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Original Article

Desmin expression during early mouse tongue morphogenesis

MARK L. MAYO, PABLO BRINGAS, Jr., VALENTINO SANTOS, LILLIAN SHUM and HAROLD C. SLAVKIN*

Center for Craniofacial Molecular Biology, University of Southern California, School of Dentistry, Los Angeles, California, USA

ABSTRACT Occipital somites provide progenitor cells for craniofacial muscle development including the tongue musculature. Serum-derived factors are assumed to be pre-requisite for myogenesis *in vitro*. To test these assertions, we designed experiments to determine whether early mouse tongue development in general, and desmin localization in particular, were expressed during the development of embryonic mouse first branchial arch explants cultured in serumless, chemically-defined medium. Immunohistochemical techniques determined the chronology and positions of desmin expression during early craniofacial development. Occipital somites expressed desmin at E9 (9 days±2 h post-fertilzation, 18-20 somites). A discrete cell migration pathway initiating in the somites and terminating in the lateral lingual processes of the tongue primordium was defined based upon desmin expression patterns in E9-E11 embryos and computer-assisted three dimensional reconstructions. The *in vitro* model system was permissive for tongue morphogenesis, allowing development and fusion of the lateral lingual processes with the tuberculum impar. During culture myoblasts were not observed to fuse into myotubes with sarcomeric assembly, even though explant myoblasts produced muscle-specific protein. E10 explants cultured for 9 days demonstrated a five-fold increase in cell number that expressed desmin ($P<0.05$) when compared to the E10 starting material. We interpret these results to indicate that the tongue myogenic cell lineage was determined between E8 and E11, and that this resident population expanded within explants cultured in serumless medium by several explanations: (i) cells other than progenitor myoblasts (e.g., satellite cells) were induced to become myoblasts, and/or (ii) progenitor myoblasts within the original explants expanded by cell division in the absence of serum factors. We postulate that endogenous autocrine and/or paracrine factors (e.g., EGF, TGF-beta, TGF-alpha) control early stages of myogenic differentiation in this simple *in vitro* model.

KEY WORDS: *mouse, tongue, myogenesis, desmin expression, in vitro*

Introduction

The expression of time- and stage-specific cytoskeletal proteins has provided significant advances towards understanding critical issues in cell differentiation. Immunohistochemical studies using antibodies against desmin and myosin isoenzymes investigating cytoskeletal proteins have been particularly useful towards the diagnosis of normal as well as neoplastic skeletal muscle cells (see reviews by Osborn and Weber, 1983; Rungger-Brandle and Gabbiani, 1983; Altmannsberger *et al.*, 1985; Hill *et al.*, 1986; Osborn *et al.*, 1986). The availability of antibodies specific for the muscle phenotype has facilitated the investigation of cytoskeletal proteins at the cellular level (Danto *et al.*, 1984; Fischman, 1985; Kaufman and Foster, 1988; Babai *et al.*, 1990).

In a previous immunohistochemical study of skeletal muscle differentiation during myogenesis in rat and mouse embryos (Babai *et al.*, 1990), a similarity in desmin and alpha-actin isoforms (alpha-sr and alpha-sm) expression in skeletal muscle cells during mouse feto-embryonic development was demonstrated. Desmin was expressed in all pre-fusion myoblasts, myotubes and in myofibers (Babai *et al.*, 1990). Further, desmin expression preceded that of alpha-actins in myoblasts. These results suggested desmin to be a specific marker for the initial phases of myogenic differentiation.

The tongue musculature is particularly suited as a model system for the study of myogenic differentiation including commitment, determination and morphogenesis. The occipital somites (2-5) of avian and mammalian embryos have been shown to be the source of myogenic lineage cells that comprise the intrinsic musculature of the tongue (Deuchar, 1958; Hazelton, 1970; Noden, 1983; Wachtler and Jacob, 1986). From the somites, precursor myogenic cells

Abbreviations used in this paper: E, embryonic; d.i.v., days *in vitro*; P.N., post natal.

***Address for reprints**: Center for Craniofacial Molecular Biology, University of Southern California, 2250 Alcazar Street, CSA 103, Los Angeles, CA 90033, USA. FAX: 1-213-342.2981

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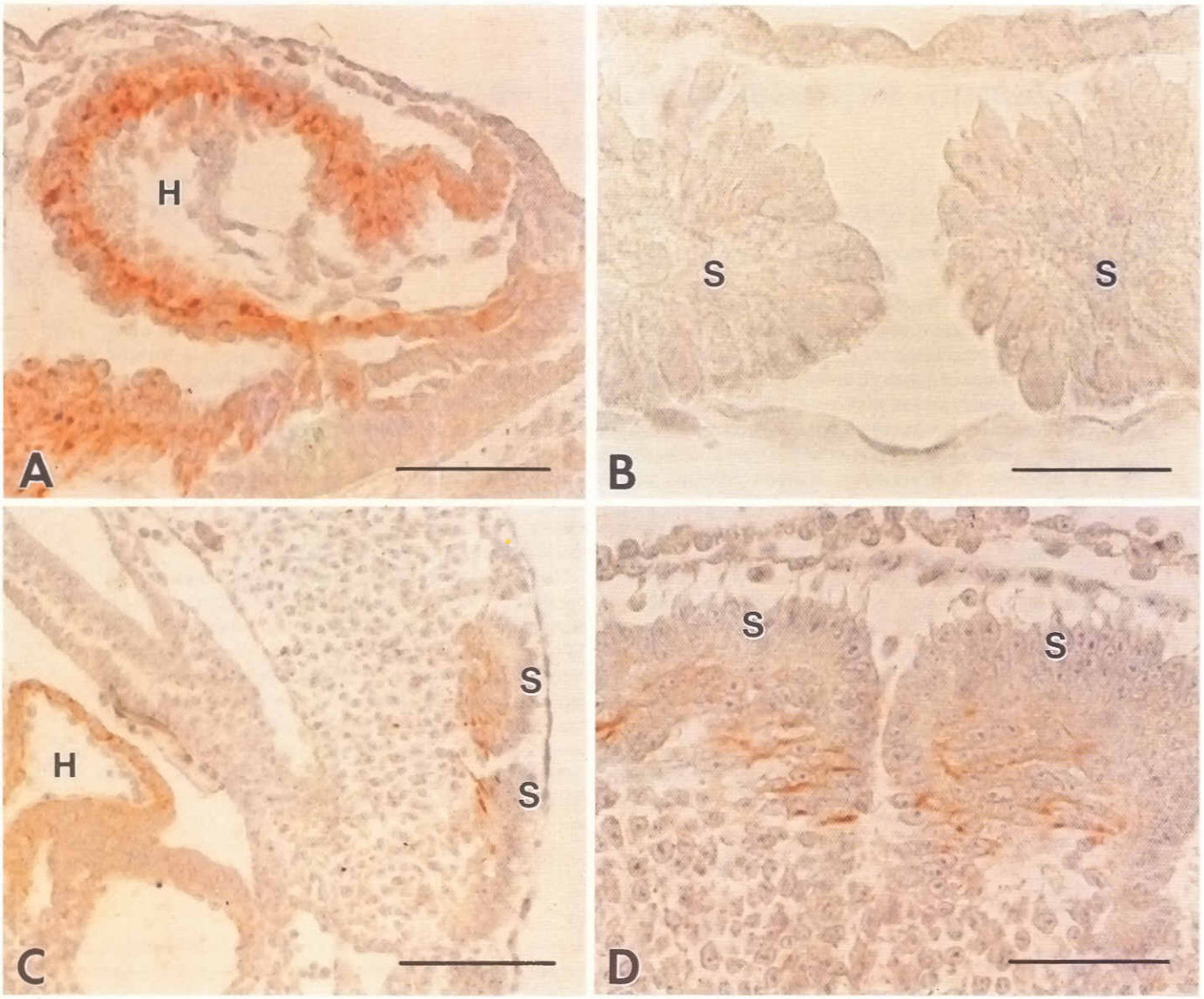


Fig. 1. Early desmin expression *in vivo*. **(A)** *E8 heart (H) with positive desmin immunolocalization within the cardiac muscle tissue.* **(B)** *Somites (S) did not stain positively for desmin in E8 embryos.* **(C)** *E9 embryo showing positive localization of desmin in both the heart (H) and somites (S).* **(D)** *Higher magnification of a different E9 somite (S) with positive desmin localization in the early myoblasts; red staining product indicates the presence of desmin epitopes. For A and B bar=50 μm; for C and D bar=20 μm.*

migrate into the developing mandibular processes (Hazelton, 1970; Noden, 1986). What is not defined, however, is when or where progenitor tongue cells become determined for the myogenic cell lineage. Further, it is not known if factors released by somitic-derived cells induce non-myogenic cells (i.e., satellite cells) in the developing tongue to become muscle cells.

The present study was designed to examine four main issues: (i) to determine whether early somitic myotome cells expressed phenotypes characteristic of muscle cells (e.g., expression of muscle-specific proteins); (ii) to test the hypothesis that the occipital somites serve as the source of myogenic units in the developing mouse embryonic tongue and to determine the pathway that somitic cells take during migration; (iii) to determine whether first branchial arches cultured in serumless, chemically-defined medium were capable of tongue morphogenesis and muscle differentiation, and (iv) to analyze cell division in the tissue compartments of the developing mandible with special emphasis on the tongue primordium. The present immunohistochemical report using antibodies directed against desmin defines the chronology and discrete pathways for early mouse embryonic tongue myogenesis *in vivo* as well as within E10 explants cultured in serumless, chemically-defined medium.

TABLE 1

DESMIN IMMUNOLOCALIZATION DURING EARLY EMBRYONIC MOUSE DEVELOPMENT

Age (Days)	Number Somites	Theiler Stage	Desmin Immunolocalization			
			Heart	Somite	Intermediate	Tongue
8	2-5	12	+	-	-	NA
8.25	7-8	12.5	+	-	-	NA
8.5	10	13	+	-	-	NA
9	18-20	14	+	+	+	NA
10	30-34	16	+	+	+	NA
11	40-44	18	+	+	+	+

Results

Desmin expression by somitic tissue

Immunolocalization was used to determine when and where the myotomes of the occipital somites first expressed desmin epitopes (Table 1). Desmin was detected in cells of the developing heart of E8 embryos, but did not localize in the somites (Fig. 1). Embryos collected at E9, E10 and E11 showed positive desmin immunostaining in both the occipital somites and in regions of the developing first branchial arch (Fig. 1). The number of myoblasts which stained varied with embryonic age and somite location. As embryonic age progressed from E9 to E11, the number of immunopositive desmin-staining cells increased; the greatest increase was observed between E10 and E11 (Table 1).

Occipital somites contribute myoblasts to the developing tongue

Isolated fusiform and small clusters of other cells expressing desmin epitopes were observed in three discrete regions: (i) occipital somites, (ii) tissues of the first branchial arch, and (iii) lateral lingual processes of the tongue (Fig. 2). Desmin-positive cells were aligned in columns extending from the occipital somites to the developing tongue (Fig. 2). The largest number of desmin immunostaining cells were distributed anterior to the tuberculum in the forming lateral lingual processes. Three dimensional reconstructions of E11 serial sections defined a discrete pathway of desmin-positive cells terminating within the first branchial arch (Fig. 3). Desmin immunopositive cells extend from the tail to the developing tongue in a continuous band.

Desmin localization during embryonic mandibular morphogenesis in vitro

First branchial arches cultured for 9 days in serumless, chemically-defined medium showed tongue morphogenesis comparable to *in vivo* development. The lateral lingual processes grew and fused at the medial sulcus (Fig. 4). The developing lateral lingual processes of E10 mandibles demonstrated that approximately 10% of the mesenchymal cells contained desmin epitopes. The immunoperoxidase-stained product was concentrated in juxtanuclear regions corresponding to the Golgi complex in most immunoreactive cells. In less than 0.5% of the myoblastic cells, multinucleated myotubes were identified; a few myogenic cells had progressed to cell fusion. The lateral lingual processes of mandibles cultured for 9 days contained approximately 49% mesenchymal cells which stained positive for desmin. Two-way ANOVA analyses compared E10 mandibular processes with E10 explants + 9 days *in vitro* (d.i.v.) and showed a significant difference ($P<0.05$). No significant difference was found within each group. Desmin immunopositive cells were located in the mesenchymal compartments; there was no epithelial immunostaining of desmin (Fig. 4). This delineation was most evident in cross-sections which had the median sulcus at the midline. Multinucleated cells were infrequent, comprising less than 1% of the myoblasts. None of the cells observed showed sarcomeric assembly characteristic of mature skeletal myotubes in both bright field or dark field microscopic examination.

^{3}H-thymidine incorporation during embryonic mandibular morphogenesis in vitro

^{3}H-thymidine incorporation experiments were conducted to determine the relative mitotic index within the mandibular processes of bone, cartilage, specialized oral epithelium, tooth epithelia and mesenchyme and tongue mesenchyme. Three ranges of mitotic activity were observed (Table 2). Highest mitotic activity was found in epithelia of the tongue, tooth epithelium, perichondrium and periosteum. Tongue epithelia showed regional variations in the level of mitotic activity. The posterior region of the tongue, which contained developing vallate papillae, demonstrated three-fold more DNA synthesis than the anterior region (Fig. 5). The lowest level of cell division occurred in compartments of bone and cartilage (Fig. 5). A third level of mitotic activity was found for tongue mesenchyme which was four-fold more than the DNA synthesis activity observed for the bone and cartilage phenotypes (Table 2). The tongue mesenchyme contained 48.8% immunoreactive myogenic cells; a significant percentage of tongue myogenic cells appeared to have replicated (see Figs. 4 and 5).

Discussion

Major processes associated with vertebrate development include pattern formation, morphogenesis and the allocation, commitment and determination of unique phenotypes. During early mouse morphogenesis, the myogenic lineage cells differentiate and fuse to form myotubes and eventually muscle fibers. One model system to investigate these issues is the developing tongue musculature.

Allocation and commitment are determined prior to tongue musculature development (see discussions by Noden 1983, 1986). Cell commitment to myogenesis occurs prior to tongue formation in the occipital somites, and the cells that form the tongue musculature migrate from the occipital somites into the forming first branchial arch. The regulatory factors which mediate myoblast differentiation within the embryonic tongue are not as yet known. In order to pursue this problem area, we have utilized an experimental strategy in which E10 mandibular processes are cultured in serumless, chemically-defined medium (Slavkin *et al.*, 1989, 1990). Under these conditions, endogenous regulatory molecules appear to control the timing and position of myogenic differentiation within the forming tongue.

Desmin expression by somites

Immunohistochemical results demonstrated the timing of a specific cytoskeletal protein expression during early mouse craniofacial development. Our observations confirmed previous work demonstrating the expression of a muscle-specific cytoskeletal protein during early embryogenesis (see Hill *et al.*, 1986; Noden, 1986; Babai *et al.*, 1990). The timing of initial desmin expression

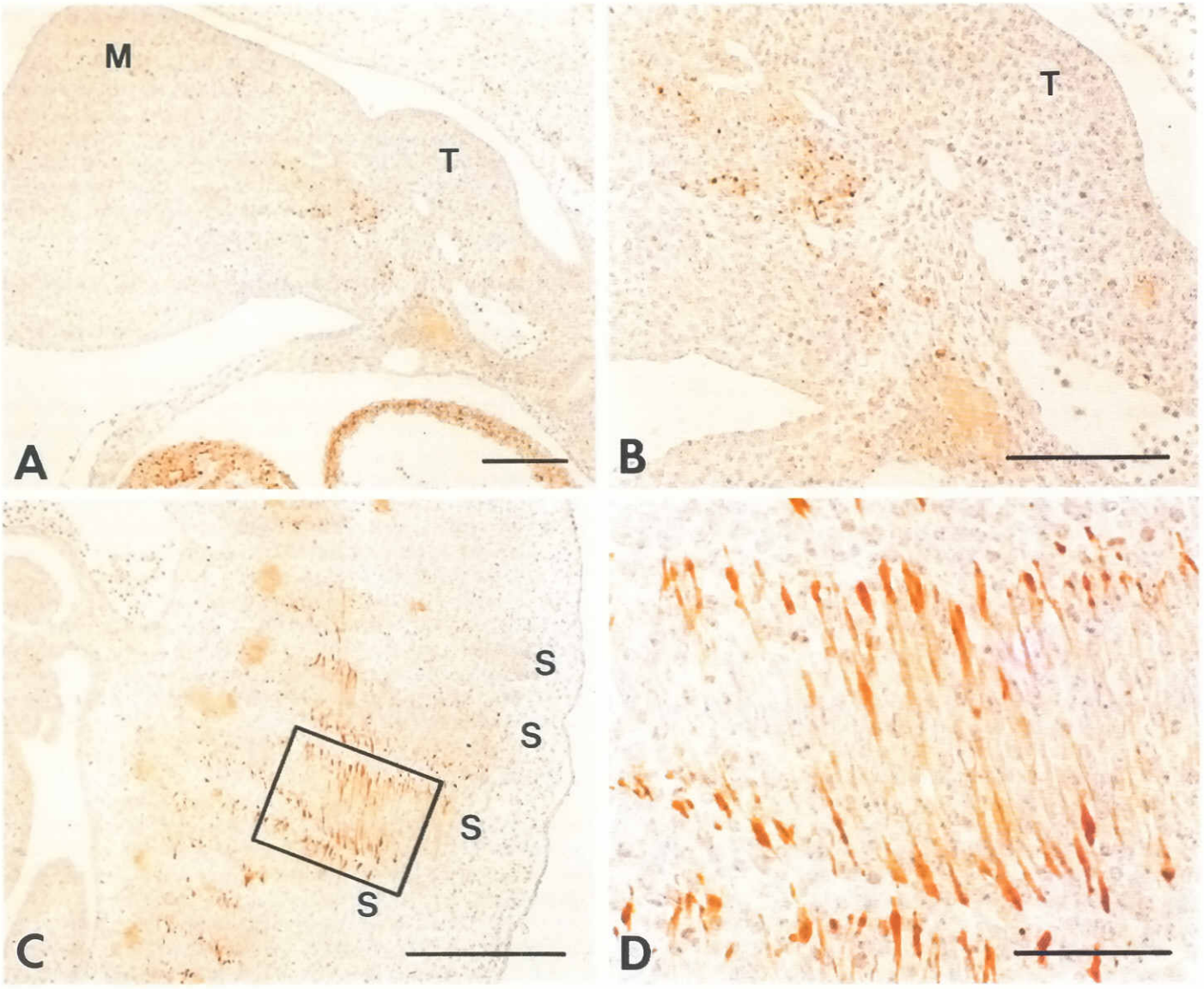


Fig. 2. Immunolocalization in tongue primordia. *Sagittal section of E11 mouse embryo stained with desmin antibodies.* **(A)** *The mandibular process (M) and the tuberculum impar (T) (bar=100 μm).* **(B)** *Higher magnification (of Fig. A) of cells inferior to the tuberculum (T) with desmin immunostaining (bar=100 μm).* **(C)** *Thoracic somites (S) with desmin localization (bar=100 μm); and* **(D)** *higher magnification (of Fig. C) demonstrating desmin staining within the myoblasts (bar=50 μm).*

in mouse somitic tissue was reported to be between E9 and E9.5 days gestation (Fuerst *et al.*, 1989). In a study of myogenesis in mouse embryos ranging from E7-E19, Babai and colleagues (Babai *et al.*, 1990) demonstrated that in E9 somites desmin appeared before alpha-actin, alpha-sr or alpha-sm in myotomes; no cells expressed desmin or alpha-actin in somites without myotome in E9 embryos. Desmin expression preceded by a few hours the expression of alpha-sr actin in myoblasts at the level of myotomes (Babai *et al.*, 1990). In the present study we observed desmin expression in the somites of all E9 mice (i.e., time 0 defined as detection of the vaginal plug ± 2 h after mating) and could not detect desmin in E8 embryos (Fig. 1).

Somitic contribution to tongue musculature

A number of investigations established that the tongue musculature originated from the occipital somites (Platt, 1897; Deuchar, 1958; Hazelton, 1970; Noden, 1983, 1986). Cell tracing techniques that employed carbon particles, autoradiography and heterologous tissue recombinations demonstrated that cells from the first four occipital somites provide the cell lineage to the intrinsic tongue muscles in amphibian, chick and mouse models (Deuchar, 1958; Hazelton, 1970; Noden, 1986). The present study during mouse embryogenesis provides additional evidence to corroborate previous studies.

Since somites appear from E8, we investigated embryos from E8

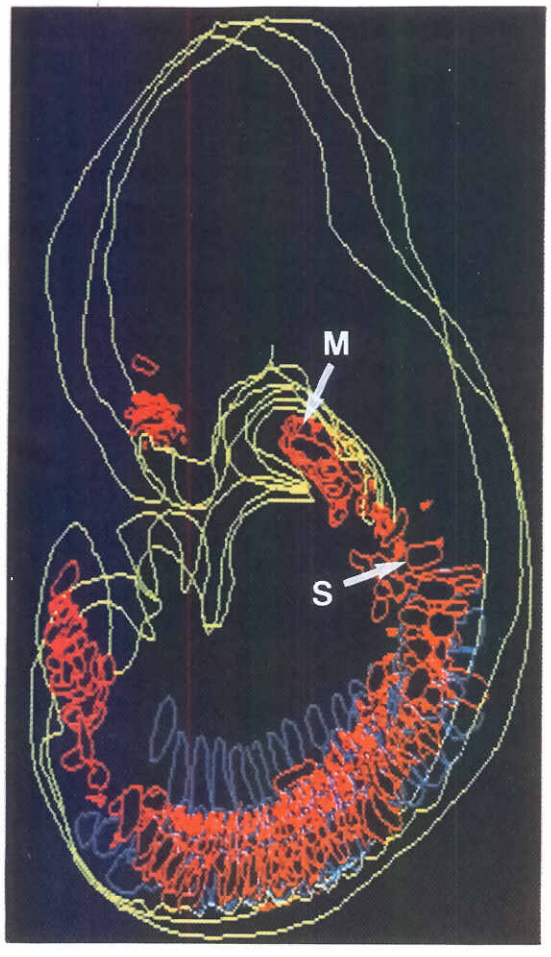


Fig. 3. Three-dimensional reconstruction of desmin immunolocalization in E11 mouse embryo. *The pattern of desmin localization between the cervical somites (S) and the mandibular process (M). Three dimensional reconstruction of desmin localization during E11 development. The image consists of 130 serial sections digitized manually from color photomicrographs using a Jandel graphics tablet (Jandel Scientific, Morte Madera, CA). The pattern of myogenic, desmin-positive cells were colored red whereas the somites were colored blue. This observation depicts only one side of the embryo as viewed from the medial aspect. Green - section outline; red - desmin staining; blue - somites.*

through E13 using sagittal and transverse serial sections. Desmin expression was studied at the level of the somites and the first branchial arch on fetuses between E8-E13. Cells from E8 fetuses did not express desmin in somites; however, desmin was present in the developing heart (Fig. 1A). Somites in E9 embryos illustrated the appearance of desmin in myotomes (Fig. 1 C,D). The three-dimensional reconstruction of whole E11 embryo serial sections showed a discrete pathway taken by migratory myoblasts traveling toward the first branchial arch (Fig. 3). The observed pattern supports a definite pathway leading from the occipital somites to the first branchial arch and the developing tongue primordia.

In vitro *tongue morphogenesis and myogenesis*

Previous reports from this laboratory demonstrated embryonic mouse mandibular morphogenesis and the cytodifferentiation of osteocytes, chondrocytes and the epithelial components of the developing tooth within explants cultured in serumless medium (Slavkin *et al.*, 1982, 1989 and 1990). In the present study we have shown that the major contributors to tongue morphogenesis were the two lateral lingual processes and the tuberculum impar which continued development during culture in a serumless, chemically-defined medium (see Figs. 2-4). The developmental pattern was analogous to *in vivo* development resulting in tongue morphogenesis. The source of the myoblasts within these tissue compartments was determined to be the first four occipital somites.

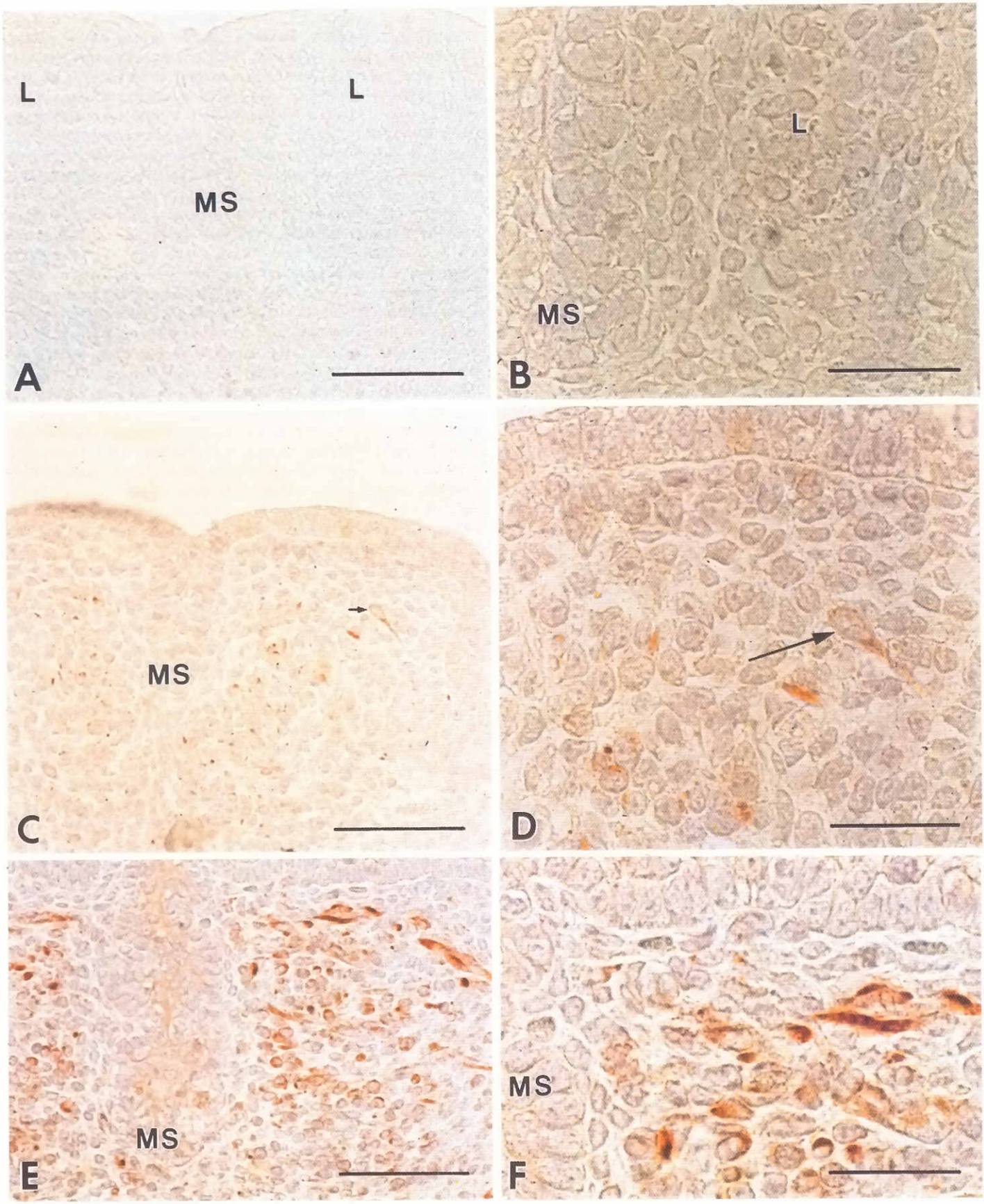
A modest number of myoblasts were determined when microdissected mandibular processes were placed in culture (Table 1). The E10 explant material contained 9.5% desmin-positive myoblasts in the tongue primordia before culture (data not shown). After 9 days *in vitro* the number of desmin-positive myoblasts increased to nearly 50%. The design of the experiment precluded the addition of migrating myoblasts from the somites, nervous stimulation or serum-derived factor stimulation. Therefore, we suggest that the five-fold increase was the result of mesenchymal cell differentiation within the mandibular explant. Several possibilities exist to explain the increase. Desmin-negative cells in the initial E10 explant were determined for the myogenic lineage, but had not differentiated to the stage of desmin expression. It is also possible that paracrine and/or autocrine stimulation of non-myoblast cells recruited or induced satellite cells to alter their phenotype to become myoblasts. Finally, it is also possible that progenitor myoblast cell division during the culture period produced the five-fold increase in desmin-positive myoblasts.

The lack of myotube formation in the tongue culture model could be caused by a variety of factors. Preliminary work has shown that laminin synthesis by mesenchymal cells within the lateral lingual processes was altered in our model system and this may be one factor related to the lack of myotube formation (data not shown). A laminin substrate has been shown to be a mitogen for early myoblasts (Darmon, 1982; Foster *et al.*, 1987). Immunodetection using E10 + 9 d.i.v. mandibular processes showed laminin to be concentrated in the mesenchymal cells adjacent, but not coincident, with the lateral lingual processes exhibiting desmin immunostaining. Although the precise role of laminin in myogenesis is unclear, it was localized around all myotubes in six month post-natal mouse hypoglossal muscles (data not shown). Whereas permissive for chondrogenesis, osteogenesis and tooth development, our serumless, chemically-defined model system was unable to ex-

TABLE 2

INCORPORATION OF ^{3}H-THYMIDINE INTO TISSUE COMPARTMENTS DURING E10 MANDIBULAR EXPLANTS CULTURED IN SERUMLESS, CHEMICALLY-DEFINED MEDIUM FOR 9 DAYS *IN VITRO*

Tissue Compartment	Labeled nuclei/2,500 μm^2
Background	0.4
Cartilage	2.5
Perichondrium	13.5
Bone	2.0
Periosteum	16.0
Tongue mesenchyme	7.0
Oral epithelium	14.0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press the entire myogenic program to continue to myoblast fusion (Slavkin *et al.*, 1990, 1989, 1982).

DNA synthesis during tongue development in serumless medium

Myogenesis in lingual skeletal muscle consists of several phases (Deuchar, 1958; Hazelton, 1970; Wachtler and Jacob, 1986). In the first phase, early somitic myoblasts increase in number resulting in a larger population of mononucleated myoblasts that produce desmin. These progenitor myoblasts migrate to the developing tongue primordia and undergo further cytodifferentiation. In the next phase, these cells withdraw from the cell cycle and fuse to become multinucleated myotubes. After fusion, the expression of characteristic muscle proteins such as myosin, troponin and titan increases markedly (Devlin and Emerson, 1978; Hill *et al.*, 1986). The fusion of myoblasts into syncytia appears to be dependent on the cell cycle; myoblasts are required to be in the G_1 phase before fusion (Bischoff and Holtzer, 1969.). Once multinucleated myotubes are formed, DNA synthesis is absent and myotubes do not incorporate tritiated thymidine (Klasing, 1990).

In the present study we observed that the tongue mesenchyme incorporated ^{3}H-thymidine at a level of activity between post-mitotic chondrocytes or osteocytes and less than mitotically active epithelia or mesenchyme adjacent to forming cartilage and bone (Table 2). The same sampling area used for mitotic activity demonstrated that approximately 50% of the mesenchymal cell population within the tongue expressed desmin. This concordance indicates that these early myoblasts were still capable of replicating and had not reached a stage of terminal differentiation. Lateral lingual processes of mandibles cultured in serumless medium showed few (<1%) multinucleated cells; these cells were never seen with the characteristic sarcomeric assembly of proteins. We argue that the myogenic developmental program was inhibited prior to terminal differentiation; the concomitant loss of mitotic activity leading to complete myotube formation was not observed. We suggest that the *in vitro* model system lacked factor(s) required to signal myoblasts to withdraw from replication and fuse into syncytia. Addition of selected exogenous factors such as the variety of IGFs, transferrin or a reduction of endogenous growth factor expression such as TGF-alpha might promote further stages of myogenesis in this simple organ culture model.

Materials and Methods

Mouse embryos

Swiss Webster (SW) strain mice were purchased and housed in a breeding colony at our institution as previously described (Bonner and Slavkin, 1975). Animals were maintained on a 12 h/12 h light-dark cycle. The temperature was maintained at 70°F. Mice were fed a diet of Breeder Blok (Wayne Pet Food, Chicago) and water ad libitum. Timed pregnancies were obtained by mating females (11-14 weeks old) with 20-week-old males for 2 hours. Day 0 was determined by the presence of a vaginal plug. Pregnant animals were sacrificed by cervical dislocation and the embryos were dissected from uterine decidua and staged by their external features according to Theiler (1972). Mandibular processes to be used as explants for organ culture were removed from embryos by microdissection (Slavkin *et al.*, 1989). All explants were isolated with the tuberculum impar. For *in vivo* studies, sequentially staged whole embryos (E8-E13) were employed. Whole E8 embryos were dissected complete with the visceral yolk sac and ectoplacental cone.

In vitro *organ culture*

Mandibular processes were cultured using a modified Trowell method as described by Slavkin *et al.* (1982). Dissected first mandibular processes (E10) were placed on Type AA, 0.8 μm pore size Millipore filter cut into discs 6 mm in diameter (Millipore, Bedford, MA) and supported by stainless steel mesh triangles. Explants were placed with the oral epithelium facing upward, opposite to the surface of the filter. Approximately 70 explants (6 explants per dish) were cultured in Grobstein Falcon dishes under optimal humidity conditions in an atmosphere containing 5% CO_2 and 95% air. The medium consisted of 800 μl of BGJb (Gibco, Grand Island New York) with supplements of 0.1 mg ascorbic acid/ml and 50 units penicillin/streptomycin (Flow Laboratories, McLean, VA). The medium was changed every other day and was kept within a pH range of 7.0 to 7.4. Cultured mandibular explants were maintained for a period of 9 d.i.v.

Immunohistochemical localization

Specimens for light microscopy were fixed in Carnoy's fixative overnight at 4°C, dehydrated in a graded series of ethyl alcohol, cleared in xylene, and embedded in paraffin. For *in vivo* and *in vitro* studies serial 5 μm thick sections were cut in the sagittal and transverse planes. Each slide contained histologic sections from each of the following: E10, E14, E10 + 9 d.i.v. and 6 month postnatal tongue. This strategy was used to reduce staining variability and provided the inclusion of positive controls. In preparation for immunohistochemistry, slides were cleared in xylene and hydrated to water through a graded series of alcohols. Slides were then treated using the Histostain-Streptavidin Peroxidase kit following the protocol provided by the vendor (Zymed Laboratories, Irvine, CA). A polyclonal antibody to desmin was purchased (ICN Laboratories, Lisle, IL). The optimal dilution for the antibody was determined to be 1:200 based on six month postnatal mouse tongue localization studies. Most slides were counter-stained with dilute hematoxylin to differentiate the nuclei. Normal rabbit serum was used in place of the primary antibody in negative control slides. All negative control slides showed no immunostain. Positive controls consisted of E14 tongue, six month postnatal tongue sections, and cardiac tissue which stained positive for desmin at E8 and throughout subsequent stages of development. Six month postnatal tongue sections showed desmin localization in the Z-line of the sarcomeres. Three dimensional reconstructions of serial sections were made using Jandel PC3D software (Jandel Scientific, Corte Madera, CA) to analyze the pathway taken by migratory immunoreactive myogenic cells. One hundred photomicrographs of matched magnification were digitized using a Jandel Scientific graphics tablet for each three-dimensional image.

Western transfer characterization of desmin antibody

Tongues were dissected from E18 Swiss Webster mice, homogenized, extracted in homogenization buffer and protein content was determined as previously reported (Slavkin *et al.*, 1988). Tongue proteins were characterized using SDS/ urea/ 7% polyacrylamide mini-gels. Gels were cut and half stained with Coomassie Blue. The remaining half was transferred to a nitrocellulose filter. Immediately after transfer the nitrocellulose sheet was probed with the polyclonal desmin antibody as previously described (Zeichner-David *et al.*, 1988) using a dilution of 1:1500. A single band of 55 KDa was observed.

Fig. 4. Immunolocalization of desmin during *in vitro* organ culture in serumless, chemically-defined medium. **(A)** *Cultured E10 negative controls for immunolocalization without red staining product in the lateral lingual process (L) or medial sulcus (MS) of the developing tongue;* **(B)** *Higher magnification (of Fig. A).* **(C)** *Cultured E10 tongue stained positively for desmin epitopes in both lateral lingual processes while the oral and medial sulcus (MS) epithelia were negative. Immunostaining was localized to the juxtanuclear region.* **(D)** *Higher magnification of Fig. C with an arrow to indicate a multinucleated cell with desmin immunolocalization;* **(E)** *E10+9 d.i.v. tongue showing desmin-positive myoblasts; and* **(F)** *higher magnification of E. For A,C and E bar=50 μm, for B, D and E bar=20 μm.*

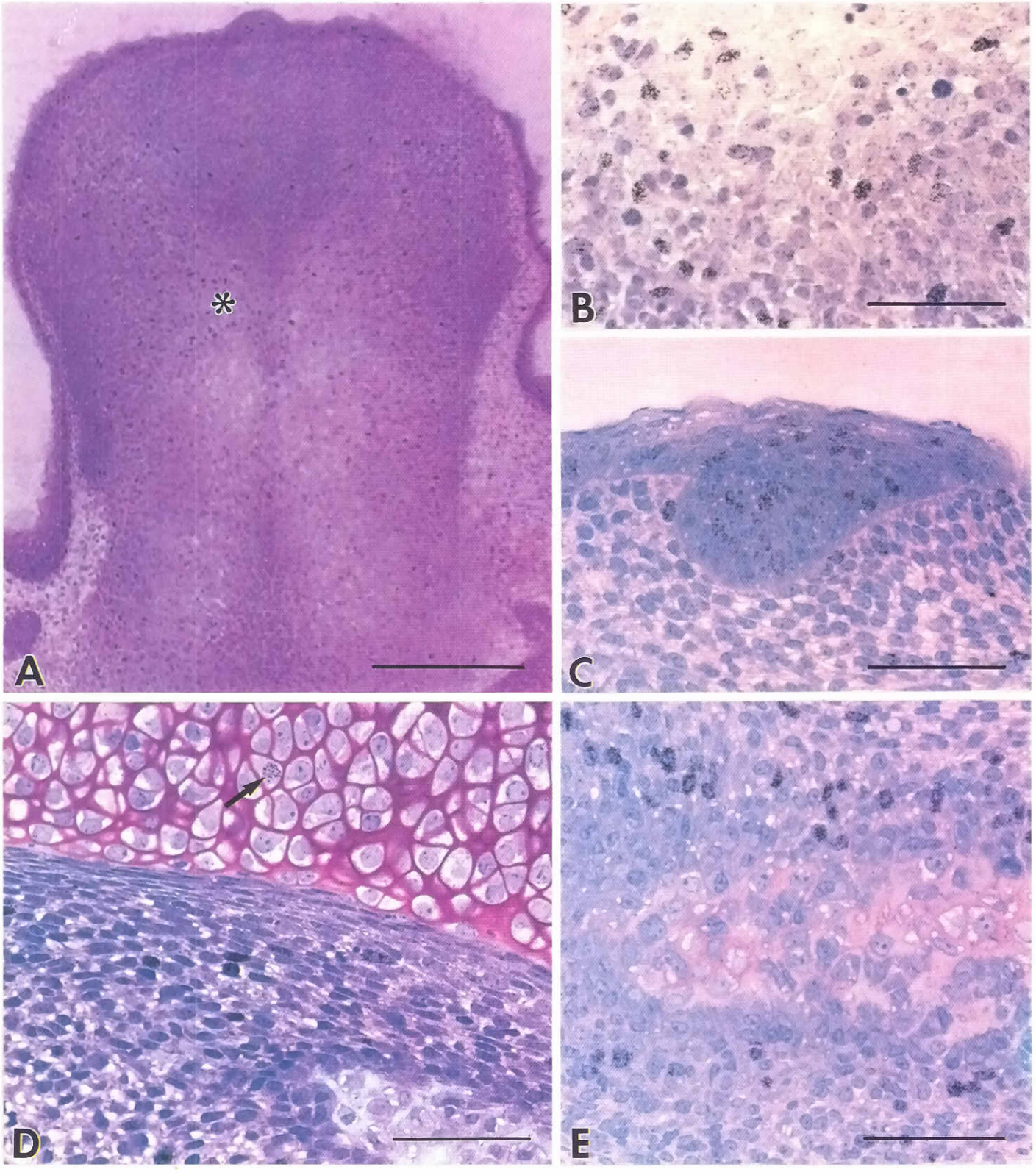

Fig. 5. Light micrographs of ^{3}H-tritiated thymidine incorporation into E10 mandibular processes cultured for nine days. (A) *Tongue showing labeled cells (black) in the epithelium and mesenchyme.* **(B)** *Tongue mesenchyme showing a region comparable to the area marked with an asterisk (*) in A.* **(C)** *Tooth bud with epithelial labeling.* **(D)** *Cartilage and perichondrium (arrow indicates a labeled chondrocyte); and* **(E)** *bone and periosteum. For photomicrograph A bar=200 μm; for B-E bar=50 μm.*

DNA synthesis and mitotic labeling index

Explants were labeled in BGJb medium supplemented with 50 uCi/ml ^{3}H-thymidine [methyl-^{3}H, 77 Ci/mmole (New England Nuclear, Boston, MA)] for 4 h and processed for autoradiography (Kopriwa and Leblond, 1962). Selected fields of cartilage, perichondrium, bone, periosteum, oral epithelium and lateral lingual tongue process mesoderm were examined using equivalent 250 μm^2 areas for analyses. Ten sample fields from each of three replicate cultures were used to determine the mean and standard error of dividing cells displaying ten or more silver grains per nucleus. Computerized grain-counting analysis was used to compare the incorporation of ^{3}H-thymidine into the various tissues of the developing mandibular processes (Imagemaster 1000, Technology Resources, Nashville, TN).

Statistical analysis

Comparison of desmin immunolocalization between E10 and E10 + 9 d.i.v. tongue processes, and ^{3}H-thymidine incorporation were analyzed by using one-way and two-way analyses of variance (ANOVA) (Sokal and Rohlf, 1969). Results were considered significant if $P<0.05$.

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