Development 109, 585–595 (1990)
Printed in Great Britain © The Company of Biologists Limited 1990

Differential expression of TGF beta isoforms in murine palatogenesis

DAVID R. FITZPATRICK[1], FABIENNE DENHEZ[2], PATURU KONDAIAH[2] and ROSEMARY J. AKHURST[1,*]

[1]*Duncan Guthrie Institute of Medical Genetics, University of Glasgow, Yorkhill, Glasgow G3 8SJ, UK*
[2]*Laboratory of Chemoprevention, National Cancer Institute, Building 41, National Institutes of Health, Bethesda, Maryland 20892, USA*

*To whom correspondence should be addressed

Summary

We have studied the expression of genes encoding transforming growth factors (TGFs) beta1, beta2 and beta3 during development of the secondary palate in the mouse from 11.5 to 15.5 days *postcoitum* using *in situ* hybridisation. The RNA detected at the earliest developmental stage is TGF beta3, which is localised in the epithelial component of the vertical palatal shelf. This expression continues in the horizontal palatal shelf, predominantly in the medial edge epithelium, and is lost as the epithelial seam disrupts, soon after palatal shelf fusion. TGF beta1 RNA is expressed with the same epithelial pattern as TGF beta3, but is not detectable until the horizontal palatal shelf stage. TGF beta2 RNA is localised to the palatal mesenchyme underlying the medial edge epithelia in the horizontal shelves and in the early postfusion palate. The temporal and spatial distribution of TGF beta1, beta2 and beta3 RNAs in the developing palate, together with a knowledge of *in vitro* TGF beta biological activities, suggests an important role for TGF beta isoforms in this developmental process.

Key words: TGF beta, *in situ* hybridisation, mouse embryo, palatogenesis.

Introduction

The murine secondary palate arises from the bilateral maxillary processes of the head. These structures are composed of migratory neural crest cells associated with craniopharyngeal ectoderm (for reviews see Greene and Pratt (1976) and Ferguson (1988)). Mesenchymal cell proliferation within the maxillary processes results in the appearance of primordia of the palatal shelves (Burdett *et al.* 1988), which grow vertically down beside the tongue. A rapid elevation of the palatal shelves brings the two processes into horizontal apposition above the tongue. This is followed by the fusion of the medial edges of the palatal shelves resulting in a continuous palate which later undergoes chondrification and ossification in its anterior portion (Greene and Pratt, 1976; Ferguson, 1988).

The epithelia that cover the palatal shelves are regionally heterogeneous. The oral, nasal and medial edge epithelia can be identified by position, morphology and cell surface markers (Ferguson, 1988). They also have different cell fates. Whereas the nasal and oral epithelia differentiate into pseudostratified and squamous epithelia, respectively, the medial edge epithelium is destined to disrupt by a combination of epithelial cell death and epithelial–mesenchymal transformation (Fitchett and Hay, 1989). Palatogenesis occurs comparatively late in embryogenesis (fusion at gestation day 14.5 in the mouse), making it an easily amenable model for studying several developmental processes, most notably epithelial–mesenchymal interactions.

The importance of epithelial–mesenchymal interactions in the developing palate was established by the tissue recombination studies of (Ferguson and Honig, 1984), who showed that the regional specification of the palatal epithelium is controlled by the underlying mesenchyme. Little is known of any reciprocal signalling from the palatal epithelium. Recently, a paracrine mechanism of transforming growth factor beta1 (TGF beta1) action was proposed in certain epithelial–mesenchymal interactions, as suggested by localisation of TGF beta1 RNA in epithelia which overlie mesenchymal tissue containing the TGF beta1 protein (Heine *et al.* 1987; Lehnert and Akhurst, 1988; Akhurst *et al.* 1990*b*). To extend this study further, particularly with respect to mammalian palatogenesis, we examined the distribution of RNAs encoding TGF beta1, beta2 and beta3, from the time of appearance of the palatal processes, at 11.5 days gestation, to completion of palatogenesis at 15.5 days *post-coitum*.

The TGF betas are a family of related polypeptides which show a high degree (65–80%) amino acid homology within the mature *C*-terminal portion of the molecule and are highly conserved throughout evolution (Sporn *et al.* 1986; Roberts and Sporn, 1990).

There are currently five vertebrate members of this family termed TGF beta1 (Derynck *et al.* 1985), TGF beta2 (Madisen *et al.* 1988; Miller *et al.* 1989*b*), TGF beta3 (ten-Dijke *et al.* 1988; Derynck *et al.* 1988; Jakowlew *et al.* 1988*b*; Denhez *et al.* 1990; Miller *et al.* 1989*a*), TGF beta4 (Jakowlew *et al.* 1988*a*) and TGF beta5 (Kondaiah *et al.* 1990), though only three have been molecularly cloned from mammalian sources.

TGF beta1 was originally identified as a mitogen (Roberts *et al.* 1981; Moses *et al.* 1981), but has since proven to have growth inhibitory effects on many cell types in culture (Moses *et al.* 1985). It also has tissue-specific effects on cellular differentiation *in vitro*, inducing differentiation of some cell types (Masui *et al.* 1986; Seyedin *et al.* 1986), whilst inhibiting differentiation of others (Massague *et al.* 1986; Ignotz and Massague, 1985). One major biological activity of TGF beta1 is its ability to induce deposition of extracellular matrix (ECM) by mesenchymal cells. This is mediated by increased biosynthesis of ECM components (Roberts *et al.* 1986; Ignotz and Massague, 1986) and of protease inhibitors and by decreased synthesis of extracellular proteases (Lund *et al.* 1987; Edwards *et al.* 1987).

TGF beta2 protein was isolated from demineralised bovine bone on the basis of its cartilage-inducing activity in culture (Seyedin *et al.* 1987). It was later found to be structurally and functionally related to TGF beta1 (Seyedin *et al.* 1987)). The spectrum of *in vitro* biological activities of TGF beta1 and TGF beta2 are very similar, though there are some marked specificities as well. TGF beta2 has potent mesoderm-inducing activity in a *Xenopus* bioassay (Rosa *et al.* 1988), whereas TGF beta1 can only act in a synergistic capacity along with fibroblast growth factor (FGF) in this assay (Kimelman and Kirschner, 1987). Conversely, of the two, TGF beta1 is the most potent growth inhibitor of endothelial cells in culture (Jennings *et al.* 1988). In the mouse embryo, TGF beta2 RNA has been shown to have a very different spatial and temporal expression pattern from that of TGF beta1 RNA, being localised to the mesenchymal components of tissues such as bone, cartilage, blood vessels and gut (Pelton *et al.* 1989). This gives further support to the proposition that these two very similar molecules serve different *in vivo* biological functions.

TGF beta3 was isolated by molecular cloning of cDNAs (ten-Dijke *et al.* 1988; Jakowlew *et al.* 1988*b*; Derynck *et al.* 1988), and there is comparatively little data available on its biological activity and role in embryogenesis.

Materials and methods

Mouse stocks

Mouse embryos were obtained from Parkes females mated with NIH males. The day on which the copulation plug was found was called day 0.5. All tissues were fixed overnight in ice-cold 4% paraformaldehyde in phosphate-buffered saline, then dehydrated and embedded in paraffin wax. The palates of embryos 14.0 days gestational age or older were dissected in ethanol to ascertain the developmental stage of palatogenesis prior to paraffin-embedding.

Probe synthesis

^{35}S-labelled riboprobes were generated to a specific activity of 10^9 disints per min μg^{-1} using either the T3 or T7 polymerase transcription systems. Probes were digested to an average of 100 nucleotides by controlled alkaline hydrolysis (Cox *et al.* 1984) and used at a final concentration of 30 pg μl^{-1} in hybridisations.

The TGF beta1-specific antisense probe was a 600 nucleotide *ApaI–KpnI* fragment subcloned into Bluescribe (Stratagene) from the full-length murine TGF beta1 cDNA (Derynck *et al.* 1986) which was kindly provided by Dr R. Derynck (Genenteck). The subclone corresponds to the precursor region of the TGF beta1 polypeptide (amino acids 68–268).

The control probe used was a full-length TGF beta1 human cDNA, kindly supplied by Dr G. Bell (unpublished). It was also subcloned into the Bluescribe vector in sense orientation with respect to the T7 promoter.

The TGF beta2 DNA probe was obtained by amplification of the reverse transcriptase product of total mouse embryo RNA using the polymerase chain reaction (Saiki *et al.* 1988). The oligonucleotide primers spanned the initiation and termination codons. This probe was identical in nucleotide sequence to that reported by Miller *et al.* (1989*b*) (F. Denhez unpublished). This study used the full-length probe; however, a TGF beta2 specific, 501 nucleotide *PstI–SacI* fragment has shown identical hybridisation pattern (data not shown).

The TGF beta3-specific probe was a 732 nucleotide fragment spanning amino acid residues 8 to 251 of the precursor polypeptide (Denhez *et al.* 1990).

Nucleotide sequence homologies between the three gene-specific probes were 42% (beta1 to beta2), 47% (beta2 to beta3) and 36% (beta1 to beta3) (Pelton *et al.* 1989; Denhez *et al.* 1990).

In situ *hybridisations*

In situ hybridisation to 7 μm tissue sections was performed according to the protocol of Wilkinson *et al.* (1987) using 60% formamide in the hybridisation mixture and a hybridisation temperature of 52°C. The slides were dipped in Ilford K5 emulsion and exposed for 3, 7 and 21 days. After development the slides were stained in haematoxylin and mounted. Photomicrography was performed on an Olympus BK2 microscope using Panatomic X film (Kodak).

Results

The differential distributions of RNAs encoding the three related growth factors, TGF beta1, beta2 and beta3 were investigated during murine palatogenesis from 11.5 to 15.5 days gestational age. *In situ* hybridisation was performed on 7 μm coronal sections of the embryonic head using radioactive gene-specific probes complementary to each transcript. As a negative control, a human TGF beta1 sense probe was employed which gave no specific hybridisation signal (data not shown).

No specific hybridisation of any probe was seen in the very early palatal shelves at 11.5 to 12.5 days gestational age (data not shown). The failure to localise transcripts at this stage may relate to the limits of detection of the

in situ hybridisation technique. The first appearance of TGF beta transcripts occurred at the late vertical shelf stage. Two patterns of RNA distribution were seen at this time and later in shelf development. TGF beta1 and beta3 are expressed in the medial edge epithelia, whereas TGF beta2 RNA is localised in the underlying mesenchyme.

Late vertical palatal shelves

At 13.5 days gestation the TGF beta1-specific probe showed strong hybridisation to the submandibular gland (Fig. 1C), as previously shown by Lehnert and Akhurst (1988). At this stage, the palatal shelves, which are growing vertically, show no specific hybridisation to this probe.

The first TGF beta gene to show high level expression in the palatal processes is that for TGF beta3 (Fig. 1G,H). This gene is expressed in the epithelial component of the palatal shelves in the region that will give rise to the future medial edge epithelium. In several embryos, examined the spatial extent of the hybridisation signal is identical. TGF beta3 expression is more extensive in the vertical epithelium facing the tongue and stops abruptly on the oral side. In the most anterior region of the oro-nasal cavity, the epithelium of the nasal septum, which is also destined to fuse with the palatal shelves, shows high level TGF beta3 expression (Fig. 1G).

There is a low level of hybridisation of the TGF beta3 probe to the mesenchyme of the tongue, mandible and the upper regions of the maxillary processes. The mesenchyme immediately adjacent to the medial edge epithelium is, however, markedly devoid of autoradiographic signal. This observation was also made at later stages (see Figs 2F,G and 3D).

At this early stage, TGF beta2 does not appear to be specifically expressed in the palatal processes, although a characteristic signal is seen in the differentiating olfactory epithelium of the nasal process (data not shown). On some sections, small regions of the medial edge epithelia appear to show hyperplasia, resulting in small blebs of stratified epithelium (Fig. 1E). The epithelial cells within these hyperplastic nodules express very high levels of TGF beta2 RNA (Fig. 1D,F), whereas there is no hybridisation with either TGF beta1 or beta3 (data not shown).

The horizontal palatal shelf

After elevation of the palatal shelves at around 14.0 days gestation, the medial edge epithelia come into almost immediate contact (Fig. 2A). At this stage, TGF beta1 is now detectable in the medial edge epithelia in the same cells that express TGF beta3 (Fig. 2B,C,D). The expression of epithelial TGF beta1 RNA is correlated with the presence of the polypeptide in the underlying mesenchyme (Heine *et al.* 1987). TGF beta1 expression is also seen in regions of membranous ossification within the developing maxilla (Fig. 2B), as previously reported by Lehnert and Akhurst (1988).

By this time the quantity of TGF beta3 RNA in the medial edge epithelium has increased considerably. There is striking hybridisation with this probe in both in the medial edge epithelia and the epithelium of the oral aspect of the prefusion anterior nasal septum (Fig. 2F,G,H). In fact, the palatal epithelium at this stage is by far the richest source of TGF beta3 RNA within the entire embryo (F. Millan and R. Akhurst unpublished).

TGF beta2 RNA is excluded from the palatal epithelium, but a gradient of expression is seen within the mesenchyme beneath the medial epithelium, with highest expression levels immediately adjacent to the epithelium (Fig. 2E).

The fusing palate

With fusion of the medial edge epithelia to form the epithelial seam, the expression patterns established in the horizontal shelves continue. TGF beta1 (Fig. 3B) and TGF beta3 (Fig. 3D,G,H) RNAs are localised to the epithelial cells of the seam. This expression is lost as the seam disrupts and the cells lose their epithelial phenotype by transformation into mesenchymal cells.

TGF beta2 transcript levels increase and are now easily seen in the mesenchyme on either side of the seam (Fig. 3C,E,F). The RNA distribution is asymmet-

Fig. 1. Localisation of TGF beta1, beta2 and beta3 in vertical palatal shelves. Coronal sections are presented from 13.5-day embryos through the anterior third of the vertical palatal shelves. A and E,F,G,H are bright-field images, B,C,D are dark-field. (A) Bright-field image showing tongue (to), palatal shelves (ps) and nasal septum (ns). (B) Dark-field images showing non-specific hybridisation with sense probe. (C) TGF beta1 probe showing hybridisation to the submandibular gland (sm). (D) Non-specific mesenchymal hybridisation of TGF beta2 probe with localised area of expression in the epithelium of the left palatal shelf. (E) High-power image of adjacent section to D showing localised hyperplasia (hy) in the epithelia of one palatal shelf (ps). (F) High-power view of epithelial expression in D. (G) Hybridisation of the TGF beta3 probe to the medial epithelia of the palatal shelves and the oral epithelium of the nasal septum. (I) High-power view of the palatal shelf (ps) epithelial expression (boxed area) in H. Scale bar (A,B,C,D,G) represents 200 micrometers, scale bar (E,F,H) 50 micrometers.

Fig. 2. TGF beta1, beta2 and beta3 expression in the horizontal palatal shelves. All sections are from 14.0-day embryos. (A) Coronal bright-field image of horizontal, middle third palatal shelve (ps) medial edge epithelia (me) with the nasal septum (ns) above and oral cavity (oc) below. (B) Adjacent section to A with TGF beta1 probe hybridising to the maxilla (ma) and medial edge epithelia (me). (C) Bright-field paramedial sagittal section through posterior medial edge epithelia (me). (D) Dark-field image of C with TGF beta1 probe specifically hybridising to the medial edge epithelium. (E) Dark-field image of coronal section TGF beta2 hybridising to the medial and nasal palatal mesenchyme. (F) TGF beta3 hybridising to the medial edge epithelia on adjacent section to A. (G) Dark-field image of coronal section through middle third of palate, posterior to F, TGF beta3 probe hybridises to the medial edge epithelia. (H) High-power view of the palatal shelves (ps) and medial edge epithelia (me) expression in G (boxed area). Scale bar (A,B,C,D,E,F,G) represents 200 μm and in (H) 50 μm.

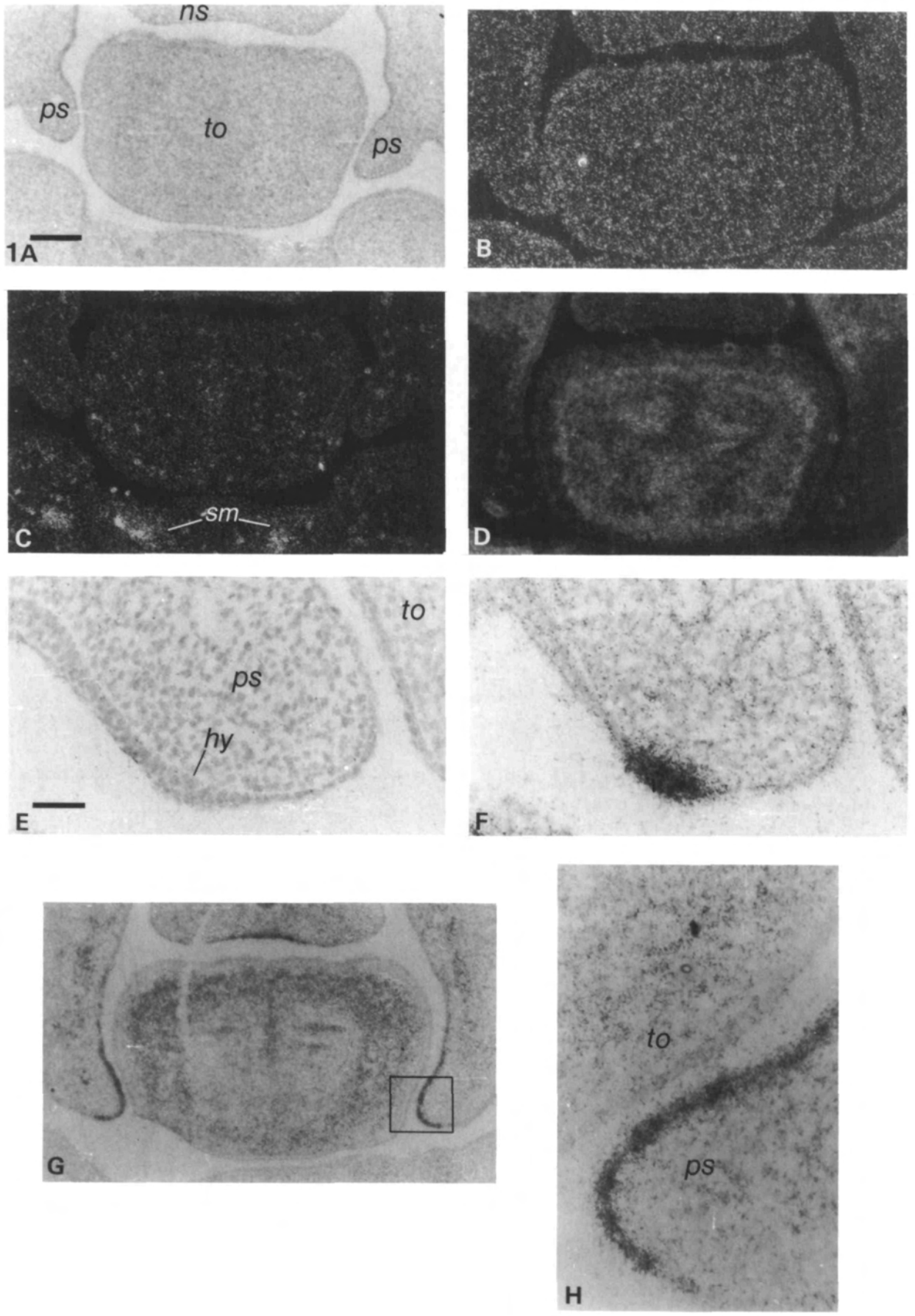

Fig. 1. For legend see p. 587

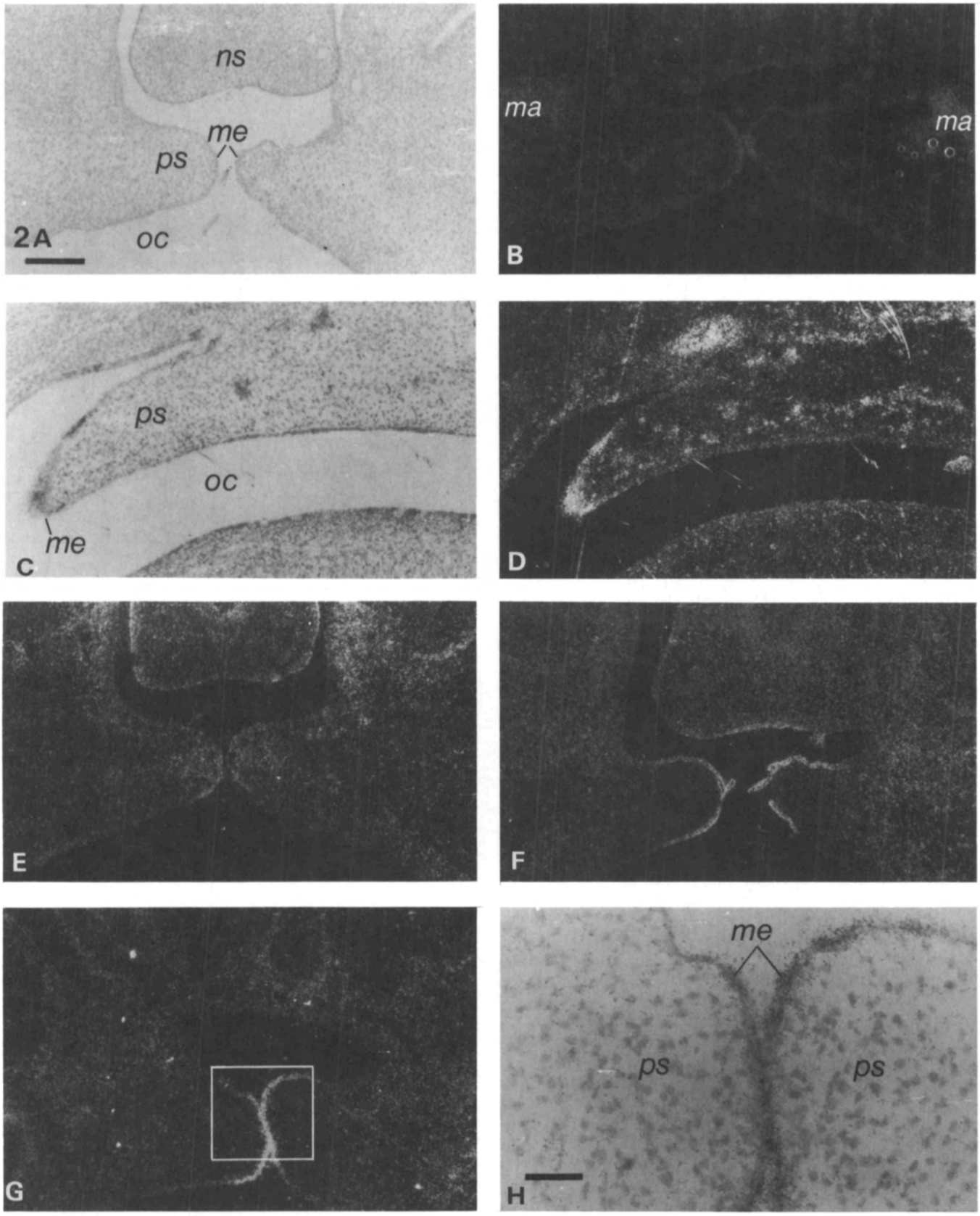

Fig. 2. For legend see p. 587

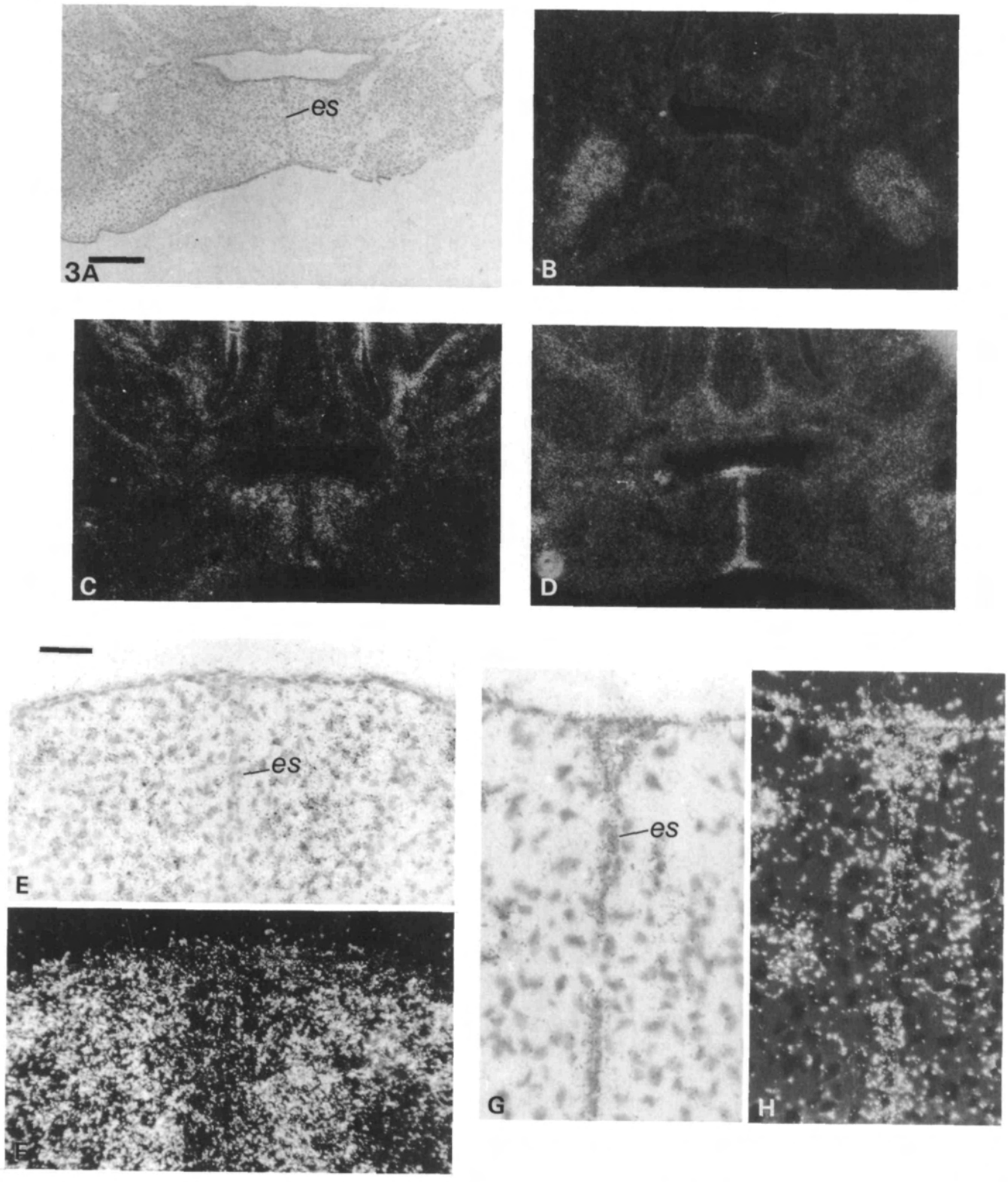

Fig. 3. Localisation of TGF beta1, beta2 and beta3 in the fusing palate. All sections are coronal through the middle third of the fusing palate of 14.5-day embryos. (A) Bright-field image of fusing palate with the midline epithelial seam (es). (B) Dark-field image showing TGF beta1 faint hybridisation to the epithelial seam. (C) Dark-field image of TGF beta2 hybridisation to the medial and nasal palatal mesenchyme. (D) Dark-field image of specific hybridisation of TGF beta3 to the epithelial seam. (E) High-power bright field image of the epithelial seam (es) in C showing absence of TGF beta2 expression in the seam. (F) Dark-field image of E. (G) High-power bright field image of TGF beta3 expression in the disrupting epithelial seam (es), showing hybridisation only where the seam is intact. (H) Dark-field image of G. Scale bar (A,B,C,D) represents 200 μm and (E,F,G,H) 50 μm.

ric with a higher concentration towards the nasal side of the palate.

The maturing palate

After fusion, the midline epithelial seam rapidly disrupts by a process of epithelial–mesenchymal transformation and cell death, thus establishing mesenchymal continuity (Fitchett and Hay, 1989). The fate of the palatal mesenchyme is regionally determined, anteriorly becoming ossified to form the bone of the hard palate and posteriorly giving rise to muscle (Ferguson, 1988).

TGF beta2 continues to be expressed in a diffuse region of the mesenchyme around the midline of the palate (Fig. 4C). Other studies from this laboratory suggest that this TGF beta2 expression may be associated with condensation of mesenchymal tissue prior to chondrification (D. Gatherer, F. Millan, D. Baird and R. Akhurst unpublished).

Postfusion, TGF beta3 expression also switches to become primarily mesenchymal. Hybridisation is seen in the perichondrium of the nasal septum as well as in the mesenchyme of the anterior secondary palate (Fig. 4D). In general, we have found that TGF beta3 expression is associated with chondrification in early mouse and human embryos (D. Gatherer, F. Millan, D. Baird and R. Akhurst unpublished).

Finally TGF beta1 expression in the maturing palate is limited to areas of ossification within the palate and around the nasal processes (Fig. 4B), as previously reported (Lehnert and Akhurst, 1988).

Discussion

Cleft palate is amongst the most common of congenital

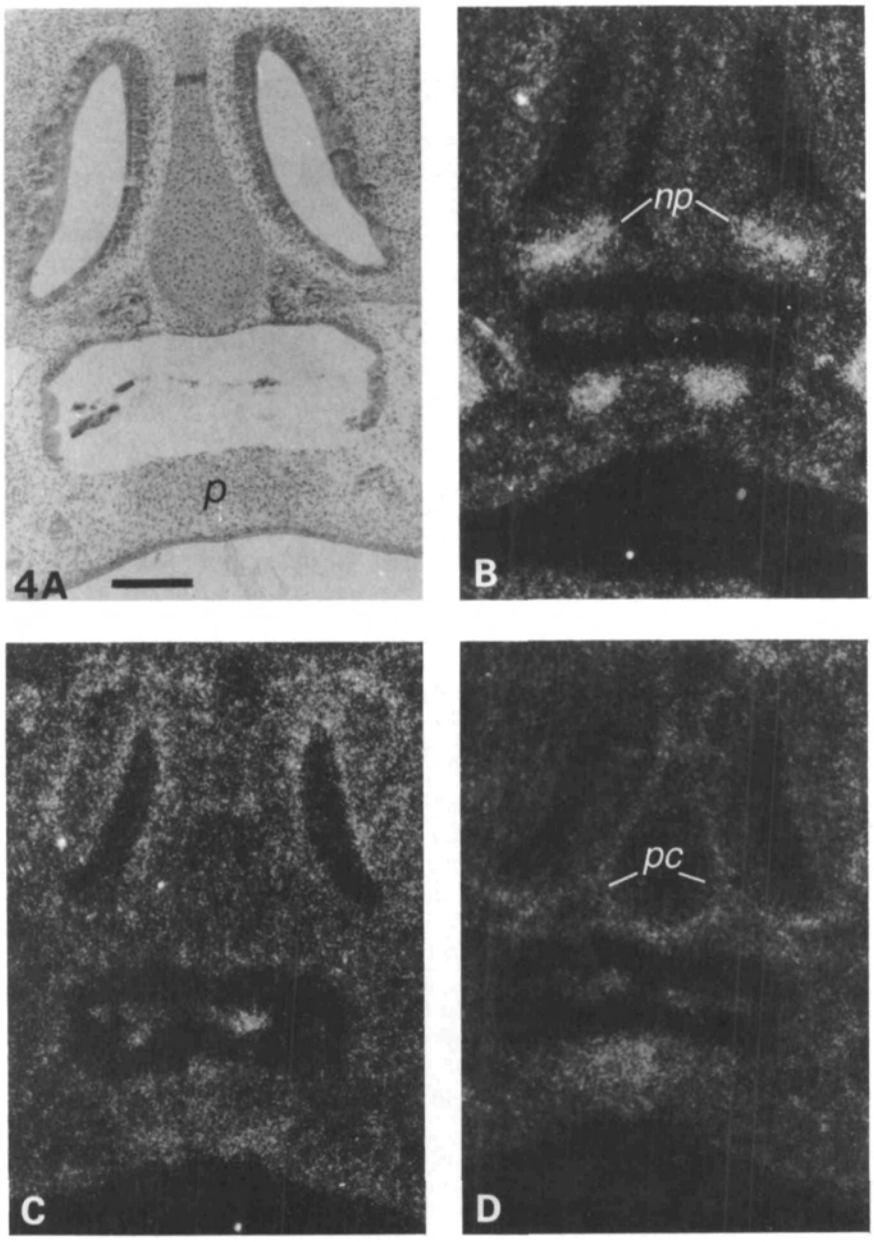

Fig. 4. TGF betas in the maturing palate. Sections have been cut coronally through the middle third of the palates of 15.5-day embryos. A is a bright-field image and B,C,D are dark-field. (A) The maturing palate (p). (B) TGF beta1 probe hybridising to the areas of ossification within the palate and around the nasal processes (np). (C) Diffuse hybridisation of the TGF beta2 probe to the mesenchyme around the midline of the palate. (D) Specific perichondrial (pc) hybridisation of the TGF beta3 probe around the nasal septum and the midline mesenchyma of the palate. Scale bar represents 200 μm.

malformations in humans (Thompson and Thompson, 1986). From a clinical standpoint, it would therefore be very important to understand the mechanisms controlling mammalian palatogenesis at the cellular and molecular level. The mouse represents a good model for such a study since palatogenesis is almost identical to that in the human, and the process can be disturbed in a controlled manner by the administration of teratogens (Morriss, 1973; Abbott *et al.* 1988). Furthermore, several genetic lines of mice are available which show increased susceptibility to this malformation (Fitch, 1957; Gasser *et al.* 1981).

The first evidence of the expression of members of the TGF beta family during palatogenesis is the epithelial expression of TGF beta3 in the vertical palatal shelves. TGF beta1 shows a similar pattern of expression to that of TGF beta3 but apparently with lower transcript prevalence. A comparison of relative transcript levels of different genes by *in situ* hybridisation is difficult to make, despite control over the size and specific activity of the radioactive probe. However, one conclusion that can be drawn regarding transcript prevalence is that the medial edge epithelial cells are the most abundant source of TGF beta3 RNA within the murine embryo at this period of development.

In addition to the epithelial expression of TGF beta1 and beta3, there is a distinct mesenchymal distribution of TGF beta2 gene expression. The temporal and spatial distribution of transcripts from these three genes is summarised in Fig. 5.

As yet there are no published reports of the polypeptide distributions of TGF beta2 or TGF beta3 during murine embryogenesis, though the mesenchymal distribution of TGF beta1 polypeptide in the palate (Heine *et al.* 1987) would be consistent with a paracrine mode of action of epithelially derived TGF beta1 on the underlying mesenchyme, as previously suggested (Lehnert and Akhurst, 1988; Akhurst *et al.* 1990*b*). From the similar pattern of epithelial TGF beta 3 gene expression one might speculate that this growth factor also has paracrine activities on the mesenchyme.

TGF beta1 (and beta3?) may act on the underlying mesenchyme in a variety of ways. TGF beta1 is known to stimulate proliferation and chemotaxis of cells of mesenchymal origin (Moses *et al.* 1985; Postlethwaite *et al.* 1987), though Sharpe and Ferguson (1988) have claimed that, in the palate, it is inhibitory to mesenchymal cell growth. Both cell migration and proliferation are essential features of palatal shelf development.

The action of TGF beta1 on the accumulation of ECM components has been the subject of extensive study. This growth factor induces synthesis of collagens and fibronectin (Roberts *et al.* 1986; Ignotz and Massague, 1986), tenascin (Pearson *et al.* 1988) and chondroitin/dermatan proteoglycans (Hiraki *et al.* 1988; Sharpe and Ferguson, 1988). Accumulation of the latter class of molecules is thought to be important in palatal shelf elevation by virtue of the rise in osmotic pressure resulting from hydration of the proteoglycan network (Pratt *et al.* 1973; Brinkley and Morris Wiman, 1987). In this respect it is interesting that high

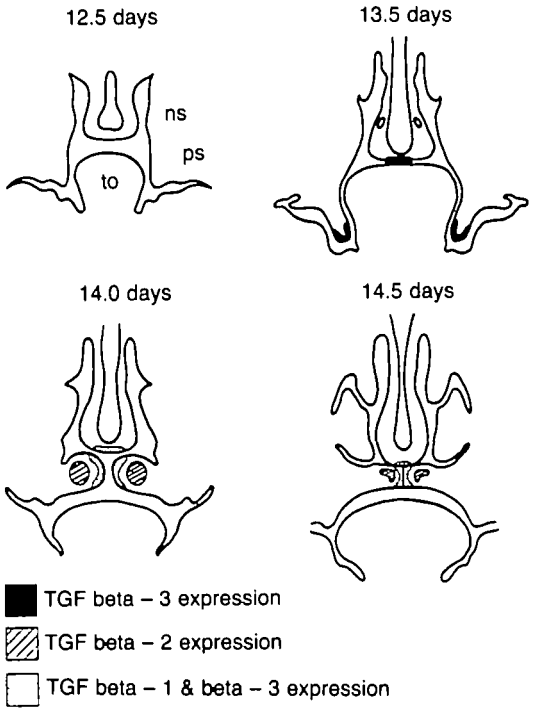

Fig. 5. Diagrammatic representation of the role of the TGF beta family in the developing palate. The line drawings represent coronal sections through the midpalate at the gestations (in days) indicated above the drawings. The diagrams show the palatal shelves (ps) growing down beside the tongue (to) and then elevating to fuse between the tongue and nasal septum (ns). The filled black areas illustrates TGF beta3 expression, diagonal striping for TGF beta2 and stippled areas for the combined expression of TGF beta1 and beta3.

levels of TGF beta3 RNA are observed 24 h prior to palatal shelf elevation.

The distributions of many extracellular matrix proteins are fairly ubiquitous within the palatal mesenchyme. Two significant exceptions are collagen IX and tenascin. Collagen IX appears on the cell surface of medial edge epithelial cells prior to shelf elevation (Ferguson, 1988). Tenascin is localised beneath the medial edge epithelium prior to and during palatal shelf fusion (Sharpe and Ferguson, 1988). Since TGF beta1 is known to induce synthesis of both of these proteins (Sharpe and Ferguson, 1988; Pearson *et al.* 1988), it is a reasonable supposition that these ECM molecules may mediate some of the effects of TGF betas. The distribution of tenascin is particularly significant since the embryonic distribution of this molecule is almost completely correlated with the presence of epithelial TGF beta1 RNA (Chiquet-Ehrismann *et al.* 1986; Lehnert and Akhurst, 1988; Akhurst *et al.* 1990*a*; Sharpe and Ferguson, 1988).

Tenascin can disrupt epithelial sheet continuity by breaking cell–cell and cell–substratum contacts (Chiquet-Ehrismann *et al.* 1989). It also specifically promotes the mobility of neural crest cells *in vitro* (Halfter *et al.* 1989). Both of these events would be necessary for

fusion along the mid-line seam, a time when the epithelial sheet disrupts, epithelial cells transdifferentiate to a mesenchymal cell phenotype and there is much cell mixing.

Terminal differentiation, or 'programmed cell death', plays an equally important role to transdifferentiation in the disruption of the midline epithelial seam (Pratt and Martin, 1975; Greene and Pratt, 1976). Cessation of epithelial DNA synthesis occurs 24 h prior to fusion (Pratt and Martin, 1975). It is accompanied by a down-regulation in epidermal growth factor (EGF) receptors (Abbott *et al.* 1988), and is not dependent on shelf contact *in vitro*. This lethal differentiation is specific to the medial edge epithelium, it is not seen in the oral or nasal components (Pratt and Martin, 1975; Tyler and Koch, 1975). The activation of TGF beta3 gene expression occurs 24 to 36 h prior to fusion and could be important in this growth inhibitory process.

TGFs beta1, beta2 and beta3 are each known to be growth inhibitory to epithelial cells, antagonising the mitogenic activities of TGF alpha and epidermal growth factor (EGF) (Massague, 1985; Like and Massague, 1986; Coffey *et al.* 1988; Grayar *et al.* 1989). It is, however, unlikely that EGF receptor down-regulation (Abbott *et al.* 1988) is directly initiated by TGF betas. Although this is a mechanism of negative growth regulation utilised by endothelial cells (Takehara *et al.* 1987), TGF beta acts distally to the EGF receptor in growth inhibition of all epithelial cells that have been examined (Massague, 1985; Like and Massague, 1986; Coffey *et al.* 1988).

TGF beta2 RNA distribution during palatogenesis is in marked contrast to that of TGFs beta1 and beta3. Its predominant localisation in the mesenchyme would agree with the observations of Pelton *et al.* (1989). It was suggested by Pelton *et al.* that mesenchymal expression of TGF beta2 might be important, not only in modulating the mesenchyme *per se*, but in supporting growth of the overlying epithelium *via* secondary events such as induction of TGF alpha. In this context, it is interesting that the TGF beta2 RNA distribution is asymmetric with respect to the nasal and oral regions. Differential concentrations of growth factors within the mesenchyme could contribute to the generation of regional heterogeneity of the overlying epithelium.

The localised high level expression of TGF beta2 in the hyperplastic nodules of the early medial edge epithelium is consistent with this growth factor acting as an inducible homeostatic regulator of epithelial growth and differentiation. Pelton *et al.* (1989) previously observed the expression of this growth factor in the suprabasal keratinocytes of the embryonic skin at a time when the rate of keratinocyte cell division would be slowing.

One point raised by these RNA localisation studies is the question of why genes encoding proteins with such similar *in vitro* biological activities should be expressed in the same developing organ, in some cases with overlapping but distinct patterns of expression, and in other cases with quite disparate transcript localisations. One explanation of the apparent temporal sequence of expression (TGF beta3 followed by TGF beta1 and beta2) would be the induction of one member of the TGF beta family by another, to amplify specific prefusion biological effects. It is known that TGF beta1 positively regulates its own expression in normal and transformed cells (Van Obberghen-Schilling *et al.* 1988) but little is known about 'cross-talk' between these individual genes. Clearly these different genes must serve some disparate *in vivo* biological functions. This is supported by the fact that some *in vitro* biological activities reported for the TGF betas do show some specificity in isoform requirement (Rosa *et al.* 1988; Jennings *et al.* 1988).

We conclude from this study that all three TGF beta isoforms must play an important role in mammalian palatogenesis. It will be interesting to see how expression of these genes is modified following treatment with teratogens that cause cleft palate, and in mutant mice with increased susceptibility to this deformity.

Thanks to Dr R. Derynck (Genentech) for the full length murine TGF beta1 cDNA probe, to Dr G. Bell (Howard Hughes Institute) for the human TGF beta1 cDNA, to Derek Gatherer and Sigrid Lehnert for instruction and advice on the *in situ* hybridisation technique and to D. Gatherer and F. Millan for quotation of results prior to publication. Thanks to Professor J.M. Connor for help and encouragement. DRF was funded by the NHS. This work was supported by grants from the MRC and the CRC.

References

Abbott, B. D., Adamson, E. D. and Pratt, R. M. (1988). Retinoic acid alters EGF receptor expression during palatogenesis. *Development* **102**, 853–867.

Akhurst, R. J., Lehnert, S. A., Faissner, A. J. and Duffie, E. (1990*a*). TGF beta in murine morphogenetic processes: the early embryo and cardiogenesis. *Development* **108**, 645–656.

Akhurst, R. J., Lehnert, S. A., Gatherer, D. and Duffie, E. (1990*b*). The role of TGF beta in mouse development. *Ann. N.Y. Acad. Sci.* (In Press)

Brinkley, L. L. and Morris Wiman, J. (1987). Effects of chlorcyclizine-induced glycosaminoglycan alterations on patterns of hyaluronate distribution during morphogenesis of the mouse secondary palate. *Development* **100**, 637–640.

Burdett, D. N., Waterfield, J. N. and Shah, R. M. (1988). Vertical development of the secondary palate in the hamster embryos following exposure to 6-mercaptopurine. *Teratology* **37**, 591–598.

Chiquet-Ehrismann, R., Kalla, P. and Pearson, C. A. (1989). Participation of tenascin and transforming growth factor beta in reciprocal epithelial-mesenchymal interactions of MCF7 cells and fibroblasts. *Cancer Res.* **49**, 4322–4325.

Chiquet-Ehrismann, R., Mackie, E. J., Pearson, C. A. and Sakakura, T. (1986). Tenascin: an extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis. *Cell* **47**, 131–139.

Coffey, R. J. Jr, Bascom, C. C., Sipes, N. J., Graves Deal, R., Weissman, B. E. and Moses, H. L. (1988). Selective inhibition of growth-related gene expression in murine keratinocytes by transforming growth factor beta. *Mol. Cell Biol.* **8**, 3088–3093.

Cox, K. H., Deleon, D. V., Angerer, L. M. and Angerer, R. C. (1984). Detection of mRNAs in sea urchin embryos by *in situ* hybridisation using asymmetric RNA probes. *Devl biol.* **101**, 485–502.

Denhez, F., Lafayatis, R., Kondaiah, P., Roberts, A. B. and Sporn, M. B. (1990). Cloning by polymerase chain reaction of a

new mouse TGF beta, mTGF-beta3. *Growth Factors* **00**, 545–572. (In Press)

DERYNCK, R., JARRETT, J. A., CHEN, E. Y. AND GOEDDEL, D. V. (1986). The murine transforming growth factor-beta precursor. *J. biol. Chem.* **261**, 4377–4379.

DERYNCK, R., JARRETT, J. A., CHEN, E. Y., EATON, D. H., BELL, J. R., ASSOIAN, R. K., ROBERTS, A. B., SPORN, M. B. AND GOEDDEL, D. V. (1985). Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells. *Nature* **316**, 701–705.

DERYNCK, R., LINDQUIST, P. B., LEE, A., WEN, D., TAMM, J., GRAYCAR, J. L., RHEE, L., MASON, A. J., MILLER, D. A., COFFEY, R. J., MOSES, H. L. AND CHEN, E. Y. (1988). A new type of transforming growth factor-beta, TGF-beta 3. *EMBO J.* **7**, 3737–3743.

EDWARDS, D. R., MURPHY, G., REYNOLDS, J. J., WHITHAM, S. E., DOCHERTY, A. J., ANGEL, P. AND HEATH, J. K. (1987). Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. *EMBO J.* **6**, 1899–1904.

FERGUSON, M. W. J. (1988). Palate development. *Development* **103 Supplement**, 41–60.

FERGUSON, M. W. J. AND HONIG, L. S. (1984). Epithelial-mesenchymal interactions during vertebrate palatogenesis. *Curr. Top. Dev. Biol.* **19**, 138–164.

FITCH, N. (1957). An embryological analysis of two mutants in the house mouse both producing cleft palate. *J. exp. Zool.* **136**, 329–361.

FITCHETT, J. E. AND HAY, E. D. (1989). Medial edge epithelium transforms to mesenchyme after embryonic palate shelves fuse. *Devl Biol.* **131**, 455–474.

GASSER, D. L., MELE, L., LEES, D. D. AND GOLDMAN, A. S. (1981). Genes in the mouse that affect the susceptibility to cortisone-induced cleft palate are closely linked to Ir genes on chromosomes 2 and 17. *Proc. natn. Acad. Sci. U.S.A.* **78**, 3147–3150.

GRAYAR, J. L., MILLER, D. A., ARRICK, B. A., LYONS, R. M., MOSES, H. L. AND DERYNCK, R. (1989). Human transforming growth factor beta3: Recombinant expression, purification and biological activities in comparison with transforming growth factors beta1 and beta2. *Molecular Endocrinology* **3**, 1977–1986.

GREENE, R. M. AND PRATT, R. M. (1976). Developmental aspects of secondary palate development. *J. Embryol. exp. Morph.* **36**, 225–245.

HALFTER, W., CHIQUET-EHRISMANN, R. AND TUCKER, R. P. (1989). The effect of tenascin and embryonic basal lamina on the behaviour and morphology of neural crest cells *in vitro*. *Devl Biol.* **132**, 14–25.

HEINE, U., MUNOZ, E. F., FLANDERS, K. C., ELLINGSWORTH, L. R., LAM, H. Y., THOMPSON, N. L., ROBERTS, A. B. AND SPORN, M. B. (1987). Role of transforming growth factor-beta in the development of the mouse embryo. *J. Cell Biol.* **105**, 2861–2876.

HIRAKI, Y., INOUE, H., HIRAI, R., KATO, Y. AND SUZUKI, F. (1988). Effect of transforming growth factor beta on cell proliferation and glycosaminoglycan synthesis by rabbit growth-plate chondrocytes in culture. *Biochim. biophys. Acta* **969**, 91–99.

IGNOTZ, R. A. AND MASSAGUE, J. (1985). Type beta transforming growth factor controls the adipogenic differentiation of 3T3 fibroblasts. *Proc. natn. Acad. Sci. U.S.A.* **82**, 8530–8534.

IGNOTZ, R. A. AND MASSAGUE, J. (1986). Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *J. biol. Chem.* **261**, 4337–4345.

JAKOWLEW, S. B., DILLARD, P. J., KONDAIAH, P., SPORN, M. B. AND ROBERTS, A. B. (1988*b*). Complementary deoxyribonucleic acid cloning of a novel transforming growth factor-beta messenger ribonucleic acid from chick embryo chondrocytes. *Mol. Endocrinol.* **2**, 747–755.

JAKOWLEW, S. B., DILLARD, P. J., SPORN, M. B. AND ROBERTS, A. B. (1988*a*). Complementary deoxyribonucleic acid cloning of a messenger ribonucleic acid encoding transforming growth factor beta 4 from chicken embryo chondrocytes. *Mol. Endocrinol.* **2**, 1186–1195.

JENNINGS, J. C., MOHAN, S., LINKHART, T. A., WIDSTROM, R. AND BAYLINK, D. J. (1988). Comparison of the biological actions of TGF beta-1 and TGF beta-2: differential activity in endothelial cells. *J. cell. Physiol.* **137**, 167–172.

KIMELMAN, D. AND KIRSCHNER, M. (1987). Synergistic induction of mesoderm by FGF and TGF-beta and the identification of an mRNA coding for FGF in the early *Xenopus* embryo. *Cell* **51**, 869–877.

KONDAIAH, P., SANDS, M. J., SMITH, J. M., FIELDS, A., ROBERTS, A. B., SPORN, M. B. AND MELTON, D. A. (1990). Identification of a novel transforming growth factor-β (TGF-β5) mRNA in *Xenopus laevis*. *J. biol. Chem.* **265**, 1089–1093.

LEHNERT, S. A. AND AKHURST, R. J. (1988). Embryonic expression pattern of TGF beta type-1 RNA suggests both paracrine and autocrine mechanisms of action. *Development* **104**, 263–273.

LIKE, B. AND MASSAGUE, J. (1986). The antiproliferative effect of type beta transforming growth factor occurs at a level distal from receptors for growth-activating factors. *J. biol. Chem.* **261**, 13 426–13 429.

LUND, L. R., RICCIO, A., ANDREASEN, P. A., NIELSEN, L. S., KRISTENSEN, P., SAKSELA, O., BLASI, F. AND DANO, K. (1987). Transforming growth factor-beta is a strong and fast acting positive regulator of the level of type-1 plasminogen activator inhibitor mRNA in WI-38 human lung fibroblasts. *EMBO J.* **6**, 1281–1286.

MADISEN, L., WEBB, N. R., ROSE, T. M., MARQUARDT, H., IKEDA, T., TWARDZIK, D., SEYEDIN, S. AND PURCHIO, A. F. (1988). Transforming growth factor-beta 2: cDNA cloning and sequence analysis. *DNA* **7**, 1–8.

MASSAGUE, J. (1985). Transforming growth factor-beta modulates the high-affinity receptors for epidermal growth factor and transforming growth factor-alpha. *J. Cell Biol.* **100**, 1508–1514.

MASSAGUE, J., CHEIFETZ, S., ENDO, T. AND NADAL GINARD, B. (1986). Type beta transforming growth factor is an inhibitor of myogenic differentiation. *Proc. natn. Acad. Sci. U.S.A.* **83**, 8206–8210.

MASUI, T., WAKEFIELD, L. M., LECHNER, J. F., LAVECK, M. A., SPORN, M. B. AND HARRIS, C. C. (1986). Type beta transforming growth factor is the primary differentiation-inducing serum factor for normal human bronchial epithelial cells. *Proc. natn. Acad. Sci. U.S.A.* **83**, 2438–2442.

MILLER, D. A., LEE, A., MATSUI, Y., CHEN, E. Y., MOSES, H. L. AND DERYNCK, R. (1989*a*). Complementary DNA cloning of murine transforming growth factor beta3 (TGF beta3) precursor and the comparative expression of TGF beta1 and TGF beta3 and TGF beta1 in murine embryos and adult tissues. *Molecular Endocrinology* **3**, 1926–1934.

MILLER, D. A., LEE, A., PELTON, R. W., CHEN, E. Y., MOSES, H. L. AND DERYNCK, R. (1989*b*). Murine transforming growth factor-β2 cDNA sequence and expression in adult tissues and embryos. *Molecular Endocrinology* **3**, 1108–1114.

MORRISS, G. M. (1973). The ultrastructural effects of excess vitamin A on the primitive streak stage rat embryo. *J. Embryol. exp. Morph.* **30**, 219–242.

MOSES, H. L., BRANUM, E. L., PROPER, J. A. AND ROBINSON, R. A. (1981). Transforminng growth factor production by chemically transformed cells. *Cancer Res.* **41**, 2842–2848.

MOSES, H. L., TUCKER, R. F., LEOF, E. B., COFFEY, R. J., HALPER, J. AND SHIPLEY, G. D. (1985). Type beta transforming growth factor is a growth stimulator and a growth inhibitor. In *Cancer Cells* (ed. J. Feramisco, B. Ozanne and C. Stiles) pp. 65–71. Cold Spring Harbor Laboratory.

PEARSON, C. A., PEARSON, D., SHIBAHARA, S. AND HOFSTEENGE, J. (1988). Tenascin: cDNA cloning and induction by TGF-beta. *EMBO J.* **7**, 2977–2982.

PELTON, R. W., NOMURA, S., MOSES, H. L. AND HOGAN, B. L. M. (1989). Expression of transforming growth factor beta-2 RNA during murine embryogenesis. *Development* **106**, 759–767.

POSTLETHWAITE, A. E., KESKI OJA, J., MOSES, H. L. AND KANG, A. H. (1987). Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor beta. *J. exp. Med.* **165**, 251–256.

PRATT, R. M., GOGGINS, J. F., WILK, A. L. AND KING, C. T. (1973). Acid mucopolysaccharide synthesis in the secondary

palate of the developing rat at the time of rotation and fusion. *Devl Biol.* **32**, 230–237.

PRATT, R. M. AND MARTIN, G. R. (1975). Epithelial cell death and cyclic AMP increase during palatal development. *Proc. natn. Acad. Sci. U.S.A.* **72**, 874–877.

ROBERTS, A. B., ANZANO, M. A., LAMB, L. C., SMITH, J. M. AND SPORN, M. B. (1981). New class of transforming growth factors potentiated by epidermal growth factor. *Proc. natn. Acad. Sci. U.S.A.* **78**, 5339–5343.

ROBERTS, A. B. AND SPORN, M. B. (1990). The transforming growth factor betas. *Handbook of Experimental Pharmacology* (In Press).

ROBERTS, A. B., SPORN, M. B., ASSOIAN, R. K., SMITH, J. M., ROCHE, N. S., WAKEFIELD, L. M., HEINE, U. I., LIOTTA, L. A., FALANGA, V., KEHRL, J. H. AND ET, A. L. (1986). Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis *in vivo* and stimulation of collagen formation *in vitro*. *Proc. natn. Acad. Sci. U.S.A.* **83**, 4167–4171.

ROSA, F., ROBERTS, A. B., DANIELPOUR, D., DART, L. L., SPORN, M. B. AND DAWID, I. B. (1988). Mesoderm induction in amphibians: the role of TGF-beta 2-like factors. *Science* **239**, 783–785.

SAIKI, R. K., GELFAND, D. H., STOFFEL, S., SCHARF, S. J., HIGUCHI, R., HORN, G. T., MULLIS, K. B. AND ERLICH, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487–491.

SEYEDIN, S. M., SEGARINI, P. R., ROSEN, D. M., THOMPSON, A. Y. AND BENTZ, H. (1987). Cartilage-inducing factor-B is a unique protein structurally and functionally related to transforming growth factor-beta. *J. biol. Chem.* **262**, 1946–1949.

SEYEDIN, S. M., THOMPSON, A. Y., BENTZ, H., ROSEN, D. M., MCPHERSON, J. M., SIEGEL, N. R., GALLUPPI, G. R. AND PIEZ, K. A. (1986). Cartilage-inducing factor-A. Apparent identity to transforming growth factor-beta. *J. biol. Chem.* **261**, 5693–5695.

SHARPE, P. M. AND FERGUSON, M. W. J. (1988). Mesenchymal influences on epithelial differentiation in developing systems. *J. Cell Sci. Suppl.* **10**, 195–230.

SPORN, M. B., ROBERTS, A. B., WAKEFIELD, L. M. AND ASSOIAN, R. K. (1986). Transforming growth factor-beta: biological function and chemical structure. *Science* **233**, 532–534.

TAKEHARA, K., LEROY, E. C. AND GROTENDORST, G. R. (1987). TGF-beta inhibition of endothelial cell proliferation: alteration of EGF binding and EGF-induced growth-regulatory (competence) gene expression. *Cell* **49**, 415–422.

TEN-DIJKE, P., HANSEN, P., IWATA, K. K., PIELER, C. AND FOULKES, J. G. (1988). Identification of another member of the transforming growth factor type beta gene family. *Proc. natn. Acad. Sci. U.S.A.* **85**, 4715–4719.

THOMPSON, J. S. AND THOMPSON, M. W. (1986). Multifactorial Inheritance. In *Genetics in Medicine* (ed. W. B. Saunders), pp. 210–225. Philadelphia.

TYLER, M. S. AND KOCH, W. E. (1975). *In vitro* development of palatal tissues from embryonic mice. I. Differentiation of the secondary palate. *Anat. Rec.* **182**, 297–301.

VAN OBBERGHEN-SCHILLING, E., ROCHE, N. S., FLANDERS, K. C., SPORN, M. B. AND ROBERTS, A. B. (1988). Transforming growth factor beta-1 positively regulates its own expression in normal and transformed cells. *J. biol. Chem.* **263**, 7741–7746.

WILKINSON, D. J., BAILES, J. A. AND MCMAHON, A. P. (1987). Expression of the proto-oncogene *int-1* is restricted to specific neural cells in the developing mouse embryo. *Cell* **50**, 79–88.

(*Accepted 6 April 1990*)