J. Anat. (1996) 189, pp. 73–86, with 10 figures Printed in Great Britain

The role of transforming growth factor alpha in rat craniofacial development and chondrogenesis

L. HUANG[1]*, MICHAEL SOLURSH[2] AND ALEXANDER SANDRA[1]

Departments of [1] Anatomy and [2] Biology, University of Iowa, Iowa City, USA

(*Accepted 20 February 1996*)

ABSTRACT

To explore the possible role of transforming growth factor α (TGF-α) in craniofacial development, its expression in the craniofacial region of rat embryos from embryonic day (d) 9 to d 20 was examined by in situ hybridisation and immunostaining. The TGF-α transcripts were first detected in the neural fold of embryonic d 9 and 10 embryos. In the craniofacial region, the TGF-α transcripts were not detected until embryonic d 16 in mesenchyme surrounding the olfactory bulb, within the olfactory bulb, the nasal capsule, vomernasal organ, and vibrissal follicle. In addition, TGF-α message was detected in mesenchyme in the vicinity of Meckel's cartilage, and in the dental epithelium and lamina. This expression pattern of TGF-α transcripts persisted until embryonic d 17 but disappeared by d 18. The presence of TGF-α protein largely coincided with TGF-α message although, unlike the message, it persisted throughout later embryogenesis in the craniofacial region. The possible function of TGF-α in chondrogenesis was explored by employing the micromass culture technique. Cartilage nodule formation in mesenchymal cells cultured from rat mandibles in the presence of TGF-α was significantly inhibited. This inhibitory effect of TGF-α on chondrogenesis was reversed by addition of antibody against the EGF receptor, which crossreacts with the TGF-α receptor. The inhibitory effect of TGF-α on chondrogenesis in vitro was further confirmed by micromass culture using mesenchymal cells from rat embryonic limb bud. Taken together, these results demonstrate the involvement of TGF-α in chondrogenesis during embryonic development, possibly by way of a specific inhibition of cartilage formation from mesenchymal precursor cells.

Key words: Craniofacial development; chondrogenesis; TGF-α; rat

INTRODUCTION

Transforming growth factor-α (TGF-α) was originally discovered in the culture fluids of various cells transformed by retroviruses or chemical agents (reviewed by Derynck, 1988). Since its expression was detected in most tumour-derived cell lines and in cells transformed by oncogenes, retroviruses, and tumour promoters, it was suggested that TGF-α is involved in the generation or progression of some forms of neoplasia (reviewed by Massague, 1990). Furthermore, TGF-α was postulated to act in an autocrine manner in the induction of the neoplastic state. More recent studies, however, have demonstrated the presence of both TGF-α mRNA and protein in certain normal tissues of the vertebrate embryo and adult, suggesting that TGF-α may also play a role in the regulation of normal cell growth and differentiation (Werb, 1990; Wilcox & Derynck, 1988; reviewed by Derynck, 1992).

The expression pattern of TGF-α in developing vertebrate embryos, including the chick, mouse and rat, has been examined at both the RNA and protein level (Twardzik, 1985; Wilcox & Derynck, 1988; Werb, 1990; Dixon et al. 1991; Diaz-Ruiz et al. 1993). In the developing mouse embryo, TGF-α mRNA could be detected as early as the 4-cell stage using a PCR-based method (Rappolee et al. 1989). TGF-α mRNA was continuously detected in the developing mouse embryo until embryonic d 10, with the highest level present at embryonic d 7–9 (Wilcox & Derynck, 1988). Similarly, in the developing rat embryo, a high

* Present address: Joint Program in Neonatology, Children's Hospital, Boston, MA 02115, USA.
Correspondence to Dr Alexander Sandra, Department of Anatomy, University of Iowa, Iowa City, IA 52242, USA.

level of expression of TGF-α mRNA was found around d 8 and 9 of gestation (Lee, 1990). In situ hybridisation studies have demonstrated the presence of a peak of TGF-α mRNA in the otic vesicle, oral cavity, 1st pharyngeal pouch, 1st and 2nd branchial arches, and developing mesonephric tubules of embryonic mouse kidney at embryonic d 9 and 10 (Wilcox & Derynck, 1988). The appearance of the mRNA peak in the developing mouse embryo coincided with that of the TGF-α polypeptide, although biochemically detectable levels were present in late gestation (Twardzik, 1985).

The possible functions of TGF-α in the formation of other organs during embryogenesis are still unclear. A number of studies have implicated TGF-α in skin development, hair growth, and keratinisation of the epidermis (Vassar & Fuchs, 1991). Mice with a null mutation of the TGF-α gene have abnormal skin architecture with a dramatic derangement of hair follicles, wavy hair and curly whiskers (Mann et al. 1993), although no craniofacial abnormality was observed in TGF-α knockout mice. Since EGF and the EGF receptors are expressed in the craniofacial region of early developing rodent embryos (reviewed by Slavkin, 1993) and since TGF-α has been implicated as the embryonic homologue of EGF (Matrisian et al. 1982; Freemark & Comer, 1987), recent attention has focused on the possible role of TGF-α in craniofacial development, especially in the formation of cartilage, bone and teeth.

The goal of this study was to investigate the possible functional role of TGF-α in craniofacial morphogenesis and chondrogenesis during craniofacial and limb formation. In situ hybridisation and immunohistochemistry were first employed to localise TGF-α mRNA transcripts and TGF-α protein in rat embryonic tissues. Two peaks of message were detected, one early in development, the other past midgestation. A similar pattern of TGF-α protein expression occurred, although the 2nd peak continued to near term. Micromass cultures were performed to analyse the role of TGF-α in chondrogenesis of the embryonic mandible and limb bud by addition of TGF-α and/or monoclonal antibody against EGF receptor. The results show that exogenous TGF-α inhibits chrondrogenesis of mesenchymal cells in micromass culture and that abrogation of the EGF receptor by an α-EGF receptor monoclonal antibody diminishes the inhibitory effect of TGF-α on chondrogenesis. These results suggest that TGF-α may regulate the size and shape of developing cartilage by inhibiting chondrogenesis through its binding to the EGF receptor.

MATERIALS AND METHODS

Preparation of tissue samples

Pregnant female Sprague-Dawley rats were killed by ether overdose, and the gravid uteri were aseptically removed in phosphate buffered saline (PBS). Individual embryos were dissected from the uterine decidua and staged based on the external features according to the descriptions of Christie (1964). Embryos older than 14.5 d were then decapitated and the heads fixed. For in situ hybridisation, tissues were fixed overnight in 4% paraformaldehyde while for immunostaining tissue were fixed overnight in Bouin's fixative or 4% paraformaldehyde. Tissues were dehydrated and embedded in paraffin wax. Sections were cut at 7 μm and mounted on Superfrost/Plus slides. For in situ hybridisation, the slides were baked overnight at 60 °C 1 d before the in situ hybridisation was carried out.

Preparation of riboprobes for in situ hybridisation

A 2.3 kb fragment from the 4.5 kb rat TGF-α cDNA was originally cloned into the pEMBL vector at the EcoR 2 site (Lee et al. 1985). A 1.3 kb insert from the 3′ end of the untranslated TGF-α cDNA was isolated by using the restriction enzyme Accl. This 1.3 kb insert was subcloned into the pBluescript vector at the Accl site. For the antisense riboprobe, the DNA template was linearised with BamH 1 and transcribed in vitro with T7 RNA polymerase in the presence of ^{35}S-UTP. The negative control sense riboprobe was synthesised with T3 RNA polymerase from the same template after linearisation with Xhol. The synthesised riboprobes were precipitated with ethanol and their size was checked by separation on agarose gels followed by autoradiography. The probes was hydrolysed to about 150 bp fragments and denatured before use by heating at 80 °C for 2 min.

In situ hybridisation

In situ hybridisation was performed according to the previously described method of Swalla et al. (1988). Briefly, sections were deparaffinised in xylene, and rehydrated through graded ethanol to water. After proteinase K (1 mg/ml) treatment at 37 °C for 30 min, sections were hybridised overnight with 50 μl hybridisation buffer (0.3 M NaCl, 20 mM NaAc, 1 mM EDTA, 0.4 M DTT, IX Denhardt's −0.02% ficoll, 0.02% polyvinyl pyrolidone, 250 μg/ml yeast tRNA, 10% dextran sulphate, 50% formamide) containing about 3.6×10^6 cpm riboprobe at 50 °C.

Stringent washes were carried out in 2 × SSC at 60 °C, followed by 0.2 × SSC at 50 °C. Sections were then treated with 20 μg of RNase A/ml and 1 U RNase Tl/ml at 37 °C min. After washing, slides were dehydrated by immersion in graded ethanol containing 0.3 M ammonium acetate. The sections were air dried, coated with NTB-2 nuclear emulsion and stored in the dark with desiccant at 4 °C for 2 to 3 wk. After development, the sections were counterstained with haematoxylin and coverslipped with Permount. In situ hybridisation experiments were performed a total of 4 times.

Immunohistochemical staining

Monoclonal antibody against rat TGF-α, purchased from Oncogene Science, was obtained by immunisation of BALB/C mice with recombinant human TGF-α and fusion of mouse splenocytes with Ag 8.653 mouse myeloma cells. This antibody was shown by immunoassay to be sensitive and specific for native TGF-α and did not detect precursor TGF-α (Sorvillo et al. 1990). Sections were deparaffinised and rehydrated through graded ethanol, followed by immersion in PBS for 10 min. The primary antibody (10 μg/ml) was applied directly to the sections and incubated at 4 °C in a humid atmosphere overnight. The sections were washed 3 times for 10 min each in PBS at room temperature, followed by incubation with goat anti-mouse IgG antibody conjugated to horseradish peroxidase (Promega) for 30 min. After rinsing with 3 changes of PBS, the antibody was visualised by development with 0.5 mg/ml diaminobenzidine (DAB) (Sigma) in PBS containing 0.01 % H_2O_2. The sections were counterstained with haematoxylin, dehydrated in graded ethanol, cleared in Histosol and mounted with Permount. In control sections, the primary antibody was omitted or competed against native TGF-α at a concentration of 10 μg/ul.

Micromass culture

Micromass cultures of rat embryonic mandibular and limb bud mesenchymal cells were performed according to previously described methods (Ahrens et al. 1977; Langille & Solursh, 1990). To obtain mesenchymal cells from mandibles of rat embryos, embryonic d 13 (stage 23A) rats were dissected in sterile PBS. Mandibles were removed from the embryos and pooled. To compare the results obtained from the micromass culture of mandibular mesenchymal cells, mesenchymal cells from d 13 rat hindlimb buds were also isolated for micromass culture.

The isolated mandible or limb bud tissues were washed in calcium and mangesium-free saline G (100 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1 mM Na_2HPO_4) and further minced with a scalpel. Tissues were then digested with 0.1 % trypsin and 0.1 % collagenase in 10 % chick serum for 10 min at 37 °C, with vigorous pipetting to loosen the epithelium and dissociate the mesenchyme from the isolated tissues. Digestion was stopped by adding culture medium containing 10 % fetal calf serum. The dissociated cells were filtered through 2 layers of no. 20 Nitex (Tetko Inc., NY) in order to obtain a single mesenchymal cell suspension. After the total cell number was calculated for the resulting cell suspensions using a haemocytometer, cells were centrifuged at 400 *g* for 6 min and then resuspended in modified essential medium (MEM) containing 10 % fetal calf serum. In order to plate cells at high density, they were suspended at 2×10^7 cells/ml. Drops of media and cells (10 μl) were carefully placed in wells of 24-well cell culture places and allowed to adhere at 37 °C for 2 h before the addition of 0.5 ml medium. The medium was changed every day. Recombinant human TGFα (R & D Systems) was added into culture media at a final concentration of 0.05 ng/ml, 0.5 ng/ml, 5 ng/ml or 10 ng/ml after cells attached to the culture plates. Both TGF-α and a monoclonal blocking antibody (diluted 1:4 in MEM) against the EGF receptor (Developmental Study Hybridoma Bank, Department of Biological Sciences, University of Iowa) (Chandler et al. 1985) were added to the media of separate wells. Micromass cultures with normal media and media containing nonrelevant hybridoma conditioned medium (NS1) (diluted 1:4 in MEM) were used as negative controls. To obtain frank chondrogenic nodules, micromass cultures of mandibular mesenchymal cells were maintained for 11 d, while the cultures of limb bud mesenchymal cells were maintained for 4 d. They were then fixed for 0.5 h in 2 % glacial acid-ethanol and rehydrated and stained overnight in 1 % Alcian blue (in 0.1 N HCl), pH 1.0. Cartilage nodule formation in each micromass culture was examined. For quantitation of cartilage matrix, the bound Alcian blue was quantitatively extracted overnight in 200 μl of 4 M quanidine hydrochloride at 4 °C. The absorbance of each extract was measured at 600 nm on a Titerkek ELISA reader. Under such conditions absorbance is linearly proportional to the amount of cartilage matrix. Each experiment was performed at least twice and data presented is the mean of triplicate samples from a single representative experiment.

RESULTS

Expression of TGF-α transcripts in the craniofacial region

In situ hybridisation was performed to detect TGF-α mRNA in the craniofacial region at different stages of rat embryo development. Since TGF-α transcripts have been detected in the 1st and 2nd branchial arches of the mouse embryo at embryonic d 9 and 10 (Wilcox & Derynck, 1988), the rat embryos used in this study were staged from embryonic d 9.5, when the neural folds are developing, to embryonic d 20. The results

Table. *Localisation of TGF-α mRNA and protein in the craniofacial region of different stage rat embryos**

Embryonic ages (day)	9.5	10.5	11.5	12.5	14.5	16.5	17.0	18.5	19.5	20.0
In situ hybridisation	+	+	−	−	−	+	++	−	−	−
Immunostaining	+	+	−	−	−	+	++	+	+	+

* −, Negative signal or staining; +, weak signal or staining; ++, strong signal or staining.

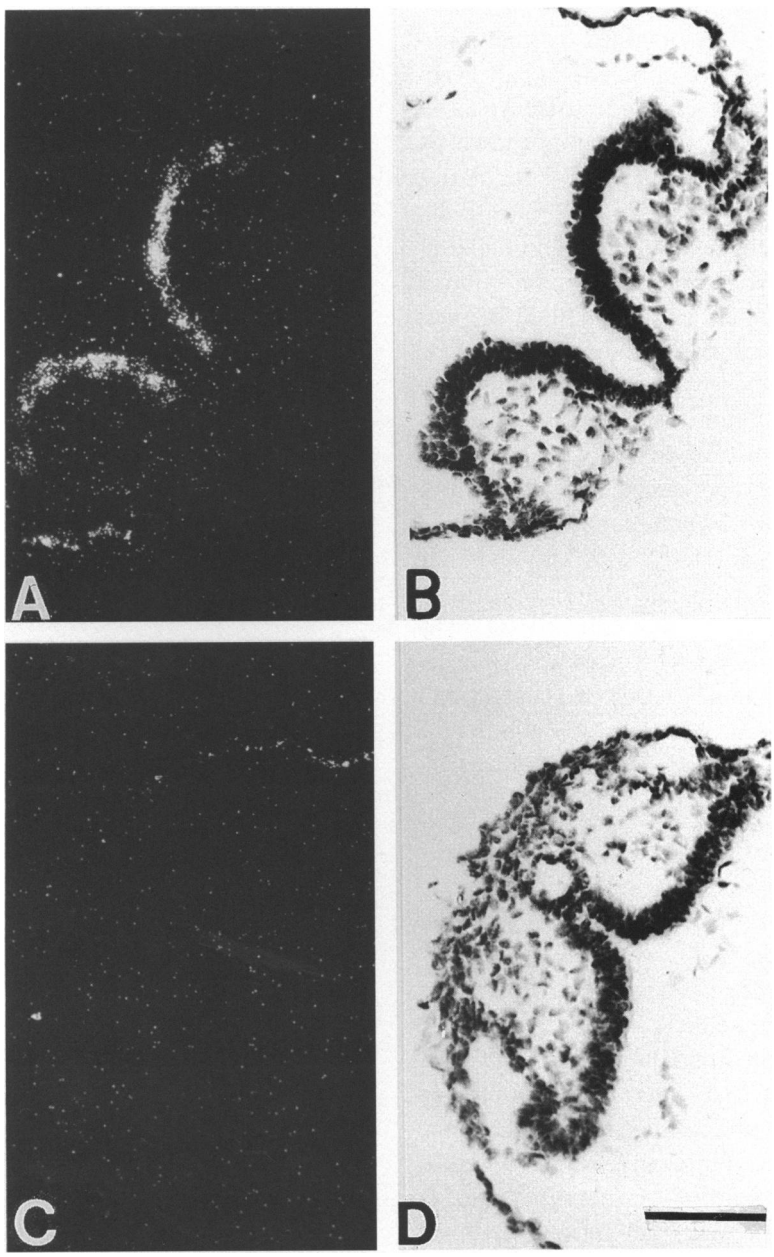

Fig. 1. Localisation of TGF-α message by in situ hybridisation in sections of the d 9.5 rat embryo. (*A*) Antisense hybridisation, dark-field; (*B*) bright-field of (*A*); (*C*) sense hybridisation, dark-field; (*D*) bright-field of *C*. Bar, 50 μm.

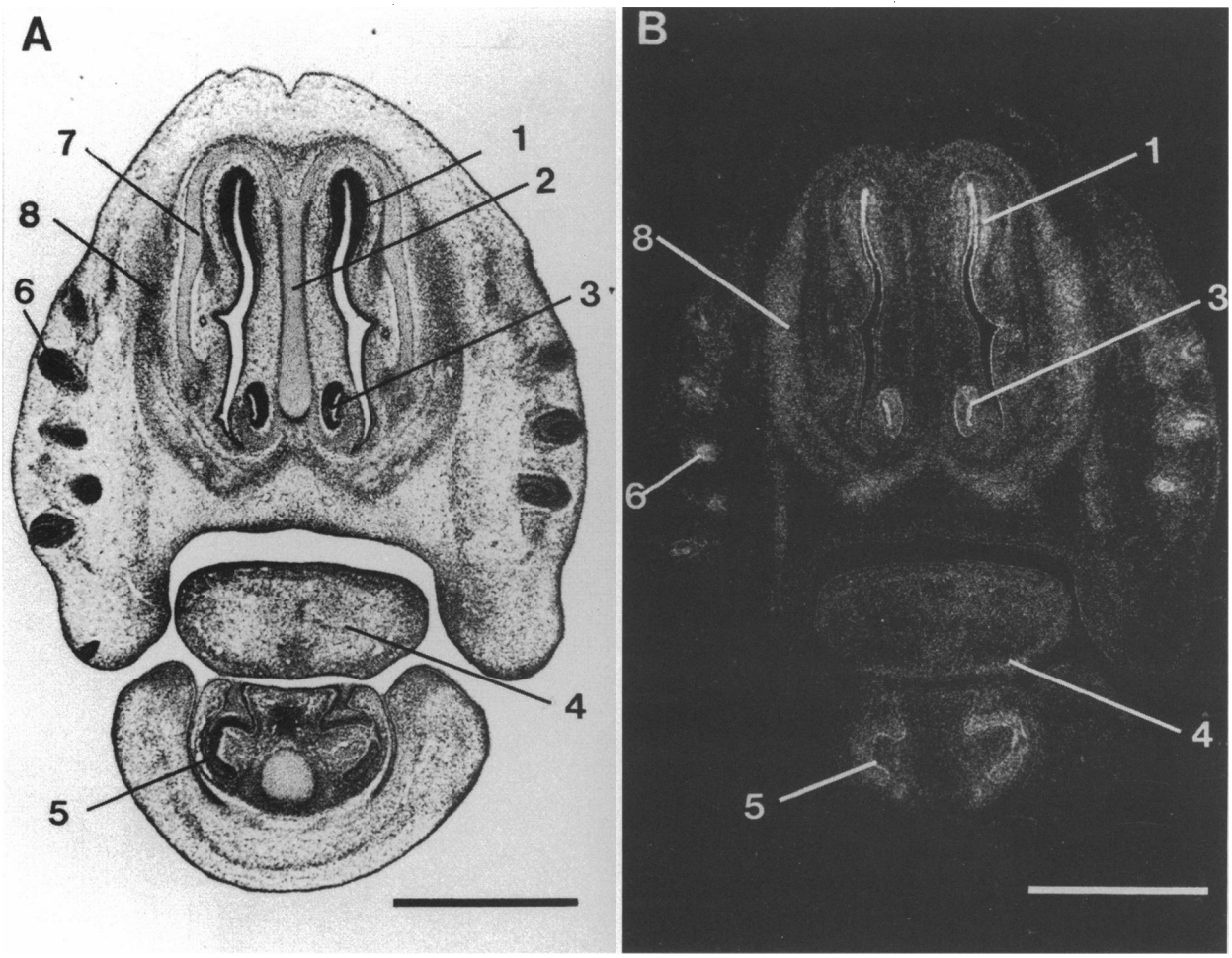

Fig. 2. Low magnification micrographs of sections of d 17 rat craniofacial region prepared for in situ hybridisation using antisense probe for TGF-α mRNA. (*A*) Bright-field micrograph of anterior frontal section through the d 17 (stage 29.5) rat head. (*B*) Dark-field image of the same section hybridised with antisense TGF-α probe demonstrating signal in the olfactory epithelium (1), vomeronasal organ (3), follicle of vibrissa (6) and the mesenchyme surrounding the nasal capsule (8). 1, Olfactory epithelim; 2, anterior extremity of cartilage primordium of nasal septum; 3, vomeronasal organ; 4, tongue; 5, lower incisor tooth; 6, hair follicle; 7, nasal capsule; 8, mesenchyme surrounding the nasal capsule. Bar, 1 mm.

are summarised in the Table. A transient and specific spatial expression of TGF-α transcripts was observed during rat craniofacial development. The expression of TGF-α transcripts in d 9.5 and 10.5 embryos was found to be localised in the neural folds of developing embryos (Fig. 1). From d 11.5 to 14.5 the craniofacial region demonstrated no detectable signal. TGF-α transcripts were again present, however, in the craniofacial region at embryonic d 16.5 and 17. These transcripts again were not detected in the craniofacial region at embryonic d 18.5 and 20.

Low-power examination of frontal sections in the craniofacial region of the d 17 embryo revealed the localisation of TGF-α mRNA in the wall of olfactory bulb, olfactory epithelium, as well as in the mesenchyme surrounding the nasal capsule and cartilage of the capsule itself at the anterior (Fig. 2) and more posterior (Fig. 3) regions of the nasal septum. Sense probes were used as controls and gave no detectable signal, indicating the specificity of the antisense probe. In addition, a positive signal was detected in several other discrete regions of the head including the vomeronasal organ and vibrissal follicle. At higher magnification (Fig. 4), the localisation of TGF-α message in the olfactory bulb and nasal cavity was clearly visible and distinctly defined. TGF-α message was also found in the mesenchyme in the vicinity of Meckel's cartilage and in the stellate reticulum, outer and inner enamel epithelia and in the dental lamina (Fig. 5). This pattern of localisation of TGF-α transcripts persisted only from d 16.5 to 17. Before and after this period, no TGF-α transcripts could be detected in the craniofacial region.

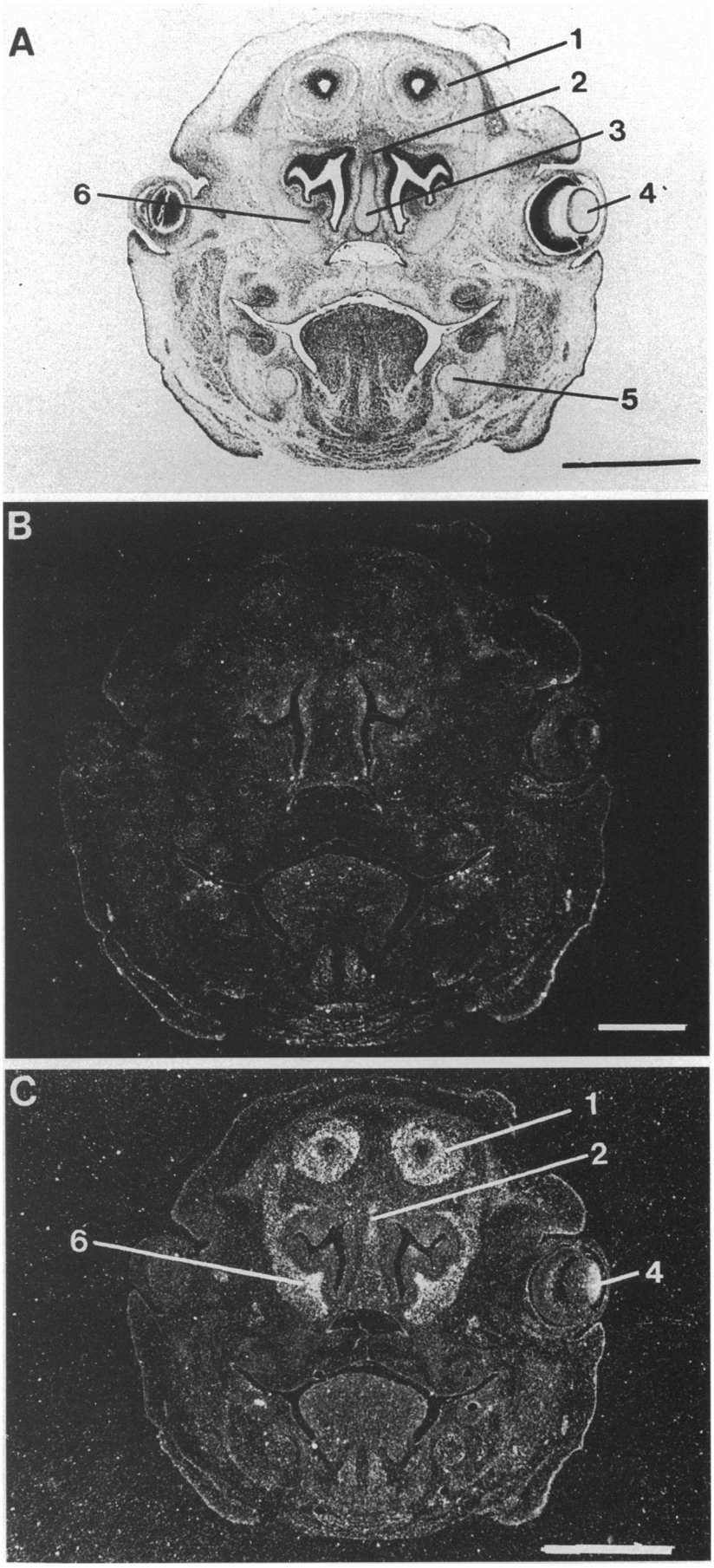

Fig. 3. For legend see opposite.

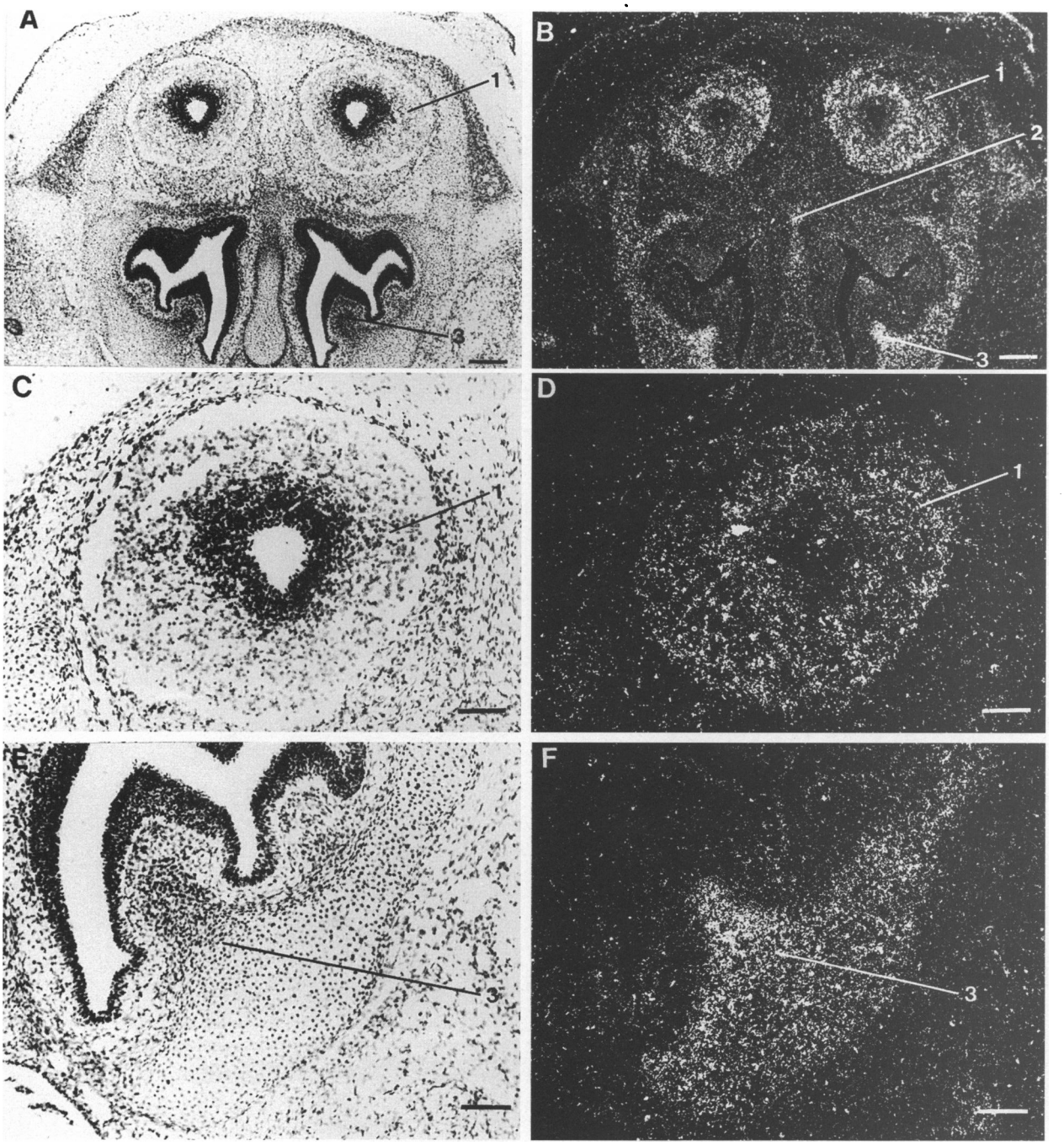

Fig. 4. Higher magnification micrograph of sections of the d 17 rat craniofacial region prepared for in situ hybridisation using antisense probe for TGF-α mRNA. (*A*) Bright-field micrograph of a frontal section through d 17 (stage 29.5) rat head. (*B*) Dark-field micrograph of (*A*) showing signal in the wall of olfactory bulb (1), mesenchyme near the nasal septum cartilage primordium (2) and mesenchyme surrounding nasal cavity (3). (*C*) Higher magnification bright-field micrograph of the wall of olfactory bulb region (1) in the same section as (*A*). (*D*) Dark-field micrograph of the wall of the olfactory bulb (1) showing a strong signal in this region. (*E*) Higher magnification micrograph of the nasal cavity region (3) in the same sections as (*A*). (*F*) Dark-field micrograph of the nasal cavity region (3) showing a strong signal in the mesenchyme surrounding this region. 1; Wall of olfactory bulb; 2, mesenchyme near the nasal septum cartilage primordium; 3, mesenchyme surrounding the nasal cavity. (*A–B*) Bar, 0.2 mm; (*C–F*) bar, 0.1 mm.

Fig. 3. Low magnification micrograph of sections of d 17 rat craniofacial region prepared for in situ hybridisation using antisense and sense probe for TGF-α mRNA. (*A*) Bright-field micrograph of a more posterior frontal section through the d 17 (stage 29.5) rat head. (*B*) Dark-field image of a frontal section through the d 17 (stage 29.5) rat head probed with TGF-α sense cRNA (control). No specifically localised signals could be detected, indicating the specificity of the antisense probe. (*C*) Dark-field photograph of an adjacent frontal section through the d 17 (stage 29.5) rat head probed with TGF-α antisense mRNA. Signal was detected in the wall of the olfactory bulb (1), the mesenchyme near the nasal septum cartilage primordium (2), and the mesenchyme surrounding the nasal cavity (6). Signal shown in the lens was nonspecific. 1, Wall of olfactory bulb; 2, mesenchyme near nasal septum cartilage primordium; 3; cartilage primordium of nasal septum; 4, lens; 5; Meckel's cartilage; 6, mesenchyme surrounding nasal cavity. Bar, 1 mm.

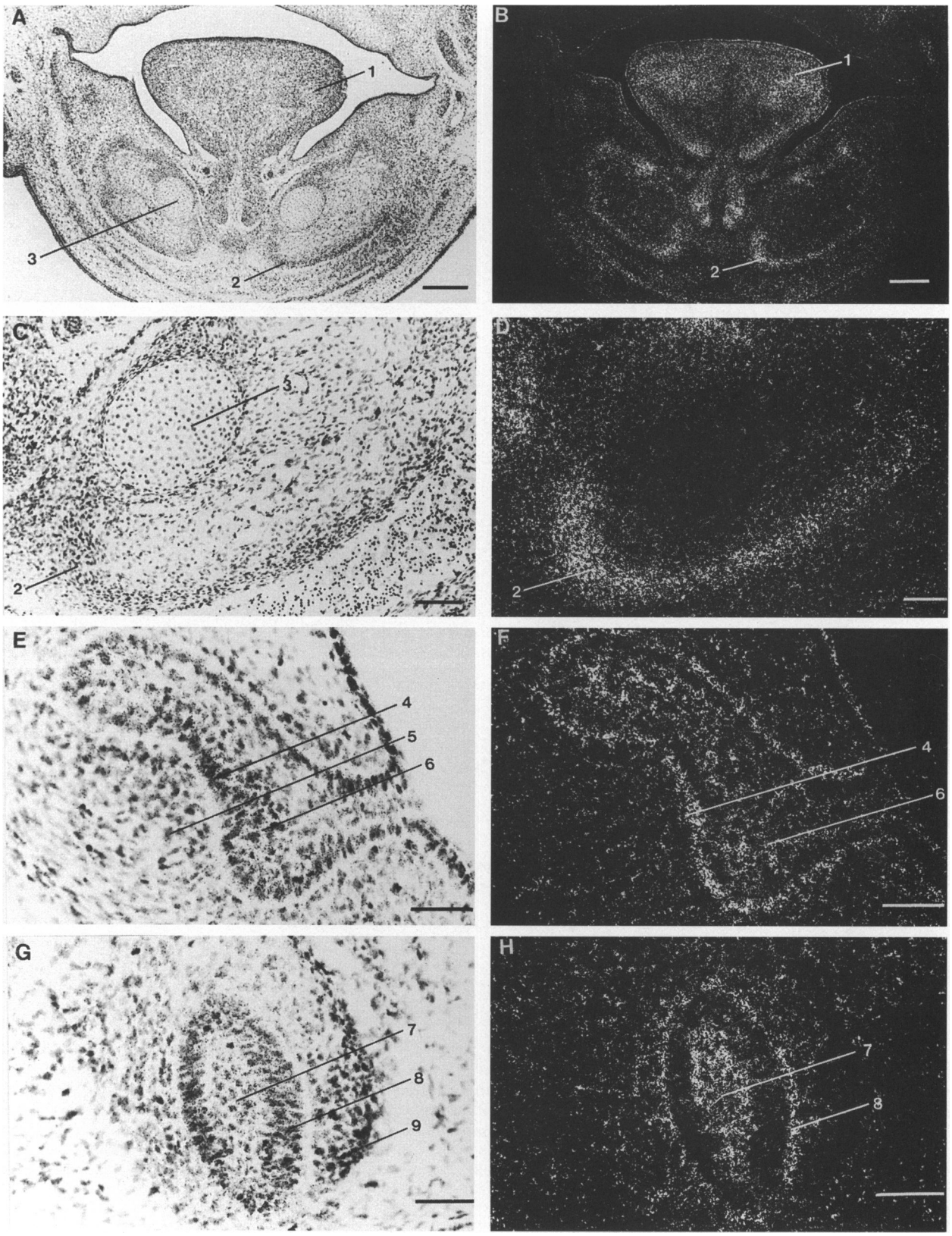

Fig. 5. Higher magnification micrographs of sections at the d 17 craniofacial region prepared for in situ hybridisation using antisense probe for TGF-α mRNA. (*A*) Bright-field photograph of a frontal section through a d 17 (stage 29.5) lower jaw. (*B*) Same frontal section as (*A*) showing inconsistent signal in tongue (1) and the mesenchyme surrounding the intramembranous bone (2). (*C*) Higher magnification bright-field of Meckel's cartilage region (3). (*D*) Dark-field image of (*C*) showing signal in the mesenchyme surrounding the intramembranous bone (2). (*E*) Higher magnification bright-field micrograph of tooth bud region. (*F*) Dark-field micrograph of tooth bud region showing signal in dental epithelium (4) and dental lamina (6). (*G*) Higher magnification bright-field of the vibrissal follicle. (*H*) Dark-field image of the

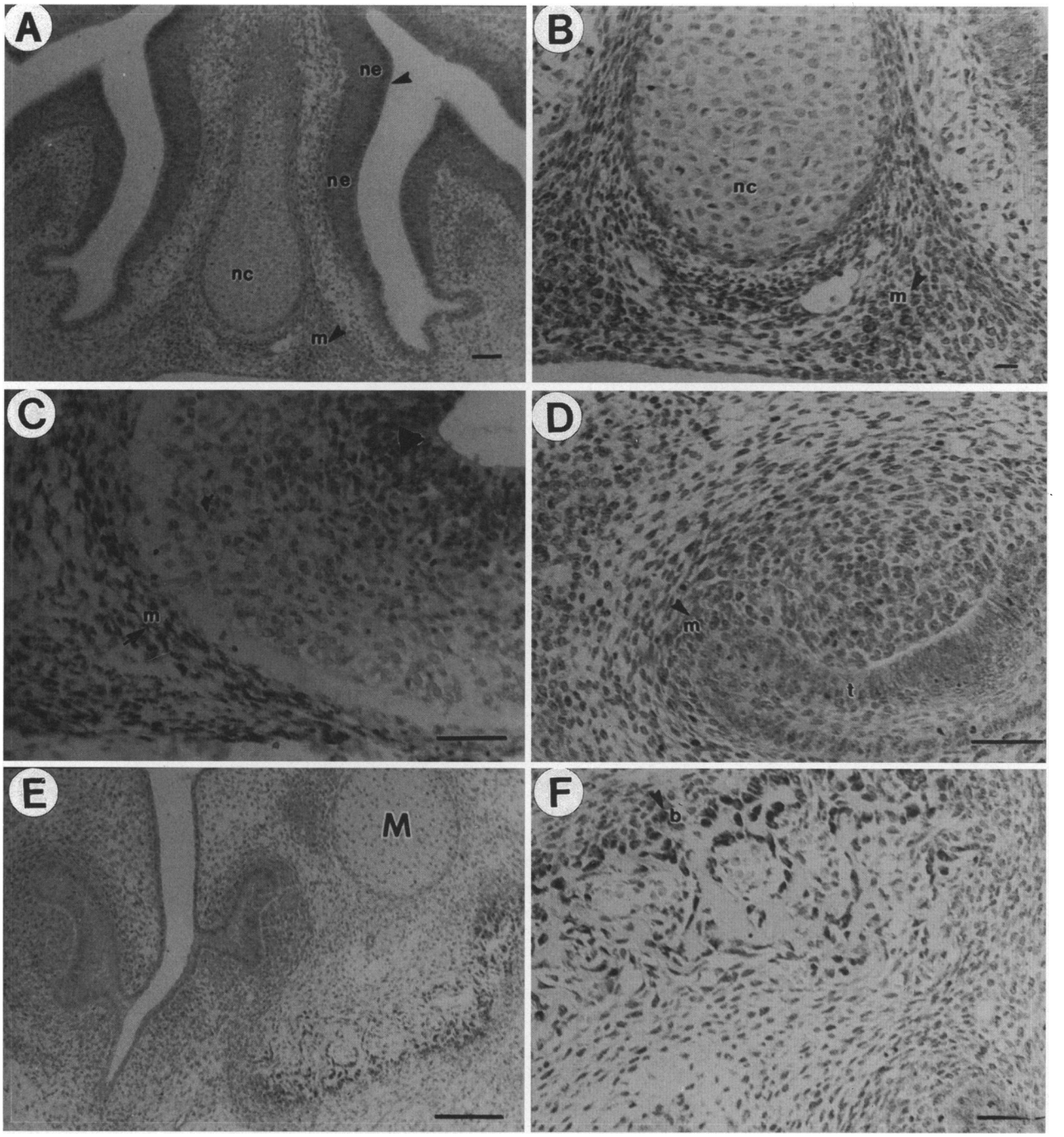

Fig. 6. Immunolocalisation of TGF-α protein using a monoclonal TGF-α antibody in the stage 29.5 (d 17) embryonic rat head (frontal section). (*A*) The nasal region showing TGF-α localisation at the olfactory epithelium (ne, arrowhead) and mesenchyme (m). (*B*) Higher magnification of nasal septum cartilage primordium and its adjacent region showing the staining of TGF-α in the adjacent nasal mesenchyme which appears to be condensing (arrowhead). (*C*) Wall of olfactory bulb showing the staining of TGF-α in its adjacent mesenchymal cells (arrow, m) and the inner cells of the bulb (arrowhead). (*D*) The developing tooth showing a low level of TGF-α staining in the mesenchyme (arrowhead m). (*E*) Lower jaw showing the TGF-α staining in the condensed dental mesenchyme and bone-forming regions near Meckel's cartilage (M). (*F*) Higher magnification showing the TGF-α staining in osteoblasts forming intramembranous bone (arrow, b) in the region of Meckel's cartilage. (*A–B*) bar, 0.5 mm, (*C–F*) 0.05 mm.

vibrissal follicle showing signal in the medulla and inner root sheath layer of vibrissal follicle. 1 Tongue; 2, mesenchyme surrounding the intramembranous bone; 3, Meckel's cartilage; 4, dental epithelium; 5, dental papilla (mesenchyme); 6, dental lamina; 7, hair papilla; 8, hair matrix; 9, root sheath of hair follicle. (*A–B*) bar = 0.3 mm; (*C–D*) bar 0.1 mm; (*E–H*) bar, 0.05 mm.

Immunohistochemical localisation of TGF-α

To assess the localisation of TGF-α translation products, immunohistochemical methods were used. The results are summarised in the Table. In the craniofacial region, similar to the expression of transcripts, immunoreactivity was detected transiently at d 9.5 and 10.5 and then beginning again at the d 16.5 embryo. This positive immunostaining persisted in the craniofacial region until d 20.5. At d 16.5, staining for TGF-α was weakly present in the epithelium, the vibrissa follicles and the bone-forming mesenchyme surrounding Meckel's cartilage (data not shown). At d 17, the immunostaining pattern of TGF-α in the craniofacial region generally coincided with the expression pattern of TGF-α transcripts revealed by in situ hybridisation (Fig. 6). TGF-α staining was present in the inner cells of the olfactory bulb and nasal epithelium and the mesenchyme surrounding the wall of the olfactory bulb. Strongly stained cells of the mesenchyme near the nasal septum cartilage and osteoblasts forming the intramembranous bone of the lower jaw were also detected. In addition, the dental mesenchyme, dental epithelium and dental lamina of the developing tooth were slightly immunopositive. Thus, while TGF-α mRNA was not found in the craniofacial region of embryonic d 18.5 and 20 by in situ hybridisation, immunochemical staining revealed the localisation of TGF-α protein in the craniofacial region of d 16.5 to d 20 rat embryos, with a pattern similar to that observed at d 17 (data not shown).

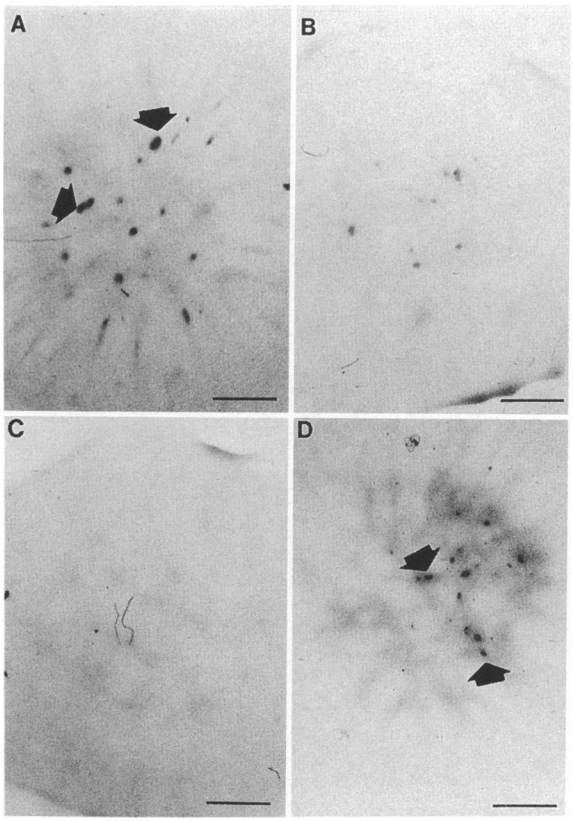

Fig. 7. Alcian blue stained rat mandible micromass cultures showing cartilage differentiation. The mesenchymal cells were isolated from d 13 embryonic mandibles and allowed to differentiate in micromass culture for 11 d. Cartilage nodules are indicated by arrowheads. (*A*) MEM+10% FCS+NS1 (1:4 dilution); (*B*) MEM+10% FCS+0.5 ng/ml TGF-α; (*C*) MEM+10% FCS+5 ng/ml TGF-α; (*D*) MEM+10% FCS+5 ng/ml TGF-α+αEGFR (1:4 dilution). (*A–D*) Bar, 1 mm.

Functional analysis of TGF-α in chondrogenesis

To analyse the possible function of TGF-α in cartilage formation, mesenchymal cells from mandibles were differentiated in vitro as micromass cultures. Embryonic d 13 (stage 23 A) rat mandibular mesenchymal cells were initially isolated for this purpose. The stage chosen was based on the observation that mandibular mesenchyme at this time becomes independent of its epithelium with respect to its subsequent production of both cartilage and bone (Langille & Solursh, 1990). In the micromass system, cells cultured with normal media or with hybridoma conditioned medium (NS1, without IgG) were used as controls. Experimental cells were cultured with medium containing different concentrations of TGF-α. In the rescue experiments, monoclonal antibody against EGF receptor was included in the medium together with TGF-α. To test any effects of EGF receptor antibody alone, cells were also cultured in media containing this reagent. Cartilage nodule formation was examined in the system and measured relative to the cartilage formation in the control cultures. The results obtained from these experiments shown in Figures 7 and 8. Mesenchymal cells from d 13 mandibles were capable of forming cartilage nodules with normal medium. Addition of hybridoma conditioned medium into the medium did not affect the formation of cartilage nodules (Fig. 7*A*). However, addition of TGF-α in the culture medium clearly inhibited chondrogenesis, as evidenced by the failure of cartilage nodule formation or the greatly reduced number and size of cartilage nodules in the micromass culture system (Fig. 7*B*, *C*). The specific inhibitory effect on cartilage nodule formation by TGF-α was demonstrated by the rescue experiment, in which monoclonal antibody against EGF receptor was added together with TGF-α. Since both TGF-α and EGF are believed to function through binding to the same receptor, the EGF receptor, antibody against the EGF receptor blocks the binding of TGF-α to its receptor and thereby prevent its function. In this

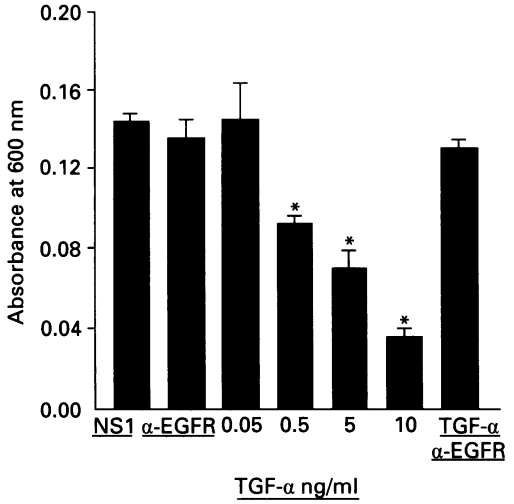

Fig. 8. Histogram showing the relative amount of cartilage matrix production as measured by Alcian blue extraction from micromass cultures of embryonic d 13 rat mandibles grown in defined medium with or without TGF-α and α-EGFR for 11 d. The data are the means of values from 3 cultures from the same cell preparation. Bars indicate standard deviation. Stars indicate statistically significant ($P < 0.001$) difference from control medium.

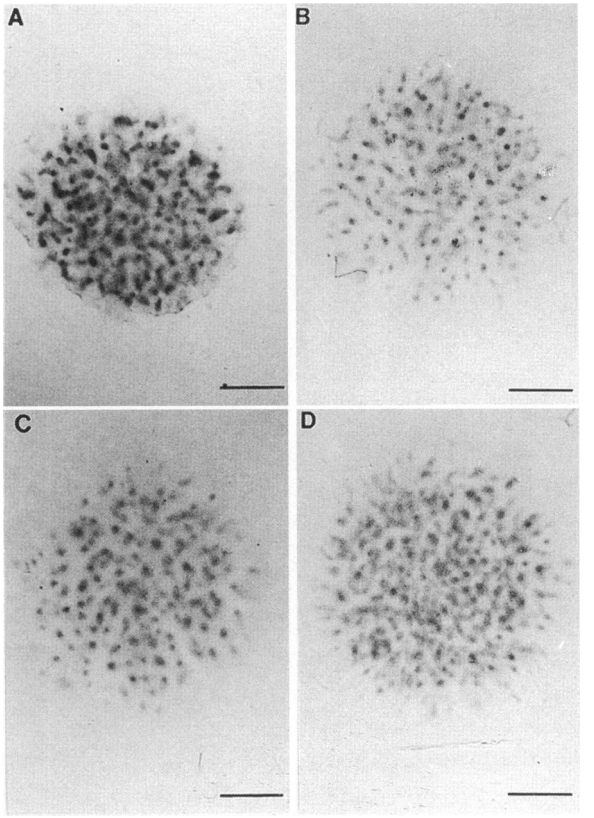

Fig. 9. Alcian blue stained 13 d embryonic rat hindlimb bud micromass cultures showing cartilage differentiation. (*A*) MEM + 10% FCS + NS1 (1:4 dilution); (*B*) MEM + 10% FCS + 0.5 ng/ml TGF-α; (*C*) MEM + 10% FCS + 5 ng/ml TGF-α; (*D*) MEM + 10% FCS + 5 ng/ml-α + α-EGFR (1:4 dilution); (*A–D*) bar, 1 mm.

experiment, cartilage nodule formation was indeed similar to that of the controls (Fig. 7*D*), indicating the rescue effect of the antibody directed against EGF receptor. The inhibitory of TGF-α on chondrogenesis of mesenchymal cells in micromass cultures were quantified by measurement of the amount of cartilage matrix production. Alcian blue bound to the cartilage matrix was extracted from micromass cultures of d 13 rat mandibular mesenchyme grown in defined medium with or without TGF-α and α-EGFR for 11 d. The amount of Alcian blue was proportionate to the amount of cartilage matrix in these cultures. Similar results were obtained in other experiments using mesenchymal cells from stage 23 chick mandibles (data not shown). It is evident that cartilage matrix production in the micromass culture system with 0.5 ng/ml and the 5 ng/ml TGF-α was reduced by about 50% compared with controls and the rescue experiment (Fig. 8). These results are statistically significant ($P < 0.001$ compared with the control and rescue experiments) by 1-way ANOVA analysis and Newman-Keuls analysis.

To further confirm the results obtained from micromass culture using mesenchymal cells from rat mandibles, mesenchymal cells from rat hindlimb bud were also used in the micromass system to test the inhibitory effect of TGF-α in chondrogenesis. Mesenchymal cells isolated from embryonic d 13 rat hindlimb buds were used in experiments designed like those described for mandibular mesenchymal cell culture. The results are shown in Figures 9 and 10. When comparing the limb bud to the mandible in the micromass system, it is obvious that chondrogenesis proceeded to a greater extent in the former. This was most probably due to the greater proportion of chondrogenic cells capable of differentiation at the time of isolation in the limb bud versus the mandible. In the control experiment, where normal culture medium was used, cartilage nodules were well-formed (Fig. 9*A*). Inclusion of the hybridoma conditioned medium in the culture media had no effect on cartilage nodule formation (data not shown). Similarly, addition of TGF-α to the culture medium (5 ng/ml) inhibited cartilage nodule formation, as indicated by the severe reduction of cartilage nodule number and size (Fig. 9*C*). This inhibitory effect appeared dose dependent and was reversed by addition of EGF receptor antibody (Fig. 9*D*). As measured by the quantification of Alcian blue extracted from the micromass cultures, 0.05 ng/ml TGF-α did not inhibit cartilage formation, while 0.5 ng/ml TGF-α inhibited cartilage formation by 30%, 5 ng/ml by about 50%,

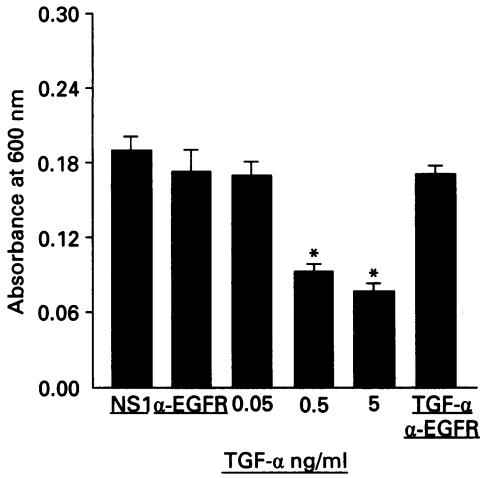

Fig. 10. Histogram showing the relative amount of cartilage matrix production as measured by Alcian blue extraction from micromass cultures of embryonic day 13 rat hindlimb buds grown in defined medium with or without TGF-α and α-EGFR for 4 d. The data are the means of values from 3 cultures from the same cell preparation. Bars indicate standard deviation. Stars indicate statistical significance ($P < 0.001$).

and 10 ng/ml by about 75% (Fig. 10). Again, specificity of the inhibitory effect of TGF-α on chondrogenesis was verified by a rescue experiment, as shown in Figure 10. These results are statistically significant ($P < 0.001$ compared with control and rescue experiments) by 1-way ANOVA and Newman-Keuls analysis. As for mandibular cells, similar results were obtained in other experiments using cultures of mesenchymal cells from stage 23 chick limb buds.

DISCUSSION

Previous studies have strongly suggested the involvement of EGF and TGF-α in craniofacial morphogenesis (Slavkin, 1993), particularly in cartilage formation (Shum et al. 1993). TGF-α transcripts were shown to be present in the murine embryo (Werb, 1990), and both TGF-α transcripts and protein have been localised to the mandible of the developing mouse (Wilcox & Derynck, 1988; Dixon et al. 1991). However, a more complete documentation of the temporal and spatial expression of TGF-α RNA and protein in the craniofacial region of the developing rat embryo has not been established.

Temporal and spatial expression patterns of TGF-α mRNA and protein

TGF-α mRNA and protein could be detected in the neuroectoderm of rat embryos as early as d 9.5. These results are basically similar to those found in the early mouse embryo, where the level of TGF-α mRNA is highest in structures of ectodermal origin (Wilcox & Derynck, 1988). It has been suggested that the major role of TGF-α mRNA and protein in the neuroectoderm and embryonic ectoderm is the promotion of cell proliferation in these rapidly growing tissues.

TGF-α is transiently and spatially expressed during rat craniofacial development. The localisation of TGF-α transcripts in the craniofacial region was primarily observed in the mesenchymal cells of the nasal and mandibular regions. In addition, TGF-α mRNA was also detected in the developing teeth, tongue and olfactory bulb. Basically, the presence of TGF-α protein coincides with the TGF-α mRNA in the craniofacial region of developing rat embryos although the protein persists longer than the message. The TGF-α containing tissues in the craniofacial region include precartilage mesenchyme, developing teeth, vibrissal follicles, olfactory bulb and nasal epithelia. In addition, in situ hybridisation data show that TGF-α mRNA was expressed in the medulla layer and inner root sheath of vibrissal follicles which are 2 layers that form soft keratin. TGF-α may regulate vibrissal follicle outgrowth by stimulating cells of the medulla and inner root sheath to proliferate and keratinise. Moreover, both TGF-α and EGF have been shown to induce accelerated tooth eruption and inhibit hair growth (Derynck, 1986). This is consistent with null mutation studies in which TGF-α minus mutants exhibit abnormal skin histology, wavy hair, curly whiskers and corneal defects (Mann et al. 1993). The fact that no other obvious anomalies occur suggests that the presence of TGF-α elsewhere during embryogeneiss is not required for normal development of those structures or is redundant relative to other growth factors or regulatory molecules.

Interestingly, after embryonic d 18.5, although TGF-α transcripts were no longer detected in the rat craniofacial region, immunostaining continued to show the presence of TGF-α protein. Possible explanations for this observation are that (1) these proteins were transported from other embryonic tissues which expressed TGF-α message and synthesised the protein; evidence at least consistent with this explanation comes from the observations that developing heart and limb continuously express TGF-α mRNA from d 16.5 to and throughout d 20 (data not shown); (2) these proteins were very stable; (3) the message was too low to detect; (4) these proteins were produced by maternal decidua and then localised to the embryo through presently unknown transport mechanisms

(Lee, 1990). Han et al. (1987) showed that a transient peak of TGF-α expression occurred in the maternal decidua of the d 8 rat conceptus. They suggested that TGF-α produced in the decidua stimulates cell proliferation not only locally though an autocrine mechanism, but may also act on the placenta and the embryo through paracrine or endocrine mechanisms.

The inhibitory effect of TGF-α in chondrogenesis

The micromass culture system was employed as a model to test the role of TGF-α in chondrogenesis of rat mandibular mesenchyme. Exogenous TGF-α in this system inhibited chondrogenesis of mandibular mesenchyme, as demonstrated by the number and size of formed cartilage nodules. This inhibitory effect of TGF-α on chondrogenesis was also demonstrated in the micromass system using rat limb mesenchyme. Furthermore, a dose-dependent effect was observed. The functional effect of TGF-α on cell growth and differentiation is generally believed to be mediated through its binding to EGF receptor and activation of intrinsic protein tyrosine kinase (Pike et al. 1982; Massague, 1983). Blockage of binding of TGF-α to the EGF receptor presumably affects the TGF-α signalling pathway, ultimately to regulate chondrogenesis.

It was shown previously that antisense ablation of EGF expression induced Meckel's cartilage dysmorphogenesis (Shum et al. 1993). Coffin-Collins & Hall (1989) reported that exogenous EGF at 10–20 ng/ml inhibited both proliferation of mesenchyme and chondrogenesis in mesenchyme cultured with or without epithelium by using [^{3}H]thymidine-labelled cells as an index of proliferative activity and chondrocyte size and relative amount of extracellular matrix as indices of chondrogenesis. All inhibitory effects of epithelium on intact tissues could be mimicked by exposing isolated mesenchyme to EGF at 10 ng/ml (Coffin-Collins & Hall, 1989). The inhibition of chondrogenesis in cultured mandibular mesenchyme by exogenous TGF-α coincides with these results, and suggests that both EGF and TGF-α have similar functions in chondrogenesis during embryonic development. Both factors exert their function by inhibiting cartilage formation and regulating the size and shape of cartilage. The actual mechanism of this inhibition is unclear and awaits further experimentation. It is possible that TGF-α simply promotes cell proliferation and therefore inhibits cell differentiation. Alternatively, TGF-α may promote the differentiation of nonchondrogenic mesenchymal cells and thus reduce the number or proximity of cells available for cartilage development.

ACKNOWLEDGEMENTS

This work has been supported by NIH grants DE09170 and AM25295. We wish to thank Ms Rebecca Reiter and Mr Jude Longo for technical assistance, and Dr Yiping Chen for critical reading and comments on the manuscript.

REFERENCES

AHRENS PB, SOLURSH M, REITER RS (1977) Stage-related capacity for limb chondrogenesis in cell culture. *Developmental Biology* **60**, 69–82.

CHANDLER LP, CHARDLER CE, HOSANG M, SHOOTER EM (1985) A monoclonal antibody which inhibits epidermal growth factor binding has opposite effects on the biological action of epidermal growth factor in different cells. *Journal of Biological Chemistry* **260**, 3360–3367.

COFFIN-COLLINS PA, HALL BA (1989) Chondrogenesis of mandibular mesenchyme from the embryonic chick is inhibited by mandibular epithelium and epidermal growth factor. *International Journal of Developmental Biology* **33**, 297–311.

CHRISTIE GA (1964) Developmental stages in somite and postsomite rat embryos, based on external appearance and including some features of the macroscopic development of the oral cavity. *Journal of Morphology* **114**, 263–286.

DERYNCK R (1986) Transforming growth factor-α: structure and biological activities. *Journal of Cell Biochemistry* **32**, 293–304.

DERYNCK R (1988) Transforming growth factor α. *Cell* **54**, 593–595.

DERYNCK R (1992) The physiology of transforming growth factor-α. *Advances in Cancer Research* **58**, 27–52.

DIAZ-RUIZ C, PEREZ-TOMAS R, CULLERE X, DOMINGO J (1993) Immunohistochemical localization of transforming growth factor-α and epidermal growth factor-receptor in the mesonephros and metanephros of the chicken. *Cell and Tissue Research* 271, 3–8.

DIXON MJ, GARNER J, FERGUSON MWJ (1991) Immunolocalisation of epidermal growth factor (EGF), EGF receptor and transforming growth factor alpha (TGF-α) during murine palatogenesis *in vivo* and *in vitro*. *Anatomy and Embryology* **184**, 83–91.

FREEMARK M, COMER M (1987) Epidermal growth factor (EGF)-like transforming growth factor (TGF) activity and EGF receptors in ovine fetal tissues: possible role for TGF in ovine fetal development. *Paediatric Research* **22**, 609–615.

HAN VKM, HUNTER III ES, PRATT RM, ZENDEGUI JG LEE DC (1987) Expression of rat transforming growth factor alpha mRNA during development occurs predominantly in the maternal decidua. *Molecular Cellular Biology* **7**, 2335–2343.

LANGILLE RM, SOLURSH M (1990) Formation of chondrous and osseous tissues in micromass cultures of rat frontonasal and mandibular ectomesenchyme. *Differentiation* **44**, 197–206.

LEE DC (1990) TGF-alpha: expression and biological activities of the integral membrane precursor. *Molecular Reproduction and Development* **27** 37–45.

LEE DC, ROSE TM, WEBB NR, TODARO GJ (1985) Cloning and sequence analysis of a cDNA for rat transforming growth factor-α. *Nature* **313**, 489–491.

MANN GB, FOWLER KJ, GABRIEL A, NICE EC, WILLIAMS RL, DUNN AR (1993) Mice with a null mutation of the TGF-α gene have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation. *Cell* **73**, 249–261.

MASSAGUE J (1983) Epidermal growth factor-like transforming growth factor. II. Interaction with epidermal growth factor

receptors in human placenta membranes and A431 cells. *Journal of Biological Chemistry* **258**, 13614–13620.

MASSAGUE J (1990) Transforming growth factor-α: a model for membrane-anchored growth factors. *Journal of Biological Chemistry* **265**, 21393–21396.

MATRISIAN LM, PATHAK M, MAGUN, BE (1982) Identification of an epidermal growth factor-related transforming growth factor from rat fetuses. *Biochemical and Biophysical Research Communications* **107**, 761–769.

PIKE LJ, MARQUARDT H, TODARO GJ, GALLIS B, CASNELLIE JE, BORNSTEIN P et al. (1982) Transforming growth factor and epidermal growth factor stimulate the phosphorylation of a synthetic, tyrosine-containing peptide in a similar manner. *Journal of Biological Chemistry* **257**, 14628–14631.

RAPPOLEE DA, WANG A, MARK D, WERB Z (1989) Novel method for studying mRNA phenotypes in single or small numbers of cells. *Journal of Cell Biochemistry* **39**, 1–11.

SHUM L, SAKAKURA Y, BRINGAS P, LUO W, SNEAD ML, MAYO M et al. (1913) EGF abrogation-induced *fusilli*-form dysmorphogenesis of Meckel's cartilage during embryonic mouse mandibular morphogenesis *in vitro*. *Development* **118**, 903–917.

SLAVKIN HC (1993) Rieger syndrome revisited: experimental approaches using pharmacologic and antisense strategies to abrogate EGF and TGF-α functions resulting in dysmorphogenesis during embryonic mouse craniofacial morphogenesis. *American Journal of Medical Genetics* **47**, 689–697.

SORVILLO JM, MCCORMACK ES, YANEZ L, VALENZUELA D, REYNOLDS JR FH (1990) Preparation and characterization of monoclonal antibodies specific for human transforming growth factor α. *Oncogene* **5**, 377–386.

SWALLA BJ, UPHOLT WB, SOLURSH M (1988) Analysis of type II collagen RNA localization in chick wing buds by *in situ* hybridization. *Developmental Biology* **125**, 51–58.

TWARDZIK DR (1985) Differential expression of transforming growth factor-α during epidermal development and differentiation. *Genes and Development* **5**, 714–727.

WERB Z (1990) Expression of EGF and TGF-α genes in early mammalian development. *Molecular Reproduction and Development* **27**, 10–15.

WILCOX JN, DERYNCK R (1988) Developmental expression of transforming growth factors alpha and beta in mouse fetus. *Molecular and Cellular Biology* **8**, 3415–3422.