$ in 200 ml 0.15 M Tris buffer (pH 9.6). Sections were counterstained with 0.1% methyl green for 2–3 min and mounted with Entellan. Negative and positive controls were performed for histochemical staining of alkaline phosphatase. As a negative control, sections were incubated with 1 mM levamisole in 0.15 M Tris buffer (pH 9.6) for 25–30 min in the dark before incubation with BCIP/NBT. Sections of adult mouse kidney were used as positive controls.

Table. Timing of skeletogenesis in the 1st arch of inbred C57BL/6 mouse embryos



DF, dental follicles; DL, dental lamina; DP, dental papillae; EP, dental epithelial cells; E, early stage; M, mid stage; L, late stage.

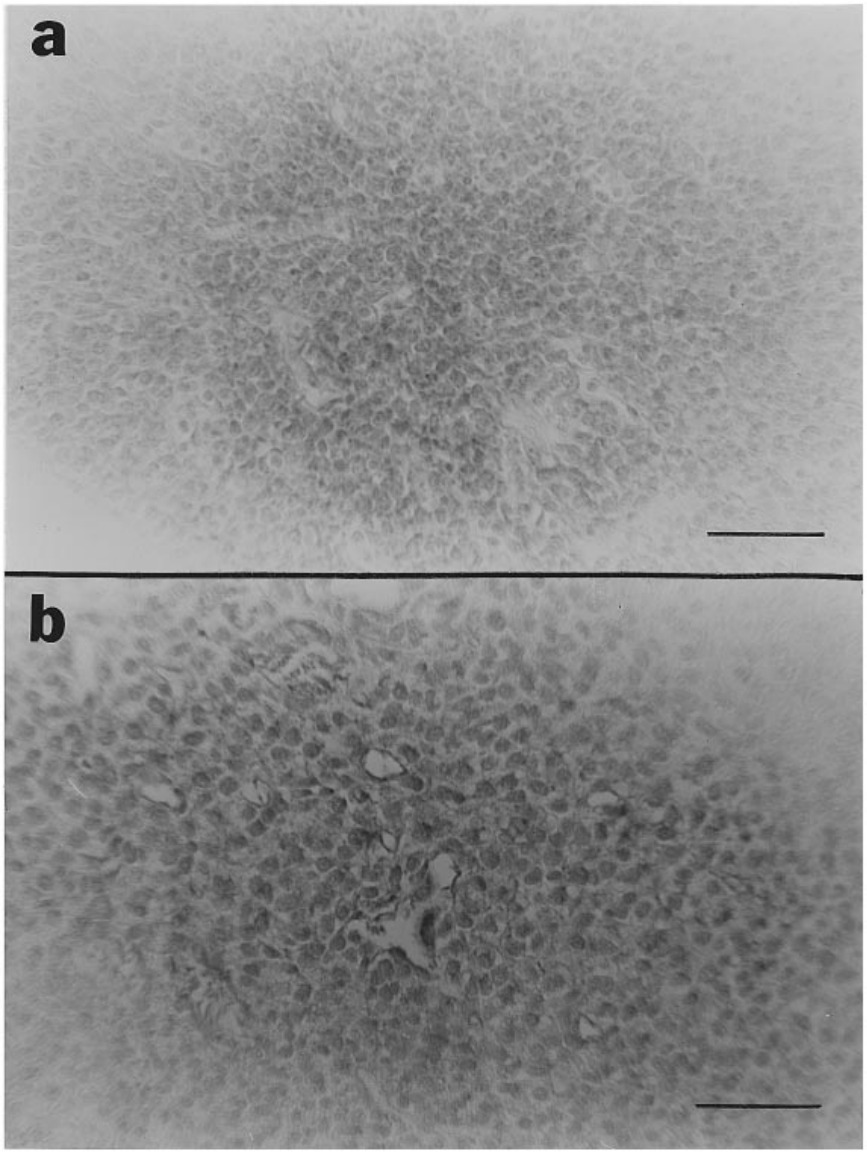


Fig. 1. Alkaline phosphatase in the first arch at stages 20.2 (*a*) and 21.25 (*b*), showing expression in the cellular membrane of mesenchyme and the extracellular matrix. Cross section. Bar, 50 μm.

Some embryos were demineralised with ethylene-diaminetetra-acetic acid disodium salt (EDTA) before histochemical preparation following Bourque et al. (1993). Before incubation with BCIP/NBT, reactivation of alkaline phosphatase was performed on histological sections (Yoshiki et al. 1972; Watson et al. 1989) by incubation with 0.2 M $MgCl_2 6H_2O$ in 0.05 M Tris-maleate buffer (pH 7.2) including 7% sucrose at 4 °C for at least 24 h, and rinsing in double distilled H_2O for 1–2 min.

Other histological stains

Some sections were stained with Hall & Brunt's quadruple (HBQ) (Hall, 1986), Mallory's triple stain (Pantin, 1960) or alizarin red S (Humason, 1979). Embryos were fixed in 80% alcohol overnight at 4 °C and embedded in low-temperature paraplast. After staining by von Kossa's method (Page, 1982), sections were counterstained with Mayer's haematoxylin for 3 min, with Alcian blue for 3 min and treated with 1% phosphomolybdic acid for 1 min. Mineralised tissues stained pale to dark blackish brown. For alizarin red S staining, sections were incubated with 0.5% alizarin red S in 0.2 M phosphate buffered saline (PBS) (pH 9.0) for 1 h, rinsed in 0.2 M PBS (pH 9.0) for 5 min and counterstained with 0.1% methyl green for 2–3 min. Some embryos were cleared and stained with Alcian blue and alizarin red S as whole mounts to visualise cartilages and mineralised bones, respectively (Miyake & Hall, 1994).

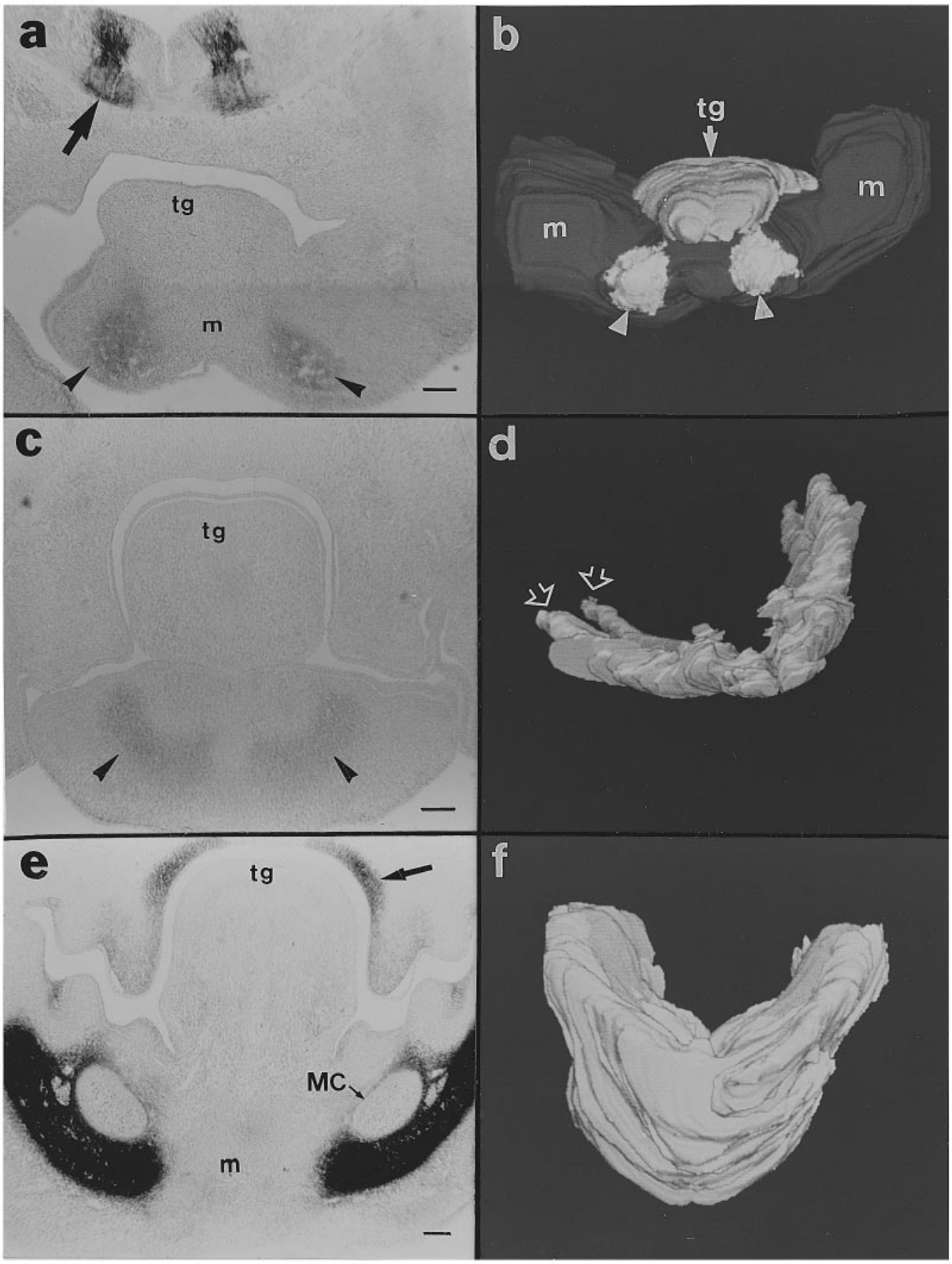


Fig. 2. (*a*, *c*, *e*) Expression of alkaline phosphatase in the 1st arch and (*b*, *d*, *f*) 3-D reconstruction of the expression viewed from the distal area of the 1st arch. Stage 20.2 (*a*, *b*): arrow, expression in the brain; arrowheads, expression in the 1st arch. Stage 21.25 (*c*, *d*): arrowheads, expression in the 1st arch; open arrows, a bifurcation of expression in the 1st arch. Stage 21.31 (*e*, *f*): arrow, expression in the presumptive palatine membrane bone. m, 1st arch; MC, Meckel's cartilage; tg, tongue. Cross section (*a*, *c*, *e*). Bar, 100 μm.

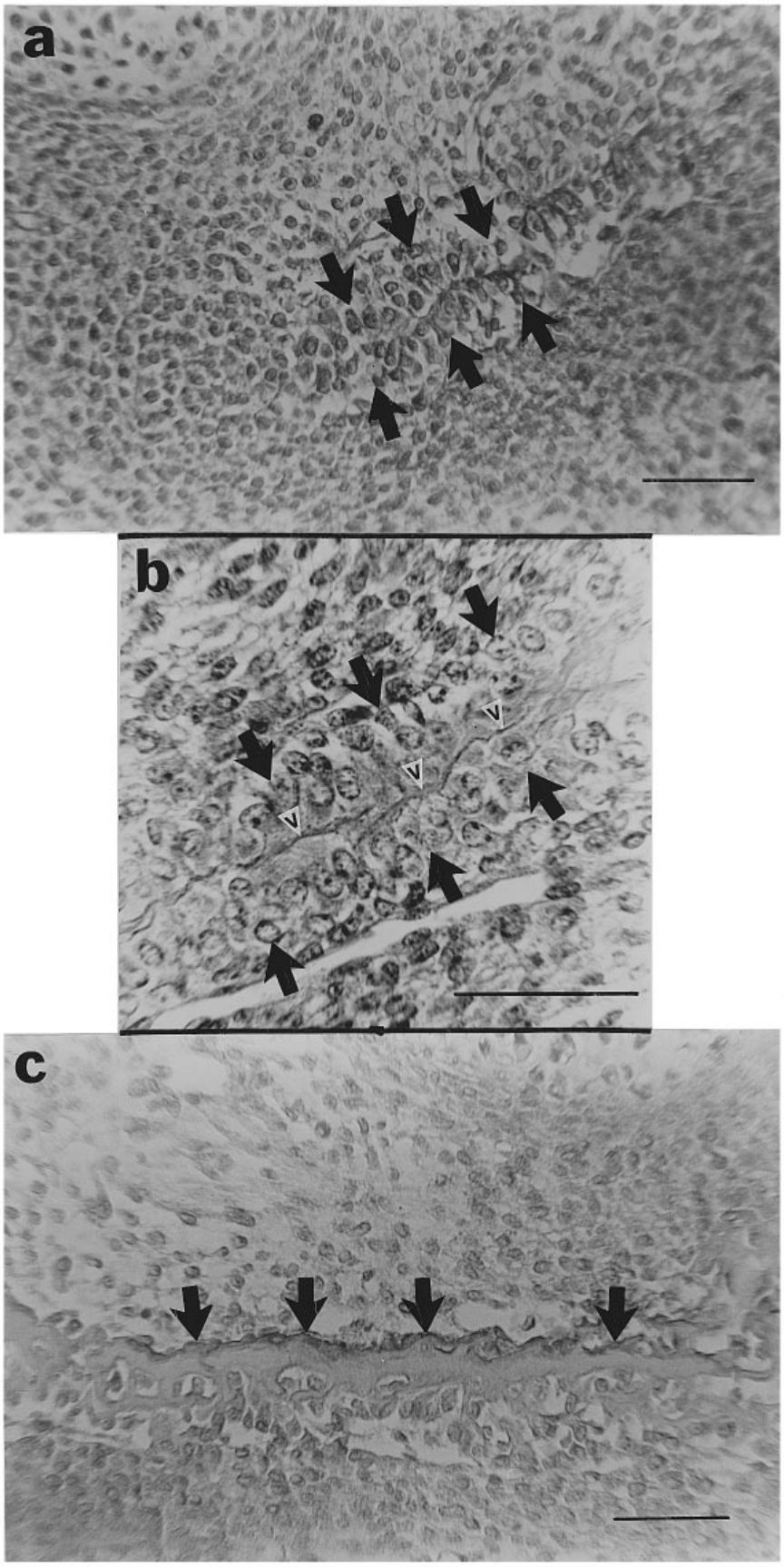


Fig. 3. (*a*) Alignment of osteoblasts (arrows) prior to formation of the osteoid in the dentary ramus at stage 21.32. Cross section. (*b*) Deposition of matrix (arrowheads) by aligned osteoblasts (arrows). (*c*) Initial mineralisation (arrows) along osteoid in the dentary ramus at stage E23. Visualised by von Kossa's method. Frontal section (*b*, *c*). Bar, 50 μm.

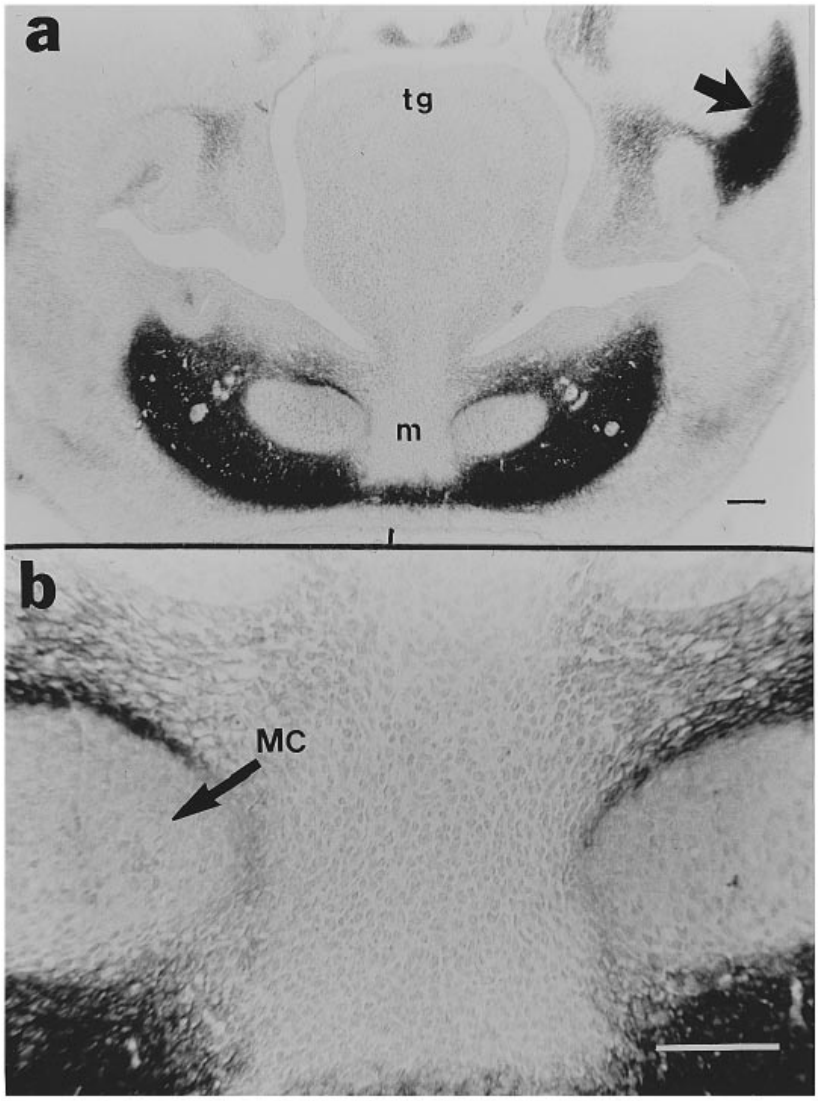


Fig. 4. (*a*) Expression of alkaline phosphatase in the 1st arch at stage E22. (*b*) High magnification of (*a*) showing the midsection of the arch under the tongue. Expression is very weak or negative at the midsection including the mediolateral perichondrium of Meckel's cartilage. An arrow indicates alkaline phosphatase expression in the presumptive maxillary membrane bone near the zygomatic process. m, 1st arch; MC, Meckel's cartilage; tg, tongue. Cross section. Bar, 75 μm.

Image analysis and 3-dimensional (3-D) reconstruction

Some sections were visualised with a Leitz Laborlux D compound microscope equipped with a monochrome camera (Cohu 4810 Series Monochrome Solid-State CCD Camera, Cohu, San Diego, California, USA). A microscopic image was captured by PixelGrabber in PixelTools (TCL-Image, Perceptics Corporation, Knoxville, Tennessee, USA), installed in a Mac IIci computer. Image software (Image 1.38), developed by Wayne Rasband at the National Institutes of Health, Bethesda, MD, USA, was used in PixelTools to capture images. Images were then printed using a monochrome laser printer. Some images were photographed directly from the computer screen using 35 mm camera with TMX 100 films. Other sections were microphotographed for 3-D constructions with TMX 100 films under a Leitz compound microscope.

3-D images were reconstructed from laser-printed images and photomicrographic negatives of histological sections. They were captured by a Hitachi CCD camera and processed in a DTK 386/25 computer with Amicus Software Version 1.0. 3-D images were then reconstructed using an ICAR 80.8 Workstation with Silicon Graphics Personal Iris 4D/25 Server and UNIX V Operating System.

RESULTS

Alkaline phosphatase expression in the dentary ramus

Timing of alkaline phosphatase in the skeleton of the 1st arch is summarised in the Table. Data on

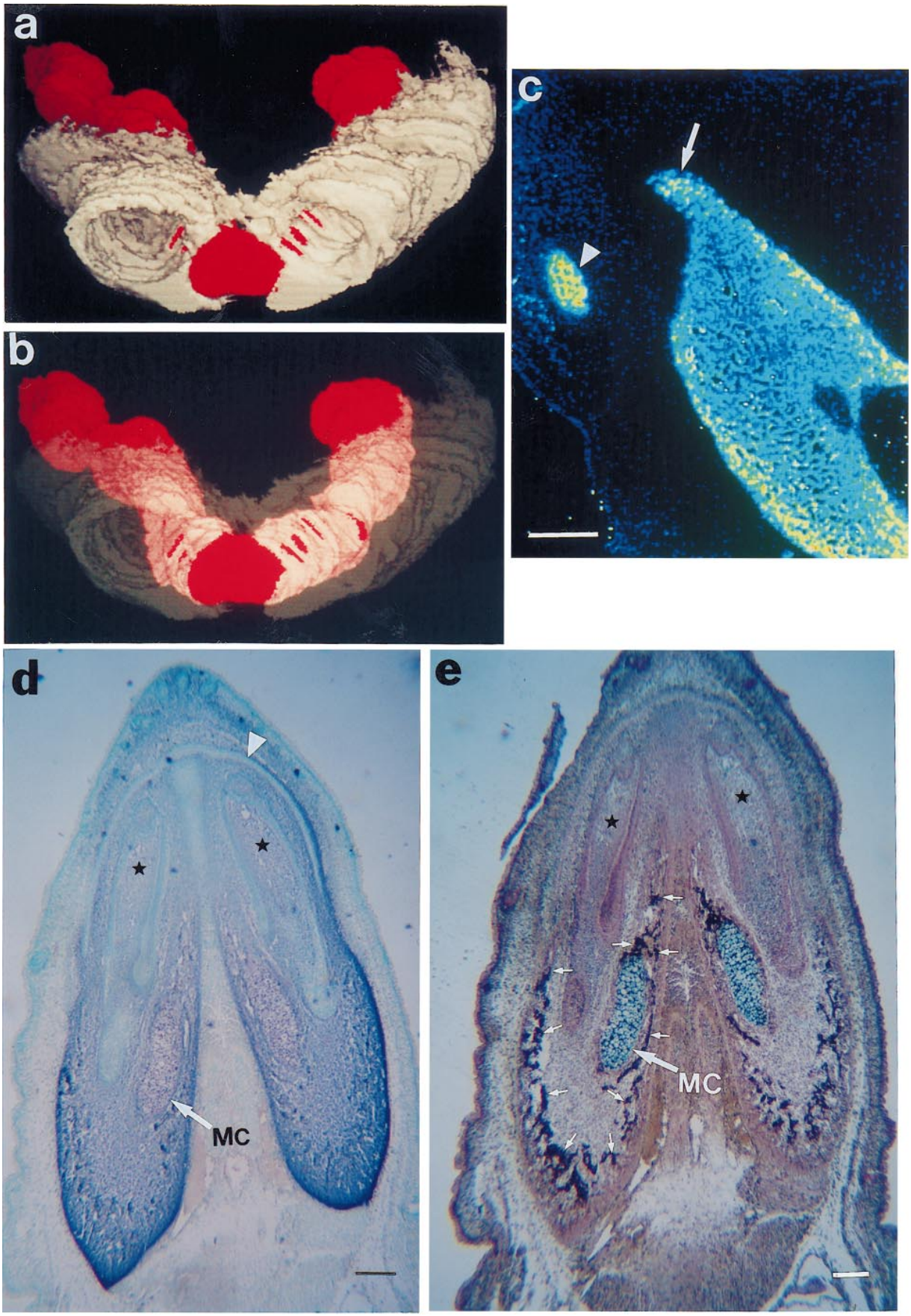


Fig. 5. For legend see opposite.

development of Meckel's cartilage in inbred C57BL/6 mouse embryos presented by Miyake et al. (1996*b*) are included in this Table.

The earliest expression of alkaline phosphatase in the 1st arch was at stage 20.2 as weak activity mostly in the plasma membrane of mesenchymal cells (Fig. 1*a*). 3-D reconstruction of expression (Fig. 2*a*) showed it to be confined to the most distal region of the 1st arch (Fig. 2*b*).

At stage 21.13, expression of alkaline phosphatase expanded caudally. Considerable mesenchyme in the distal region of the 1st arch was weakly positive both in plasma membranes and extracellular matrix (ECM). Towards the end of stage 21.2, expression extended laterally. The most proximal extent of expression was near the junction of the 1st arch and the tongue.

Expression of alkaline phosphatase was slightly upregulated from stage 21.2 onwards (Fig. 1*b*). Expression expanded further into the proximal region of the 1st arch (Fig. 2*d*). Mesenchyme in the periphery of the distal region expressed alkaline phosphatase weakly. Expression was stronger towards the midsection. Expression towards the junction of the 1st arch and tongue was confined to the midsection of the 1st arch. Peripheral cells were negative (Fig. 2*c*).

Stage 21.3 marked deposition of osteoid matrix in the dentary ramus. Alkaline phosphatase activity was stronger in the 1st arch than at previous stages. Activity distally was more diffuse towards the periphery, but most mesenchymal cells were positive (Fig. 2*f*). Expression in the proximal region was confined to the midsection, and was intense (Fig. 2*e*). The strongest expression was peripheral in the midsection where osteoid formed at stage 21.32.

Osteoblasts began to deposit unmineralised matrix (osteoid) along the midregion ventrolateral to Meckel's cartilage at stage 21.32 (Fig. 3*a*, *b*). A group of tightly packed osteoblasts condensed and lined up proximodistally ventrolateral to Meckel's cartilage before matrix deposition (Fig. 3*a*). Matrix was deposited in the middle of this condensation in a proximodistal direction (Fig. 3*b*, arrowheads). Each side of the matrix consisted of at least 3 or 4 cells along the osteoid. In some cases matrix deposition extended laterally along this midsection of the osteoid. Activity was strongest in these condensed cells. Using a simple regression of days of gestation on substages and divisions ($Y = 0.073X + 11.201$) produced by Miyake et al. (1996*a*), the time between onset of alkaline phosphatase expression and matrix deposition of the dentary ramus was estimated to be 28.0 h.

Stage M22 marked initial elevation of the palatal shelf. Expression of alkaline phosphatase was upregulated after stage 21. Almost all mesenchymal cells and ECM were positive in the distal region, strongest in the midsection and weaker towards the periphery. The symphysis was negative. Where the tongue expanded dorsally, expression was defined with clear-cut medial and lateral expression boundaries. (Fig. 4). The entire midsection under the tongue was negative (Fig. 4*b*).

Mineralisation of the dentary ramus began at the beginning of stage 23 in a small area of osteoid in the midregion of the arch. Von Kossa's method (Fig. 3*c*) and alizarin red S stain recognised this mineralised matrix. The ramus contained marrow spaces where alkaline phosphatase was still expressed. Almost all mesenchymal cells and ECM in the most distal region were positive. Activity was much weaker medially but stronger peripherally. The alkaline phosphatase-positive area was surrounded by fibrous cell layers, also strongly positive. The marrow space of the dentary ramus and mineralised matrix were also strongly positive.

Expression in the ramus tended to be slightly weaker in the marrow space at stage 24 except peripherally, as seen in 3-D reconstruction of expression in the 1st arch (Fig. 5*a*,*b*). Most of the distal region except the symphysis and midregion of Meckel's cartilage were surrounded by the alkaline phosphatase-positive dentary ramus (Fig. 5*a*, *b*). This expression pattern continued at stage 25 (Fig. 5*d*). As the marrow space of the dentary ramus expanded, mineralisation appeared to be confined to the periphery and excluded from the marrow space (Fig. 5*e*).

Alkaline phosphatase expression in the 3 processes of the dentary

At the mid and proximal regions of the 1st arch, the alkaline phosphatase-positive area of the dentary

Fig. 5. (*a*, *b*) 3-D reconstruction of expression of alkaline phosphatase and Meckel's cartilage in the 1st arch at stage E24, showing expression (white) (*a*) and its relation to Meckel's cartilage (red) (*b*). (*c*) The presumptive coronoid process (arrow) identified by alkaline phosphatase expression in the right side of the dentary ramus at stage E23. Expression was enhanced by computer imaging analysis. Yellow colour indicates the strongest activity of alkaline phosphatase. Arrowhead, alkaline phosphatase expression in the presumptive squamosal membrane bone. Cross section. (*d*) Alkaline phosphatase expression and (*e*) mineralisation in the 1st arch at stage E25. The incisor dental papillae (stars) and all chondrocytes of Meckel's cartilage are alkaline phosphatase-positive. Arrowhead, alkaline phosphatase-negative vestibular lamina; arrows, mineralised area in the dentary ramus. Mineralisation was visualised by von Kossa's method. Frontal section. Bar, 150 μm.

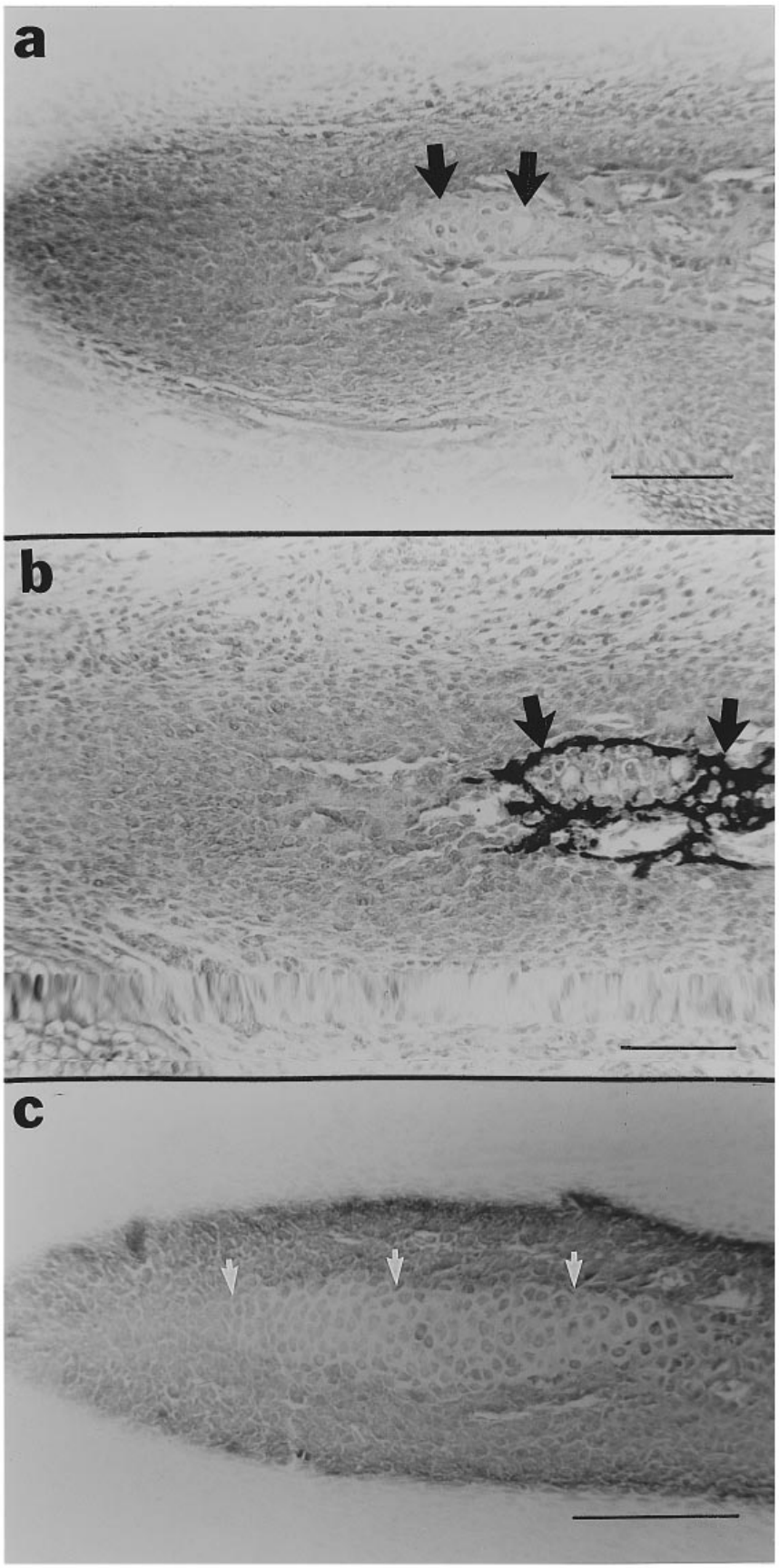


Fig. 6. (*a*) Alkaline phosphatase expression and matrix formation of the secondary cartilage and (*b*) mineralised matrix of the secondary cartilage in the condylar process at stage M24. (*c*) Alkaline phosphatase expression in the angular process at stage M24. In a proximal to distal direction, immature to mature chondroblasts (arrows), all alkaline phosphatase-positive, are surrounded by alkaline phosphatase-positive bone-forming cells. Frontal section. Bar, 100 μm.

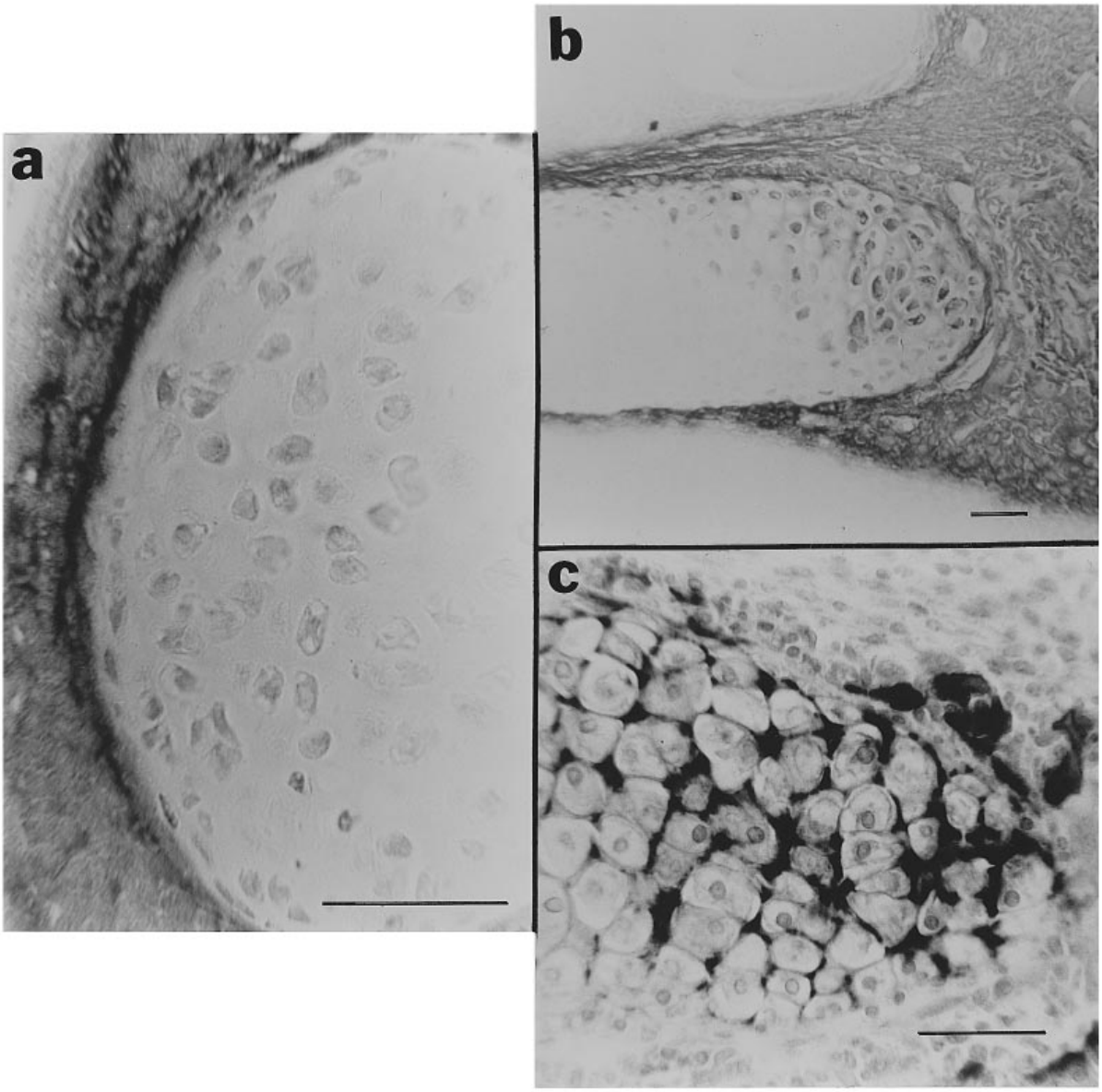


Fig. 7. Expression of alkaline phosphatase of Meckel's cartilage in the 1st arch of mouse embryos at stages M23 (*a*) and L24 (*b*). Nonhypertrophied chondrocytes are positive along the alkaline phosphatase-positive perichondrium. Hypertrophied chondrocytes are strongly positive at stage L24. (*c*) Mineralisation of Meckel's cartilage in the midregion of the 1st arch at stage E25. Visualised by von Kossa's method. Cross section (*a*); frontal section (*b*, *c*). Bar, 50 μm.

ramus expanded dorsolaterally at stage 23 as the presumptive coronoid process (Fig. 5*c*). The presumptive masseter and temporalis muscles lay laterally and mediodorsally, respectively. Secondary cartilage was not formed in the coronoid process at any of the embryonic stages examined.

The presumptive condylar and angular processes were not clearly observed by alkaline phosphatase staining at early stages, but their locations were discernible in relation to Meckel's cartilage, other cranial cartilages and presumptive muscles by stage 23.

Secondary cartilages formed in the presumptive condylar and angular processes by stage M24 (Fig. 6). A well developed matrix was present (Fig. 6*a*), and mineralised distally (Fig. 6*b*). In a proximodistal direction, immature to hypertrophied chondrocytes all expressed alkaline phosphatase and were surrounded by alkaline phosphatase-positive and highly condensed cuboidal cells (Fig. 6*a*, *c*). All were surrounded by the periosteum. Although most chondrocytes in the distal area of the matrix hypertrophied at stage 25, they still expressed alkaline phosphatase.

Alkaline phosphatase expression in the 1st arch cartilages

The core condensation of Meckel's cartilage was initiated at stage 20.12, and the condensation of the

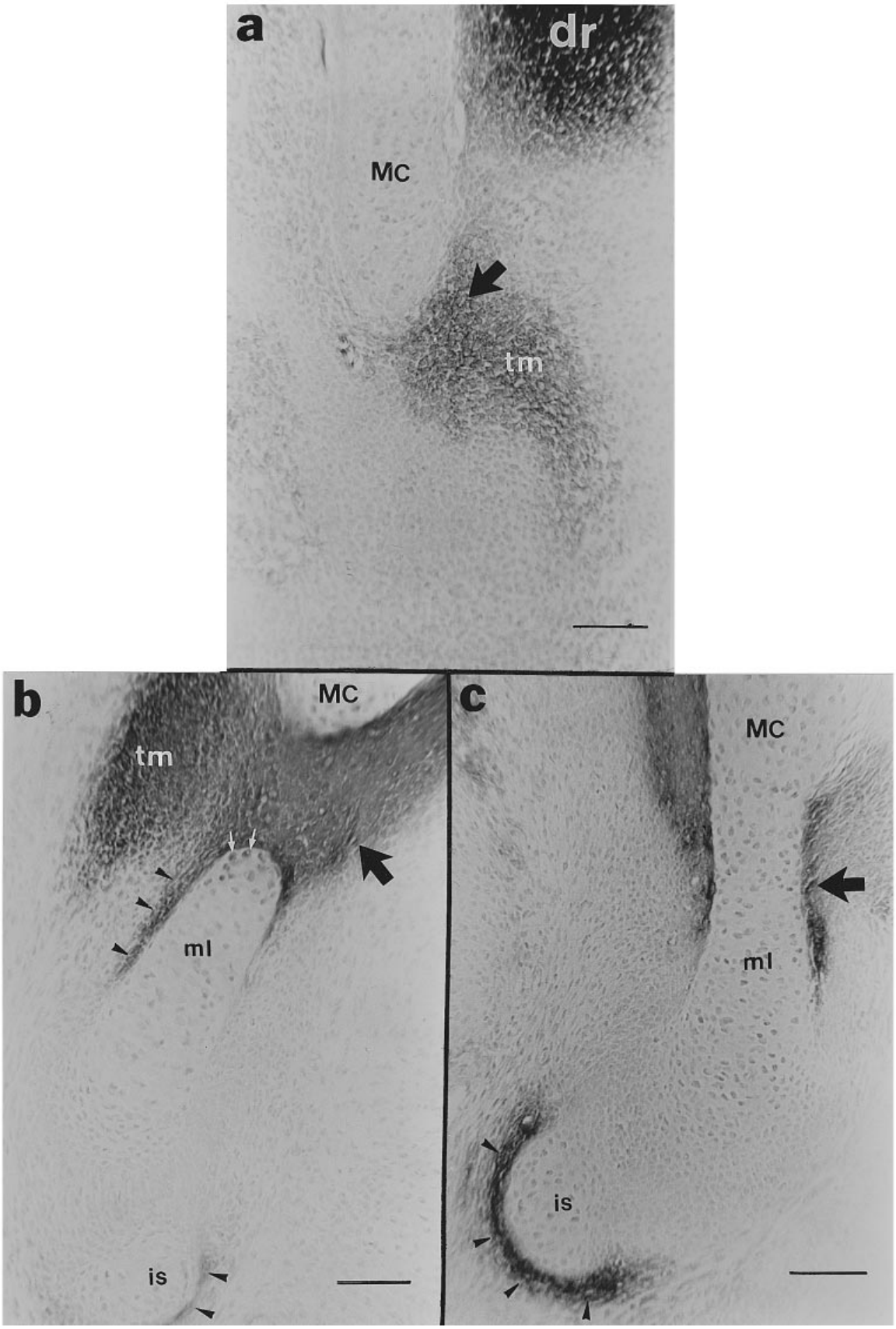


Fig. 8. Expression of alkaline phosphatase in the most proximal region of Meckel's cartilage (MC), malleus (ml) and incus cartilages (is) and gonial (arrows) and ectotympanic (tm) membrane bones. (*a*) Left side of the 1st arch at stage E22; (*b*) left side of the 1st arch at stage M24; (*c*) right side of the 1st arch at stage E25. Some nonhypertrophied chondrocytes in the malleus (small arrows) at stage M24 and in the incus at stage E25 are positive. Arrowheads indicate alkaline phosphatase-positive perichondrium of the incus cartilage. Cross section (*a*); frontal sections (*b*, *c*). Bar, 75 μm.

symphysis cartilage at stage 20.2 in C57BL/6 mouse embryos (Miyake et al. 1996*b*). No condensed or epithelial cells expressed alkaline phosphatase.

Meckel's cartilage was negative at stages 21.1 and 21.2. Matrix formation in Meckel's cartilage began at stage 21.14 (Miyake et al. 1996*b*). The alkaline phosphatase-positive area of the presumptive dentary ramus in the proximal region split dorsoventrally and bisected the proximal area of Meckel's cartilage (Fig. 2*d*).

The perichondrium of Meckel's cartilage distally was already alkaline phosphatase-positive at stage 21.3 (Fig. 2*e*). Under the tongue, the medial side of the perichondrium of Meckel's cartilage was weakly positive or negative (Fig. 4*b*), although the lateral side was strongly positive. Expression of the presumptive dentary ramus extended proximally along the lateral side of Meckel's cartilage, but the proximal half of the medial side of Meckel's cartilage was negative.

Alkaline phosphatase was expressed in peripheral chondrocytes along the lateral border of the distal region of Meckel's cartilage by stage M23, but the majority of the chondrocytes were negative (Fig. 7*a*). Those which were positive were not yet hypertrophied. The perichondrium along the most anterolateral border of Meckel's cartilage except medially was positive. The perichondrium was negative proximally (Fig. 8*a*) until stage M24. More chondrocytes in the midregion of Meckel's cartilage were positive by stage L24 (Fig. 7*b*), as was the matrix. The mineralised area of the ramus extended proximally at some distance along the perichondrium or both sides of Meckel's cartilage.

The perichondria in the distal area of the malleus and in the proximal area of the incus cartilages began to express alkaline phosphatase at stage M24 (Fig. 8*b*). The positive area of the malleus perichondrium was continuous with expression in the gonial (prearticular bone) and ectotympanic membrane bones and fibrous mesenchymal cells. Some chondrocytes, not yet hypertrophied, along this perichondrium expressed alkaline phosphatase (Fig. 8*b*).

Expression in Meckel's cartilage was confined to chondrocytes in the matrix which was surrounded by the alkaline phosphatase-positive perichondrium and presumptive dentary ramus at stage 25. The area was visualised in a 3-D reconstruction (Fig. 5*a*, *b*). Most chondrocytes in the distal areas except in the area of the symphysis became positive by the beginning of stage 25 (Fig. 5*d*). Mineralisation occurred in the midregion of Meckel's matrix at the beginning of stage 25 (Fig. 7*c*) where hypertrophied chondrocytes along the perichondrium were strongly positive. The proximal region of Meckel's cartilage hypertrophied by stage 25, but did not express alkaline phosphatase except for those overlying the alkaline phosphatase-positive area of the gonial membrane bone. The perichondria of malleus and incus cartilages were strongly positive and some chondrocytes of the incus along the alkaline phosphatase-positive perichondrium were also positive (Fig. 8*c*). Initial expression of alkaline phosphatase in chondrocytes of Meckel's cartilage, malleus and incus cartilages was thereby preceded by expression in their adjacent perichondria. This was true of chondrocytes in all other cranial cartilages examined in our study.

Alkaline phosphatase expression in teeth

Dental laminae of the 1st molar teeth were recognised as an epithelial thickening at stage 20.11. Definitive dental laminae of both molar and incisor teeth were observed by stage 20.12. None of these tissues were alkaline phosphatase-positive at these earliest stages.

Incisor primordia developed at the dorsal apex of the mandibular symphysis. The dental laminae thickened at the beginning of stage 21.1 and invaginated proximally into the middorsal area of the symphysis towards the end of stage 21.1. At stage 21.16 alkaline phosphatase expression was present in the superficial epithelial cells of the dental laminae. Deep cells were weakly positive. The tooth primordia reached the early bud stage at the beginning of stage 21.2, when the superficial and deep cells of the dental laminae were strongly positive (Fig. 9*a*) but only distally in the incisor primordia (Fig. 9*b*). Expression in the superficial epithelial cells of the laminae extended to the vestibular lamina. Presumptive dental mesenchyme was negative, although the mesenchyme of the presumptive dentary ramus ventral to the dentary mesenchyme was positive. The 1st molar tooth primordia reached the early bud stage at the beginning of stage 21.2, but did not express alkaline phosphatase.

The incisor and the 1st molar tooth primordia reached the bud stage at the beginning of stage 21.3 and later bud stage by the end of stage 21.4. Superficial and deep epithelial cells associated with the incisor dental laminae were strongly positive (Fig. 9*c*). Intercellular space of some inner and outer dental epithelial cells was also positive. A small number of superficial and deep epithelial cells of the molar dental laminae were weakly positive at stage 21.32 (Fig. 10*a*), but were not as strong as those of the incisor teeth until the end of stage 21.3. Activity in the laminae,

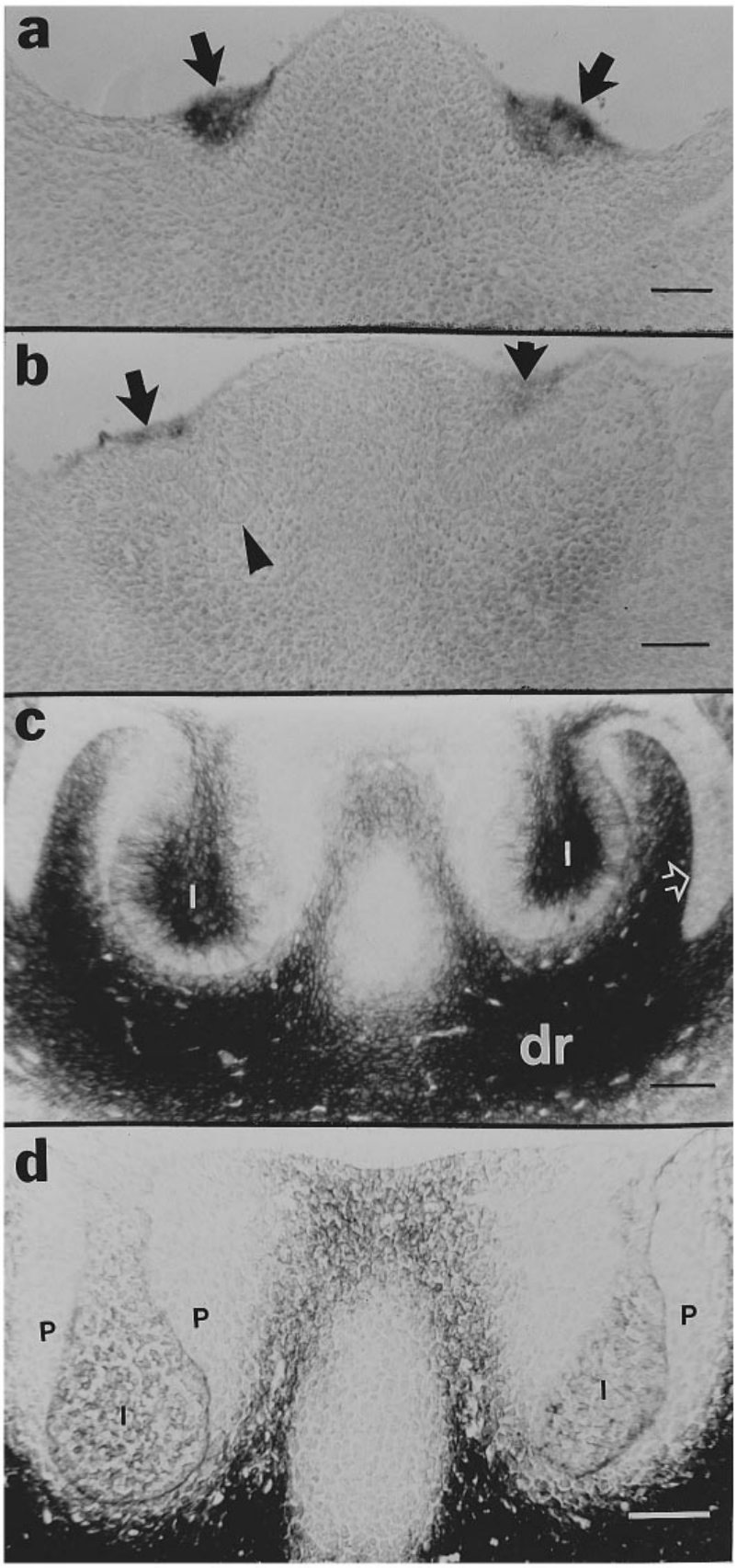


Fig. 9. Expression of alkaline phosphatase in the presumptive incisor teeth of the mouse embryos at stages 21.25 (*a*, *b*), 21.32 (*c*) and E22 (*d*). (*a*) and (*b*) are micrographs taken from consecutive sections showing expression in the dental lamina (arrows) of the incisor teeth. Arrowhead indicates an invaginating dental lamina. A strong alkaline phosphatase expression in the dental lamina (l) and dentary ramus (dr) is seen at stage 21.32 (c). Open arrow indicates the vestibular lamina showing a weak alkaline phosphatase expression. Alkaline phosphatase is not yet expressed in the dentary papillae (p) at stage E22 (d). Cross section. Bar, 50 μm.

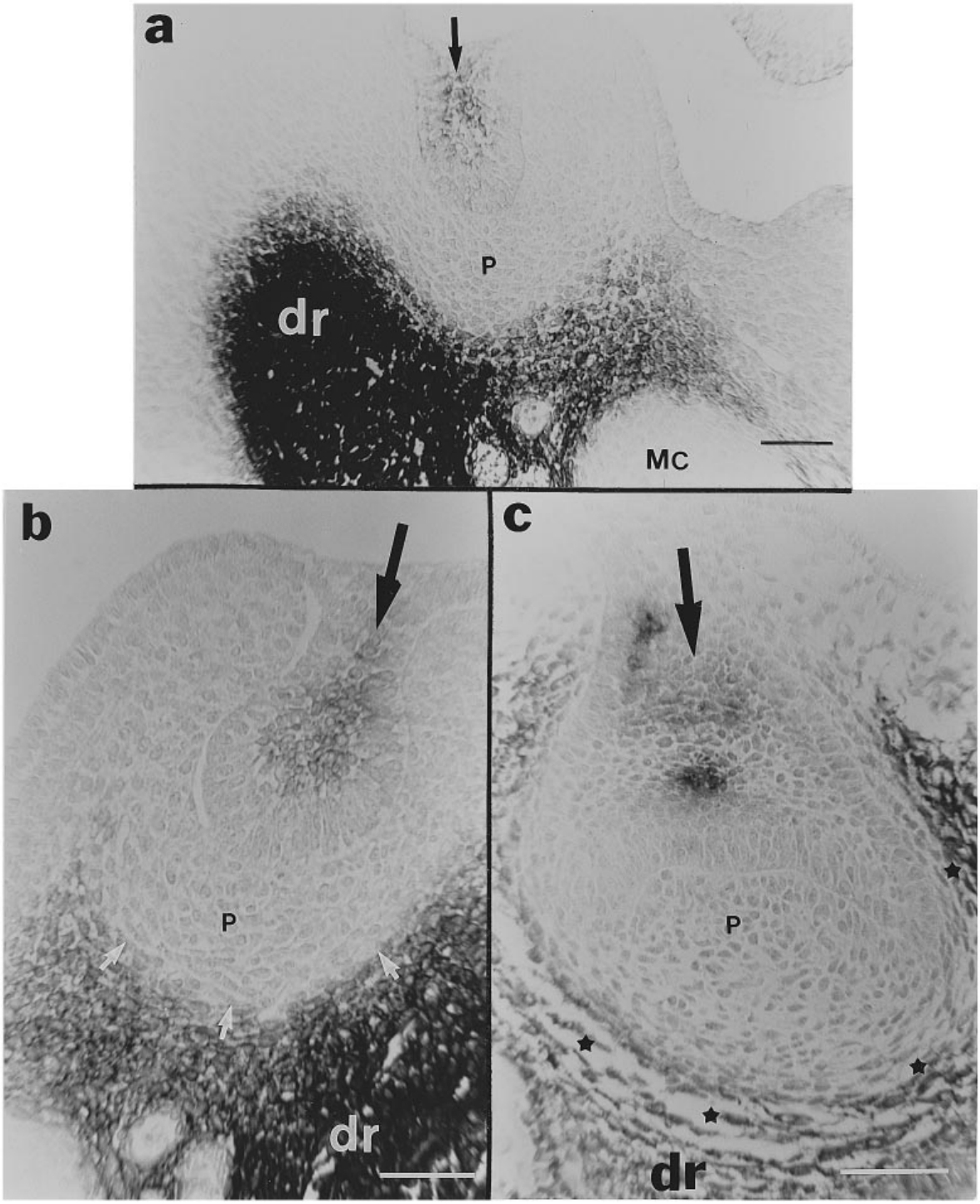


Fig. 10. Expression of alkaline phosphatase in the presumptive 1st molar teeth of the mouse embryos. (*a*) Right side of the 1st arch at stage 21.32; (*b*) left side of the 1st arch at stage E22; (*c*) right side of the 1st arch at stage M23. Arrows indicate alkaline phosphatase expression in the dental lamina. A boundary between the presumptive dental papillae (p)/follicles and periosteum of the dentary ramus (dr) is shown at stage E22 in (*b*) micrograph (white arrows) and (*c*) micrograph (stars). The periosteum and probably some dental follicular cells are alkaline phosphatase-positive at stage mid-23. Cross section. Bar, 50 μm.

however, varied among individual embryos. In some it was totally absent. Others were weakly positive. The condensed dental mesenchyme of both molar (Fig. 10*a*) and incisor teeth was devoid of alkaline phosphatase.

The incisor and the 1st molar tooth primordia moved from the late bud to cap stage at stage 22. Epithelial cells of the incisor dental laminae, particularly presumptive stellate reticulum, were strongly positive whereas the condensed dental mesenchyme was negative (Fig. 9*d*). Activity in the deep layer of epithelial cells of the 1st molar dental laminae was

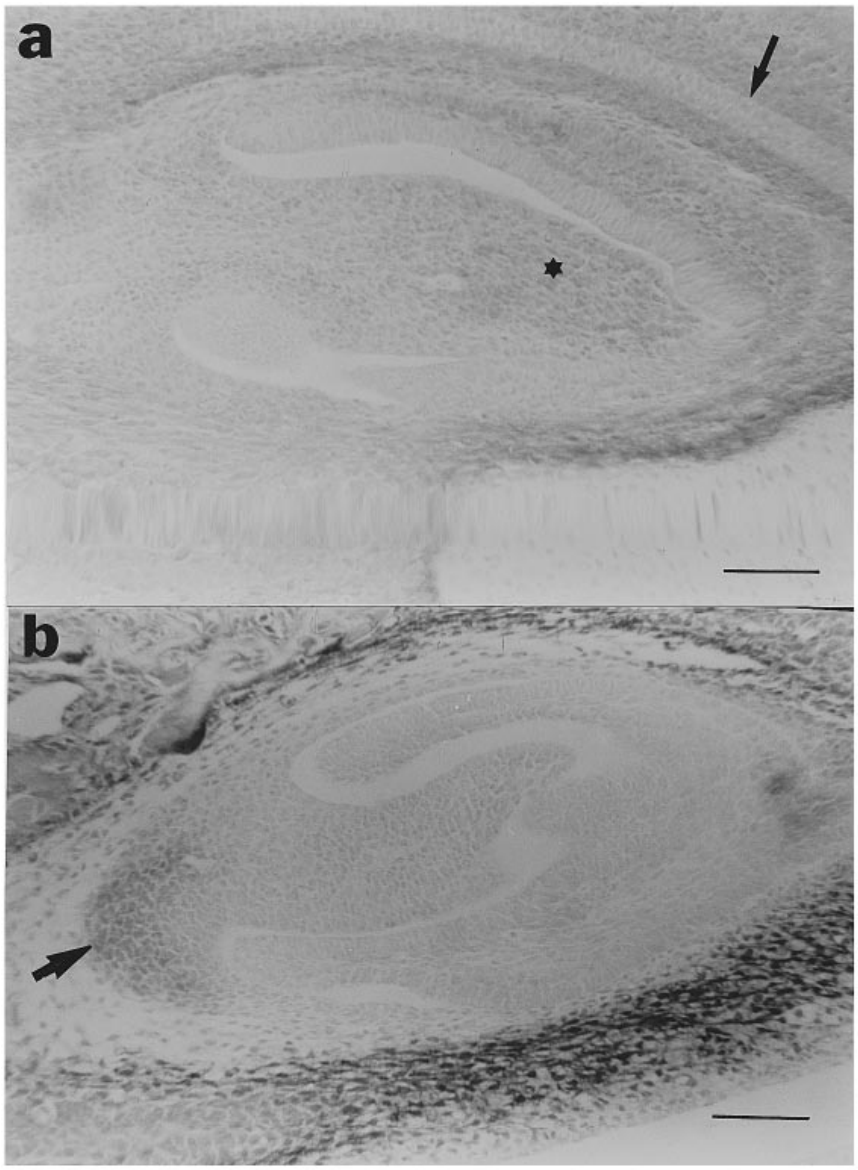


Fig. 11. Expression of alkaline phosphatase in the dental papilla of the incisor tooth (*a*, star) and the dental follicle of the first molar tooth (*b*, arrow) at stage M24. Arrow in (*a*) shows the vestibular lamina. Frontal section. Bar, 75 μm.

stronger than at stage 21.3 (Fig. 10*b*). The condensed dental mesenchyme was negative (Fig. 10*b*).

Both the incisor and the 1st molar tooth primordia reached the cap stage at stage 23. A well defined inner dental epithelial layer was present. The presumptive stellate reticulum of the incisor teeth was strongly positive. The dental papillae contained condensed mesenchymal cells surrounded by a fibrous layer of cells next to the periosteum of the dentary ramus, but its boundary was not clearly demarcated at this stage. The outer layer of the papillae differentiated into the dental follicle.

Alkaline phosphatase was present in dental epithelial cells of the 1st molar teeth (Fig. 10*c*) at stage 23 but, again, expression varied between embryos. Two areas were notably positive and lay near the enamel neck and enamel knot, which were connected by weakly positive cells in the presumptive stellate reticulum (Fig. 10*c*). At this stage, the periosteum of the dentary ramus was continuous with the outer fibrous cell layer of the condensed mesenchyme of the molar teeth, so that the most dorsal area of the periosteum was indistinguishable from the outer fibrous cell layer of the tooth mesenchyme (Fig. 10*c*, stars). Since the boundary between dental follicles and periosteum could not be distinguished, alkaline phosphatase may be expressed in the follicles but not be clearly observed at this stage (Fig. 10*c*).

The dental follicles and papillae of the incisor teeth and the dental follicles of the 1st molar teeth expressed alkaline phosphatase at stage M24. Expression extended to preodontoblasts, but was very weak (Fig.

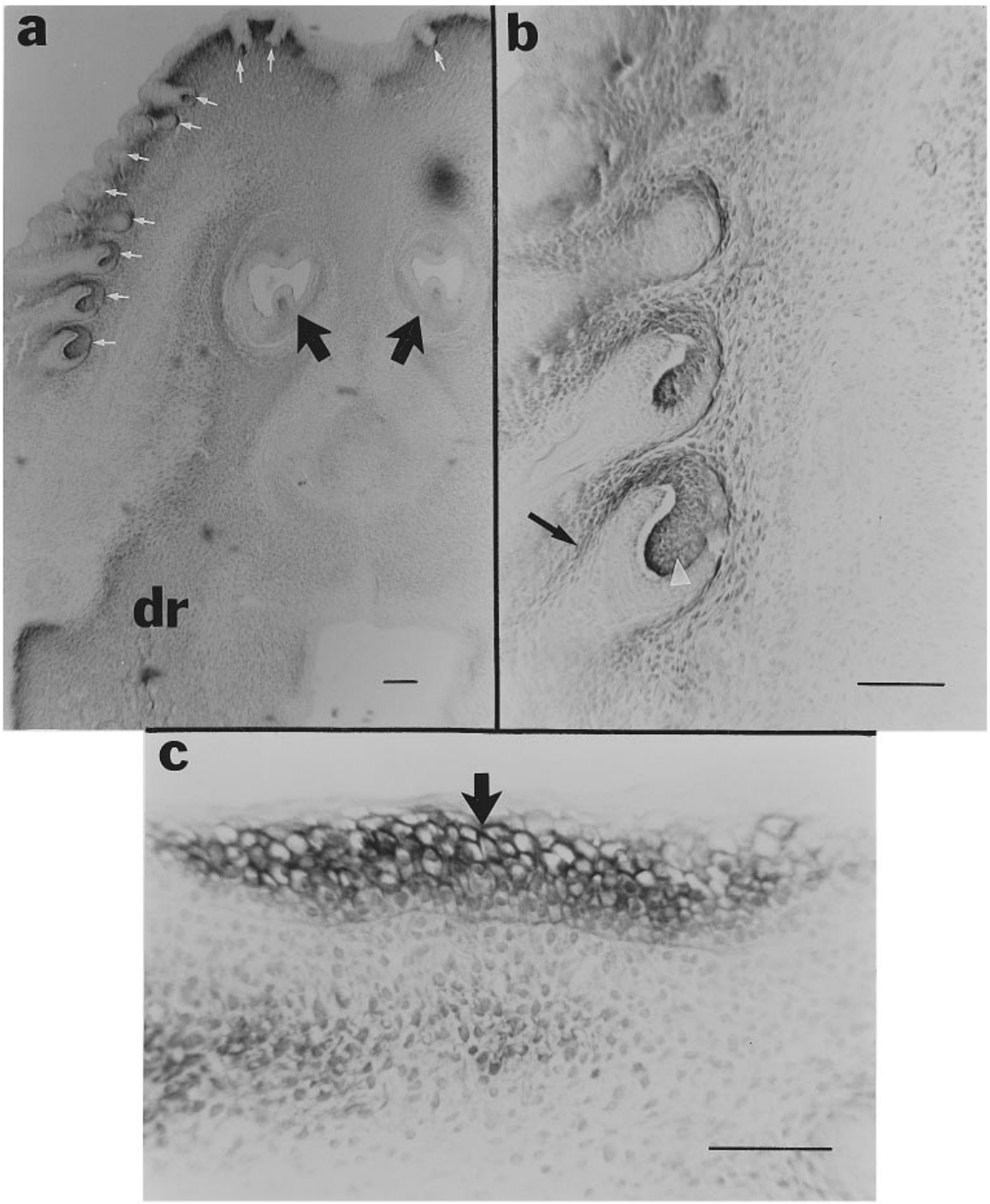


Fig. 12. Expression of alkaline phosphatase in hair primordia (*a*, *b*) at stage M24 and oral lingual epithelia (*c*) at stage E25. (*a*) shows a frontal section of the distal region of the 1st arch with alkaline phosphatase expression in hair primordia (small arrows) and surrounding dermis. Expression in the dentary ramus (dr) extends distally and surrounds the incisor teeth (large arrows). Expression is strongest in hair papillae (arrowhead) and sheath (arrow) in (*b*). The surrounding dermis is also alkaline phosphatase-positive. Hypertrophied epithelial cells in the oral lingual area (arrow) are strongly alkaline phosphatase-positive in (*c*). Frontal section. Bar, 75 μm.

11*a*). In addition, the peripheral cells—the presumptive dental follicles—expressed alkaline phosphatase, but only in the cervical area of the tooth primordia (Fig. 11*b*). The cells in the enamel cap of the 1st molar teeth upregulated alkaline phosphatase activity at stage M24.

The incisor teeth reached the early bell stage at stage 25 when presumptive columnar odontoblasts

lined up with the inner dental epithelia and deposited dentine. Almost all papillar cells including odontoblasts were alkaline phosphatase-positive (Fig. 5*d*). The 1st molar teeth reached the late cap stage. There was not yet a clear alignment of presumptive odontoblasts along the inner dental epithelia of the molar teeth. The dental papillae initiated expression at the beginning of stage 25.

Alkaline phosphatase expression in other skeletal elements and soft tissues

Expression of alkaline phosphatase was initiated in the presumptive squamosal membrane bone between stages 21.24 and 21.25 in an area extending lateral to the positive area of the dentary ramus (Fig. 5*c*). The extreme proximal alkaline phosphatase-positive area of the squamosal began to lay down osteoid at stage mid-23.

A group of alkaline phosphatase-positive cells extended from the dorsomedial corner of the alkaline phosphatase-positive area towards the distal corner of the external auditory meatus, beginning at stage 21.31. These were differentiating preosteoblasts for the ectotympanic membrane bone (Fig. 8*a*) and were connected with mesenchymal cells of the dentary ramus and the gonial membrane bone by weakly alkaline phosphatase-positive cells (Fig. 8*b*). The ectotympanic bone deposited osteoid towards the end of stage 23 and mineralised by stage 24.

Expression was also observed around the medioventral area of the junction of Meckel's cartilage and malleus cartilage in the otic region at stage 23. The area became the gonial membranous bone (Vázquez et al. 1991) and was connected with the alkaline phosphatase-positive area of the ectotympanic membrane bone (Fig. 8*b*). The perichondrium of the malleus cartilage above the presumptive gonial bone was also positive (Fig. 8*b*).

As observed by Kwong & Tam (1984) and Narisawa et al. (1994) localised expression of alkaline phosphatase was present in the brain at stage 20 (Fig. 2*a*). Although facial nerves innervated the alkaline phosphatase-positive area of the 1st arch, they were negative. Almost all mesenchymal cells in the distal region of the first arch became positive by stage 21.3 (Fig. 2*f*). Some of these differentiated into hair follicle mesenchyme and surrounding dermis (Fig. 12*a*). At stage 24 expression was confined ventrally to condensed hair follicle papillae, sheath, mesenchyme in the dermis surrounding hair buds (Fig. 12*b*) and presumptive dentary ramus. Expression was strongest in the hair follicle papillae and sheath. Surrounding mesenchyme showed weak expression (Fig. 12*b*). The area between these structures became more fibrous, contained peripheral nerves and was devoid of expression as was the vestibular lamina.

The oral lingual epithelial cells became alkaline phosphatase-positive by stage 21.3 and the expression extended more proximally at stage 22. These cells, connected with the incisor dental laminae, hypertrophied along the oral surface (Fig. 12*c*) by stage M24 and were strongly positive whereas the vestibular lamina was negative.

DISCUSSION

Our study demonstrates the timing and pattern of alkaline phosphatase expression of different skeletal components and soft tissues in the 1st arch of inbred C57BL/6 mouse embryos. Expression is stage-specific as is skeletal development (Miyake et al. 1996*b*). Initial expression is limited to the distal region at stage 20.2, and weaker towards the periphery. During stage 21.1 expression expands to the mid and proximal regions. This expression pattern is maintained at later stages.

Expression, which is present throughout the distal region of the 1st arch between stages 22 and 23, is confined to several developing tissues at later stages. By the time mineralisation begins, the distal region contains hair follicles, incisor teeth and dentary ramus. Activity is confined to condensed hair follicle papillae and sheath and surrounding dermis, incisor dental papillae and follicles and dentary ramus. Since these structures undergo epithelial–mesenchymal interactions (Hall, 1980; Thesleff et al. 1991; Hardy, 1992), these changes of organisation and expression result from temporal regulation of alkaline phosphatase expression during and after the interactions required for differentiation and morphogenesis of mesenchyme.

The pattern of alkaline phosphatase in the mid and proximal regions is restricted to the midsection of the 1st arch, where expression is only found in presumptive preosteoblasts and periosteum, even before matrix formation at stage 21.32. The periphery is always negative and is occupied mostly by mandibular muscles. As Hall (1982) showed in developing chick 1st arch, mesenchymal cells with different potentials may be distributed in different areas at earlier stages of skeletogenesis. A specific localisation of mesenchymal cells in the 1st arch may be crucial to interactions with epithelia to undergo differentiation and morphogenesis into skeletal tissues.

Dunlop & Hall (1995) showed that alkaline phos-

phatase is activated before condensation in osteogenesis of the chick 1st arch. The same is true of the dentary ramus in C57BL/6 mouse embryos. Although epithelial-mesenchymal interactions are required for condensation and to initiate differentiation of mouse and chick 1st arch bones (Hall, 1980; Dunlop & Hall, 1995), this is different from condensation of primary cartilages. Prechondrogenic condensation marks activation of cartilage-specific genes (Hall & Miyake, 1992; Miyake et al. 1996*b*). In Meckel's cartilage of C57BL/6 embryos, condensation begins at stage 20.12 and matrix deposition begins at stage 21.14 (Miyake et al. 1996*b*). The estimated duration between these stages is 12.3 h (Miyake et al. 1996*b*). As described in this study, alkaline phosphatase expression begins at stage 20.2 (1 stage later than stage 20.12) and matrix deposition begins at stage 21.32. The estimated duration between these stages is 28.0 h (Miyake et al. 1996*b*). Different durations of early chondrogenesis and osteogenesis in the 1st arch, therefore, may reflect different cellular and molecular regulations of cartilage and bone differentiation and morphogenesis.

Development of the 3 presumptive mandibular processes—coronoid, condylar and angular processes—is recognised by alkaline phosphatase expression, especially in the coronoid process at stage E23. Expression is continuous with the dentary ramus. Development of secondary cartilages in the condylar and angular processes begins at stage 24. Secondary chondroblasts, chondrocytes and hypertrophied chondrocytes all express alkaline phosphatase. These cells are surrounded by cuboidal cells (alkaline phosphatase-positive) which are continuous with the periosteum of the ramus. This suggests that cartilaginous cells in secondary cartilage originate from alkaline phosphatase-positive cuboidal cells. The earliest differentiation of these secondary cartilages may undergo molecular and cellular changes similar to those described in chick secondary cartilages by Fang & Hall (1995).

Since the periosteum of the dentary ramus marks the boundary of the ramus and 3 processes, all may share a common cellular origin. As described and summarised by Livne & Silbermann (1990), the 3 processes undergo extensive cellular organisations. Their proper development is mediated by mechanical loading at subsequent stages (Herring, 1993*a*, *b*). In this sense, each process develops as a separate unit after the initial differentiation of the processes from a common cellular pool (Atchley & Hall, 1991) and interacts with other mandibular structures for proper development (Atchley, 1993).

Richany et al. (1956) and Granström et al. (1988) described the fate of Meckel's cartilage in human and rats. Chondrocytes in the distal region of the 1st arch hypertrophy, the matrix is mineralised and the cartilage is replaced by bone, whereas chondrocytes in the mid to proximal regions disappear without any role in endochondral ossification. Alkaline phosphatase expression appeared to coincide with hypertrophy of chondrocytes in rats (Granström et al. 1988). Our study found similar fates for chondrocytes in the distal and mid-regions of Meckel's cartilage. However, a causal relationship between expression of alkaline phosphatase and hypertrophy and mineralisation remains unclear. Positive cells are confined to the region of Meckel's matrix surrounded by an alkaline phosphatase-positive perichondrium and presumptive dentary ramus, where chondrocytes express alkaline phosphatase before they hypertrophy. Hypertrophy occurs just before mineralisation. That the chondrocytes in the proximal region of Meckel's cartilage of C57BL/6 mouse embryos hypertrophy but never express alkaline phosphatase suggests that alkaline phosphatase expression may not be causally linked to hypertrophy. Entry of chondrocytes into hypertrophy may be regulated by the interplay of growth factors (Ohya & Watanabe, 1994; Böhme et al. 1995).

Expression in chondrocytes may be induced by adjacent alkaline phosphatase-positive perichondria and may be a prerequisite for mineralisation. Positive chondrocytes are always associated with adjacent alkaline phosphatase-positive perichondria in the head of C57BL/6 mouse embryos. *All perichondria express alkaline phosphatase prior to the expression in adjacent chondrocytes*. Our study, therefore, indicates that nonhypertrophied chondrocytes begin to express alkaline phosphatase after adjacent perichondria express alkaline phosphatase. These chondrocytes hypertrophy and the matrix undergoes mineralisation. Expression of alkaline phosphatase is thereby causally linked to mineralisation (de Bernard et al. 1986; Tenenbaum et al. 1989; Morris et al. 1992). Although expression of type X collagen has been suggested to play a role in mineralisation (Schmid & Linsenmayer, 1985*a*, *b*), expression of type X collagen by chondrocytes may not always be a prerequisite for mineralisation. Chung et al. (1995) reported that none of the chondrocytes in rat Meckel's cartilage except those in the malleus and incus express mRNA for type X collagen. The distal region of Meckel's cartilage mineralises in rats (Granström et al. 1988).

The dental laminae of the 1st molar and incisor teeth begin to form at stage 20.11 and 20.12 respectively in C57BL/6 mouse embryos. Contrary to

Väkevä et al. (1990) but like Pourtois (1961), dental superficial and deep epithelial cells begin to express alkaline phosphatase at stage 21.16 in the incisor teeth and at stage 21.32 in the 1st molar teeth. Expression in hypertrophied superficial epithelial cells associated with the dental laminae is noticeably strong at stages 24 and 25. However, activity differs considerably between incisor and molar teeth: in the dental laminae of the molar teeth it is very weak, tends to vary between embryos and is not upregulated until stage 22. Expression is also confined to the cells in the enamel neck and knot as development proceeds to the cap stage, unlike the wider expression in the stellate reticulum of the incisor teeth. Väkevä et al. (1990) detected alkaline phosphatase only in dental papillae of the bell and later stages of the incisor and molar teeth. That the BCIP/NBT method is more sensitive than the naphthol-AS-BI-phosphate/fast red method (Miyake et al. unpublished data; Zernik et al. 1990) may account for our detection in the dental laminal epithelial cells of the molar teeth.

Condensed mesenchymal cells of the incisor and molar teeth do not express alkaline phosphatase until stage M24. Both incisor and molar teeth reach the cap stage at the beginning of stage 23. The dental mesenchyme invests an outer fibrous layer, the precursor of the dental follicle. Expression begins at stage 24 or 25 in this outer layer, suggesting that expression may coincide with differentiation of dental follicle from dental papilla. However, the alkaline phosphatase-positive periosteum of the dentary ramus is intimately associated with the outer fibrous layer of condensed dental mesenchyme at earlier stages. Since the dorsal area of this periosteum is continuous with the outer layer of the dental mesenchyme, the dental mesenchyme is not clearly demarcated histologically from the underlying dentary ramus mesenchyme. Many molecules, e.g., homeobox containing genes *Msx-1* and *2* (MacKenzie et al. 1991*a*, *b*, 1992), surface proteoglycan syndecan (Thesleff et al. 1988; Vainio et al. 1991), lymphoid enhancer factor 1 (LEF-1) (van Genderen et al. 1994) and BMP-2 and 4 (Bitgood & McMahon, 1995), have recently been localised in condensed dental mesenchyme at bud and cap stages. However, since the roles of these molecules in dental formation remain unclear, detailed work is needed to elucidate the expression of these molecules during differentiation of dental follicles and the role of alkaline phosphatase in both the dental laminae and dental mesenchyme.

Recent molecular characterisation of 3 different isoforms of alkaline phosphatase in embryonic mice suggests that each isoform is localised in specific tissues at a specific time of development (Hahnel et al. 1990; Manes et al. 1990; Terao et al. 1990; Studer et al. 1991; Bossi et al. 1993; Narisawa et al. 1994). One of the isoforms, tissue nonspecific alkaline phosphatase (TNAP), characterised by Terao et al. (1990), Hahnel et al. (1990) and Studer et al. (1991), is expressed from the 2-cell stage to 9.5 d in primordial germ cells, placenta, testis, thymus, kidney, bone, liver and brain (Hahnel et al. 1990; Terao et al. 1990; Narisawa et al. 1994). Along with our study, these studies suggest that alkaline phosphatase may have multiple functions in skeletal and soft tissues, and that the enzyme may play a different role in different phases of osteogenesis. Alkaline phosphatase expression and active involvement in Ca^{2+} and/or metabolism, regulated both by PTH and vitamin D, found in bones, liver, kidney and probably intestine may thus not be coincidental (Rockwell et al. 1993; Martin et al. 1994) and may share common regulatory mechanisms in alkaline phosphatase function.

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