Development 127, 217-224 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV2450

Independent regulation of *Dlx2* expression in the epithelium and mesenchyme of the first branchial arch

Bethan L. Thomas[1,2,*], Jen Kuei Liu[3,‡], John L. R. Rubenstein[3] and Paul T. Sharpe[1]

[1]Department of Craniofacial Development and [2]Department of Oral and Maxillofacial Surgery, GKT Dental Institute, King's College, University of London, London, SE1 9RT, UK
[3]Nina Ireland Laboratory of Developmental Neurobiology, UCSF, CA 94143-0984, USA
*Author for correspondence (e-mail: bthomas@hgmp.mrc.ac.uk)
‡Present address: Sentinel Biosciences, 855 California Ave., Suite C, Palo Alto, CA 9430, USA

Accepted 26 October; published on WWW 20 December 1999

SUMMARY

***Dlx2*, a member of the *distal-less* gene family, is expressed in the first branchial arch, prior to the initiation of tooth development, in distinct, non-overlapping domains in the mesenchyme and the epithelium. In the mesenchyme *Dlx2* is expressed proximally, whereas in oral epithelium it is expressed distally. *Dlx2* has been shown to be involved in the patterning of the murine dentition, since loss of function of *Dlx1* and *Dlx2* results in early failure of development of upper molar teeth. We have investigated the regulation of *Dlx2* expression to determine how the early epithelial and mesenchymal expression boundaries are maintained, to help to understand the role of these distinct expression domains in patterning of the dentition.**

Transgenic mice produced with a *lacZ* reporter construct, containing 3.8 kb upstream sequence of *Dlx2*, led to the mapping of regulatory regions driving epithelial but not mesenchymal expression in the first branchial arch. We show that the epithelial expression of *Dlx2* is regulated by planar signalling by BMP4, which is coexpressed in distal oral epithelium. Mesenchymal expression is regulated by a different mechanism involving FGF8, which is expressed in the overlying epithelium. FGF8 also inhibits expression of *Dlx2* in the epithelium by a signalling pathway that requires the mesenchyme. Thus, the signalling molecules BMP4 and FGF8 provide the mechanism for maintaining the strict epithelial and mesenchymal expression domains of *Dlx2* in the first arch.

Key words: *Dlx2*, homeobox, branchial arch, mandible, regulation, mouse

INTRODUCTION

The *distal-less* family of homeobox genes contains six members, *Dlx1*, *Dlx2*, *Dlx3*, *Dlx5*, *Dlx6* and *Dlx7*, arranged in convergent pairs within the genome of mammals. Both genes of a given pair (*Dlx1* and *Dlx2*, *Dlx3* and *Dlx7*, *Dlx5* and *6*) have been shown to have similar domains of expression (Qiu et al., 1997). *Dlx1* and *Dlx2* are expressed in the epithelium and mesenchyme of the maxillary and mandibular divisions of the first branchial arch in similar domains. Likewise, *Dlx5* and *Dlx6* share very similar expression domains and their expression patterns in the mandibular division of the first branchial arch are similar to those of *Dlx1* and *Dlx2*, although they are not expressed in the maxillary division of the first arch (Qiu et al., 1997).

The *Dlx* genes had been suggested to be involved in a homeobox code patterning the ectomesenchyme of the first arch with respect to tooth development (Sharpe, 1995). Loss of function of *Dlx1* and *Dlx2* results in failure of development of upper molars (Thomas et al., 1997), supporting their role in odontogenic patterning. The effect on the upper jaw only, with mandibular molars developing normally, has been explained by the expression of other *Dlx* genes, such as *Dlx5* and *Dlx6* in the mandible, compensating for the loss of *Dlx1* and *Dlx2* in mandibular molar development (Qiu et al., 1997; Thomas et al., 1997).

Prior to the initiation of tooth development, *Dlx1* and *Dlx2* are expressed in the proximal mesenchyme of both the maxillary and mandibular divisions of the first arch (Thomas and Sharpe, 1998). This mesenchyme contributes to the development of molar teeth. At this stage the proximal epithelium does not express these *Dlx* genes, but epithelial expression is present towards the distal tips of the maxillary and mandibular divisions of the first arch, that is, in epithelium not overlying *Dlx1* and *Dlx2* expressing mesenchyme. Thus, expression of these two genes resides in distinct domains in the epithelium and mesenchyme; their domains of expression abut approximately midway along the arches (Bulfone et al., 1993; Qiu et al., 1997; Thomas and Sharpe, 1998) (Fig. 1F-I).

This early expression of *Dlx2* in the mesenchyme in the proximal, future molar regions is consistent with a role for the *Dlx* genes in dental patterning resulting from its mesenchymal expression domain, as predicted in the odontogenic homeobox code (Thomas and Sharpe, 1998; Sharpe, 1995). This has been confirmed by tissue recombination experiments using *Dlx1/Dlx2* mutant mice in which mutant epithelium

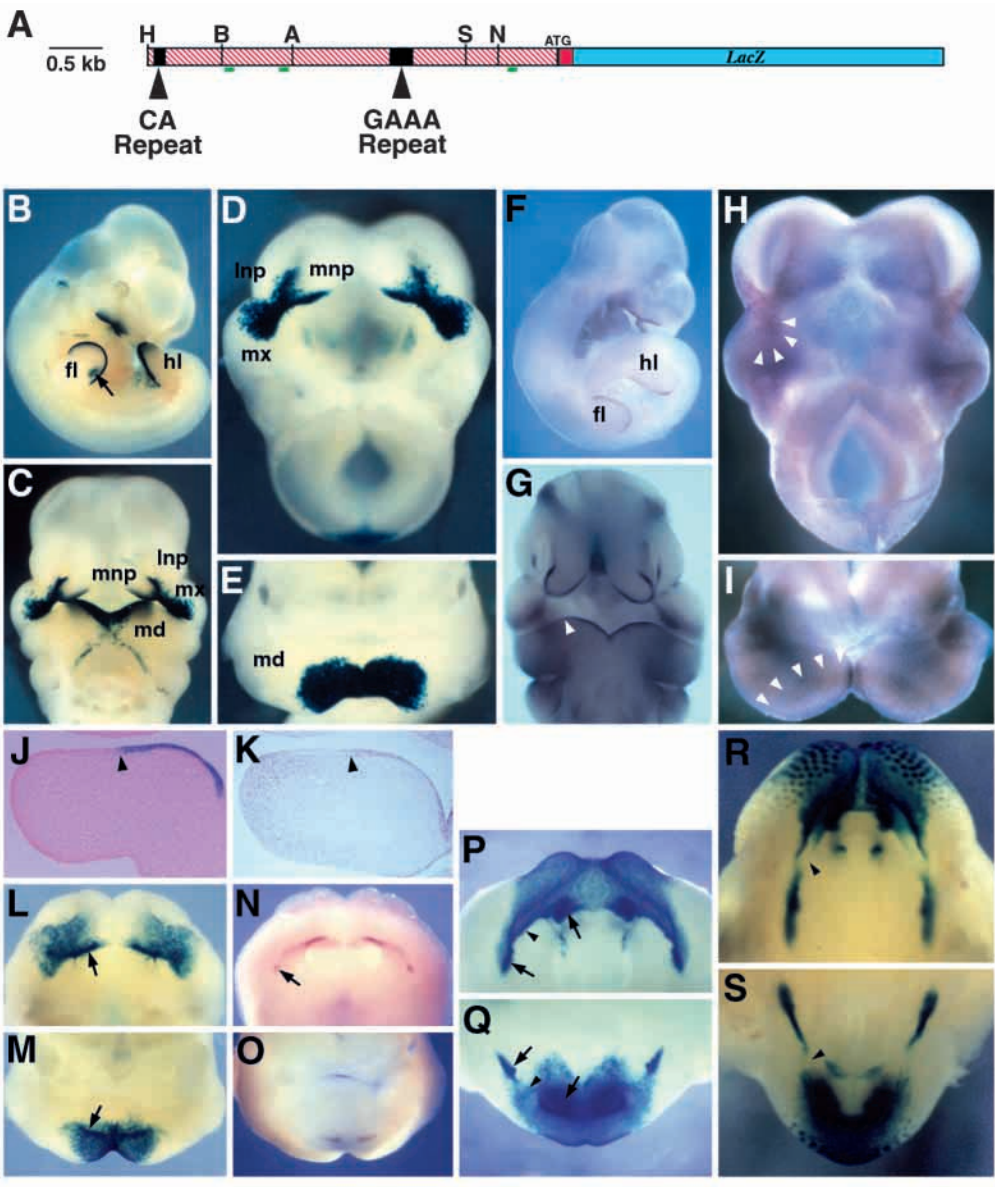

Fig. 1. Expression of the *Dlx2-lacZ* transgene during embryogenesis. (A) Diagram representing the construct used to produce *lacZ* transgenic mice. Restriction sites used to produce the deleted constructs for production of transient transgenic embryos are shown; H, *Hin*dIII; B, *Bgl*II; A, *Apa*I; S, *Spe*I; N, *Not*I. Positions of putative Smad binding sites (Jonk et al., 1998) are indicated by green dashes. (B-E) *LacZ* expression in an E10.5 embryo showing well defined domains in the oral epithelium of the maxilla, medial and lateral nasal processes (D) and mandible (E), as well as in the apical ectodermal ridge (AER) of the fore- and hindlimb buds, and also in a small area of mesenchyme on the posterior aspect of the limb buds indicated by an arrow (B). (F-I) *Dlx2* endogenous expression identified by in situ hybridisation showing the mesenchymal and epithelial expression domains in the maxillary division (H) and mandibular division (I) of the first branchial arch, and also in the limb buds (F). (J,K) Frontal sections of E10.5 embryos showing the limit of *lacZ* expression correlating with that of the endogenous gene expression at the border with the expression domain in the mesenchyme. (L,M,P-S) *Dlx2-lacZ* transgene expression and (N,O) *Shh* expression in epithelium on the oral aspect of the maxilla (L,N,P,R) and mandible (M,O,Q,S) of E11.5 (L-O), E13.5 (P,Q) and E15.5 (R,S) embryos. The invaginating epithelium of the incisor tooth germs (L,M,P,Q) and first molar tooth germs (N,P,Q) are indicated by arrows. Large arrowheads in H-K indicate the limits of epithelial expression in the mandible and maxilla. Small arrowheads in P-S indicate the initial *lacZ* expression in the epithelial thickening of the diastema region (P,Q), which is subsequently downregulated (R,S). md, mandibular division of first branchial arch; mx, maxillary division of first branchial arch; mnp, medial nasal process; lnp, lateral nasal process; fl, forelimb bud; hl, hindlimb bud.

recombined with wild-type mesenchyme enabled tooth development to proceed, whereas in the converse experiment in which mutant mesenchyme was recombined with wild-type epithelium, tooth development did not proceed beyond the epithelial thickening (Thomas et al., 1997). It would seem likely that given this role for *Dlx2* in determining molar tooth development it would be imperative that it's mesenchymal domain is restricted to the proximal region.

Recently it has been shown that misexpression of another proximally restricted homeobox gene, *Barx1*, in the distal mesenchyme results in transformation of incisors into molars (Tucker et al., 1998a). Interestingly, *Barx1* expression is specifically lost in maxillary molar odontogenic mesenchyme in *Dlx1/Dlx2* mutant mice, implying that *Dlx1* and *Dlx2* lie upstream of *Barx1* transcription (Thomas et al., 1997). It is not clear how the *Dlx* genes and *Barx1* are maintained in a proximally restricted pattern in the mesenchyme and thus maintain the dental pattern. The sharp boundary in expression from epithelium to mesenchyme of *Dlx2* suggests that this junction may represent a boundary that is not visible morphologically but may be responsible for determining the proximal molar region and the distal incisor region; thus, an understanding of the regulation of this junction is important for understanding regulation of the dental pattern.

While studying the regulation of mouse *Dlx2* expression, we found that a 3.8 kb genomic fragment upstream of the *Dlx2* coding region could drive expression of a *lacZ* reporter gene in a pattern identical to the ectodermal expression of *Dlx2* in the first branchial arch. We subsequently produced deletion constructs to map the locations of regulatory regions directing expression to the different domains. Using these transgenics we found that the BMP and FGF signalling pathways control

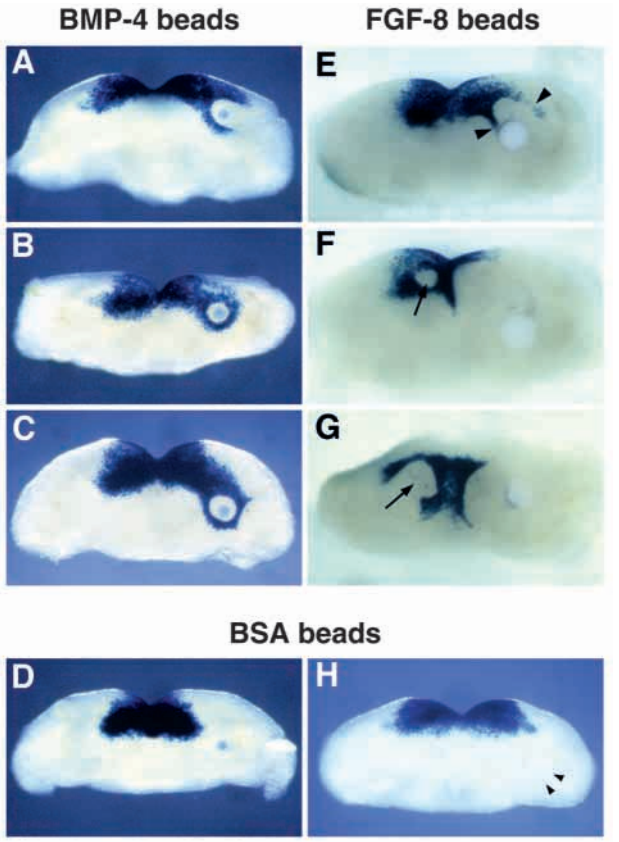

Fig. 2. Effects of BMP4 and FGF8 on expression of *Dlx2* epithelial expression. β-Gal staining showing expression of *lacZ* in E10.5 mandibles cultured for 20 hours. (A-C) Oral epithelium showing varying degrees of induction of *lacZ* expression by BMP4. (E-G) Oral epithelium showing repression of *lacZ* expression by FGF8. (F,G) More extensive effect with loss of expression seen on the contralateral side indicated by arrows. (D,H) Oral epithelium showing no change in expression pattern following application of BSA beads. Small arrowheads indicate the position of the bead. (G) Mandible has rotated so that the most ventral (whisker pad) epithelium is fully visible.

expression in the epithelium and in the mesenchyme, and propose a mechanism by which the expression in both tissues is maintained in mutually exclusive domains.

MATERIALS AND METHODS

Construction of *Dlx2-lacZ* reporter construct

A *Dlx2* genomic clone, obtained from a genomic library from strain 129 mice (Qiu et al., 1995), was used to clone a fragment directly upstream of the *Hin*cII site in the first exon, in-frame with *lacZ*. Release from the vector using *Hin*dIII produced a construct in which 3.77 kb upstream of the translation start site, including the endogenous *Dlx2* promotor, directs expression of a Dlx2-lacZ fusion protein, containing 132 amino acids of Dlx2. This construct was used to produce transgenic mice by microinjection, at a concentration of 4 ng/μl, into one-cell embryos derived from superovulated FVB/N females and transfer of eggs into pseudopregnant females (B6/CBA).

Further transgenic mice were produced using the same construct but with progressive deletions from the 5′ end using suitable restriction sites: *Bgl*II deleting 675 bp, *Apa*I deleting 1.33 kb, *Spe*I deleting 2.8 kb and *Not*I deleting 3.24 kb.

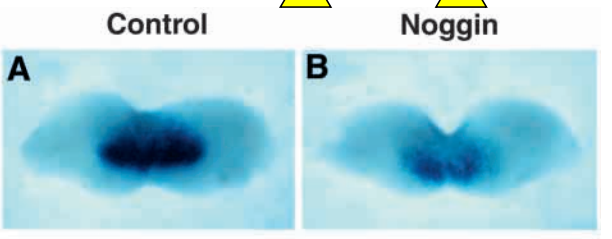

Fig. 3. Noggin represses *Dlx2* epithelial expression. β-Gal staining showing expression of *Dlx2* in E10.5 mandibles cultured for 20 hours. Oral epithelium showing that Noggin represses expression of *Dlx2* (B) when compared to expression in control cultures (A).

Analysis of transgenics

Embryos, from both transient transgenics and transgenic lines, were harvested in PBS and assayed for β-gal activity by staining with X-Gal (Sanes et al., 1986).

Genotyping was performed using Southern blots with DNA obtained from the bodies of embryos (transient transgenics) or tail snips (from mice bred to generate lines).

In situ hybridisation

Whole-mount in situ hybridisation was performed as previously described by Wilkinson (1992). The *Dlx2* antisense probe was transcribed using T3 RNA polymerase from a mouse cDNA that had been linearised with *Eco*RI.

In vitro culture

Cultures were carried out using embryos harvested between embryonic day (E)10 and E10.5. Mandibles were dissected out in D-MEM. For experiments requiring either epithelium or mesenchyme alone, the tissues were separated by incubating in Dispase at 2 U/ml in calcium- and magnesium-free phosphate-buffered saline (PBS). Following incubation at 37°C for 10 minutes the tissues were separated by careful dissection and the tissue required was washed in D-MEM with 10% fetal calf serum. Epithelium for culture was placed on small droplets of set Matrigel (Collaborative Biomedical Products). Tissue was placed on membrane filters supported by metal grids according to the modified Trowel technique (Saxén, 1966) and beads were placed as required.

BMP4 beads were prepared using BMP4 (R&D Systems) at 100 μg/μl on Affi-Gel-blue beads (Bio-Rad). The beads were washed and dried, then incubated in the protein at 37°C for 1 hour. FGF8 beads were prepared using FGF8 (R&D Systems) at 100 μg/μl on heparin acrylic beads (Sigma). The beads were washed in PBS and incubated overnight in the protein. BSA control beads (Affi-Gel-blue and heparin acrylic beads) were made in a similar manner. Beads were stored for up to 2 weeks at 4°C.

Noggin was obtained by transfecting chicken 0-line embryonic fibroblasts with an RCAS Noggin retrovirus, culturing the cells for 7 days, replacing the medium and collecting the Noggin-containing supernatant 24 hours later. As a control, cells were transfected with an RCAS GFP retrovirus and cultured in parallel. Noggin or control supernatant was used in place of D-MEM/10% fetal calf serum to culture whole mandibles as described above.

Following 18 hours in culture the explants were prepared for in situ hybridisation or X-Gal staining.

RESULTS

Expression of *Dlx2* in the first branchial arch

Regulation of *Dlx2* was examined by producing transgenic mice carrying a *lacZ* reporter construct in which an upstream

region of mouse *Dlx2*, as well as part of the first exon, were cloned in-frame upstream of *lacZ*. Expression of this construct was examined in first arch tissues of E10.5 embryos. The construct, containing 3.77 kb upstream from the ATG start site, produced expression of *lacZ* in the epithelial domain of the first branchial arch, but not in the mesenchymal domain (Fig. 1B-E). This pattern was seen in four separate transgenic lines as well as four transient transgenic embryos examined.

The expression in the epithelium of the mandibular division of the first arch was present as a large domain at the distal aspect, with distinct limits on the dorsal and proximal extents on the oral surface (Fig. 1E). Expression was also present in the distal region of the maxillary division of the first arch and extended as projections around the medial and lateral nasal processes (Fig. 1D). Expression in these areas correlated precisely with that of endogenous *Dlx2* epithelial expression, as revealed by comparison with both whole-mount in situ hybridisations (Fig. 1F-I) and sections of in situ hybridisations (Fig. 1K) in which the epithelial expression is found to terminate at the midpoint of the arch, at the site where it abuts the mesenchymal expression of the proximal domain.

Expression of this transgene was examined through various stages of first arch development using the lines produced. Expression was first detected at E9.0 in a small patch of cells at the very distal extent of the first branchial arch (data not shown). Expression extended proximally from this patch during the following 36 hours to the pattern described above at E10.5. From E11.5 onwards, the time at which patterning events of the dentition are superceded by cytodifferentiation events, expression of the *Dlx2-lacZ* reporter construct extended proximally (Fig. 1L,M,P-S). Expression was detected in a band of thickened epithelium within which the tooth germs will form. This band included the dental lamina of the diastema (arrowheads in Fig. 1P,Q), although this subsequently lost expression of *Dlx2* (arrowheads in Fig. 1R,S). *Shh*, an early marker for the dental lamina of individual tooth germs, was found to be expressed in epithelium that is simultaneously expressing *Dlx2* (Fig. 1N,O) at the sites of development of the future incisor and molar tooth germs. This pattern of expression suggests that *Dlx2* may be part of a signal responsible for determining the dental epithelium of the oral cavity.

Localisation of epithelial regulatory elements

To map the regulatory domains within the *Dlx2* promotor region, deletions of the *lacZ* construct from the 5′ end were carried out using appropriate restriction enzymes and used to produce transient transgenic embryos. Transient transgenic embryos produced using restrictions as far as the *Apa*I site expressed *lacZ* in exactly the same pattern as produced using the entire construct (data not shown); deletion down to the *Spe*I or *Not*I sites resulted in loss of all first arch epithelial expression (data not shown), thus identifying a 1.47 kb sequence containing the necessary elements to direct expression to the first arch epithelium (Fig. 1A). This region was located 0.97 kb upstream of the ATG start site of *Dlx2*.

Sequencing of the 1.47 kb region revealed a 200 bp GAAA repeat (not shown). Interestingly, three potential Smad binding sites (Jonk et al., 1998) are present in the 3.77 kb sequence. Two of these sites are located within the region between the *Bgl*II and the *Apa*I site, and the third is located between the *Not*I site and the translation start site (Fig. 1A). Although these potential Smad binding sites maybe involved in a Bmp signalling pathway, deletion of two of the sites in the *Apa*I transient embryos did not abolish expression, and deletion down to the *Spe*I site, thus maintaining the third Smad binding site, did not support *lacZ* expression. It therefore seems unlikely that these sites represent the major enhancer elements responsible for expression of *Dlx2* in the epithelium.

Regulation of *Dlx2* in the first branchial arch epithelium

We studied the ability of candidate secreted proteins to spatially regulate *Dlx2* via this specific epithelial regulatory domain in order to test how its expression may be regulated in vivo. Several signalling molecules are expressed in the first arch, including Sonic hedgehog (Hardcastle et al., 1998), BMPs (Vainio et al., 1993; Tucker et al., 1998a,b), activin (Ferguson et al., 1998), FGFs (Grigoriou et al., 1998; Tucker et al., 1999) and Wnts (Parr et al., 1993; Christiansen et al., 1995; Wang and Shackleford, 1996).

Bmp4 is expressed in the epithelium of the first branchial arch at E10.5 in a domain coincident with the *Dlx2* epithelial expression (Tucker et al., 1998b). Since BMP4 is known to regulate other homeobox genes in the first arch (Vainio et al., 1994; Tucker et al., 1998a) it seemed a likely candidate to play a role in the regulation of *Dlx2*. *Fgf8* is expressed in patches of proximal oral epithelium of the first arch overlying the *Dlx2* mesenchymal expression domain (Grigoriou et al., 1998; Tucker et al., 1999). FGF8 has also been shown to regulate homeobox gene expression in the first arch (Grigoriou et al., 1998; Tucker et al., 1999). The temporo-spatial patterns of expression of *Shh* and *Activin βA* are not consistent with a role in regulating *Dlx2* expression in epithelium or mesenchyme at the stages examined in this study (Hardcastle et al., 1998; Ferguson et al., 1998; Dassule and McMahon, 1998).

BMP4 and FGF8 were therefore the most likely candidates to regulate *Dlx2* expression in the first arch. BMP4 soaked beads placed on the mandible, laterally, outside the *Dlx2* epithelial expression domain resulted in ectopic expression of *lacZ* around the bead (Fig. 2A-C). In all cases the epithelium directly around the bead did not express *lacZ*, resulting in a translucent zone similar to those seen in mesenchyme explants following application of ectopic protein (Vainio et al., 1994), and also observed in the epithelium of whole mandible cultures by other workers (I. Thesleff, personal communication).

In contrast, addition of FGF8 soaked beads placed at the edge of the *Dlx2* epithelial expression domain resulted in loss of *lacZ* expression in the epithelium directly around the bead (Fig. 2E) and in some cases also resulted in loss of expression on the contralateral side (Fig. 2F). Loss of expression on the contralateral side was seen when there was a large area of loss of expression on the ipsilateral side (Fig. 2G). In some cultures ectopic expression of *lacZ* could be seen around this *lacZ* negative epithelium (Fig. 2E, arrowheads). FGFs bound to heparin-coated beads are known to diffuse a distance of only a few cell diameters away from the beads (Storey et al., 1998) so it is unlikely that the long-range effects of FGF8 on *Dlx2* expression were due to diffusion of the protein. Similar long-range effects of FGF8 have been observed in regulating mesenchymal gene expression in the mandible (Tucker et al., 1999), although the mechanism is not yet known.

BSA soaked beads were used in parallel cultures for each

experiment and showed no change in the *lacZ* expression domain (Fig. 2D,H). These results suggested that *Dlx2* epithelial expression is induced by BMP4 and repressed by FGF8 in the first arch. Additionally, culture of whole mandibles in the presence of Noggin protein resulted in inhibition of *lacZ* expression (Fig. 3B) compared to control cultures (Fig. 3A), suggesting that BMP4 is the endogenous inducer of *Dlx2* expression in this epithelium.

To determine whether signalling by BMP4 and FGF8 occurred within the epithelium or required mesenchyme, epithelium was separated from mandibular mesenchyme at E10.5 and cultured on Matrigel for 18 hours. The effects of addition of BMP4 and FGF8 beads were compared separately with control cultures since β-galactosidase activity remained in the epithelium following separation from the mesenchyme. This was most probably due to the presence of BMP4 protein in the epithelium and the stability of β-galactosidase. Addition of BMP4 beads to epithelial cultures produced an increase in *Dlx2* expression compared with controls (Fig. 4A,C). Cultures treated with FGF8 beads showed no effect on the expression of *Dlx2* compared with controls (Fig. 4B,C). This was in contrast to the results seen following culture of whole mandibles, where FGF8 repressed *Dlx2* expression in the epithelium (Fig. 2E-G). This suggests that the induction of *Dlx2* by BMP4 can occur directly within the epithelium, whereas the repression by FGF8 cannot, and presumably requires the presence of mesenchyme. Interestingly, neither protein produced translucent zones seen in the whole mandible cultures, suggesting that the translucent zone is a feature of the presence of mesenchymal cells; one possibility is that it is formed as a result of local expression of signalling inhibitors that are known to be expressed in mesenchymal cells (P. T. S., unpublished data).

Regulation of *Dlx2* in the mesenchyme of the first branchial arch

Dlx2 epithelial and mesenchymal expression abut in non-overlapping domains (Fig. 1F-I). *Fgf8* expression overlies the mesenchymal domain (Tucker et al., 1999) and *Bmp4* expression coincides with the epithelial domain (Tucker et al., 1998a,b). Having shown that these signalling molecules have repressing and inducing properties that could reflect their roles in maintaining the epithelial expression domain of *Dlx2*, we investigated whether they may also play a role in the mesenchymal expression of *Dlx2*. Mesenchyme from E10.5 mandibles was cultured in the presence of either BMP4 or FGF8 soaked beads. Following culture for 20 hours *Dlx2* expression was examined by in situ hybridisation. Removal of the epithelium resulted in loss of *Dlx2* expression in the mesenchyme (Fig. 4D,E), as reported with other homeobox genes (Vainio et al., 1993; Grigoriou et al., 1998). FGF8 was found to induce expression of *Dlx2* in a localised area around the bead (Fig. 4E). BMP4 did not induce *Dlx2* expression in the mesenchyme (Fig. 4D). This finding is in contrast to previous reports, which have shown that BMP4 will induce *Dlx2* in first arch mesenchyme at later stages of development (Bei and Maas, 1998).

DISCUSSION

Dlx genes are expressed in the epithelium and mesenchyme of the branchial arches and limb buds. Each *Dlx* gene pair studied to date has shown almost identical expression patterns. Examining the function of *Dlx* genes has proved complicated by functional redundancy between the pairs (Qiu et al., 1995, 1997). Mutation of either *Dlx1* or *Dlx2* results in craniofacial skeletal abnormalities, but tooth development is unaffected. Mice mutant for *Dlx1* and *Dlx2* exhibit arrest of upper molar tooth development at the earliest stage of tooth development, that is as the epithelial thickening forms, indicating complete functional redundancy for tooth patterning. Expression patterns at the time of arrest and tissue recombination experiments suggest that the functionally important domain of *Dlx* expression for tooth patterning is the mesenchymal expression domain (Thomas et al., 1997). In the mandibular primordia, *Dlx5* and *Dlx6* are expressed in almost identical mesenchymal domains to *Dlx1* and *Dlx2* (Qiu et al., 1997) and have been suggested to compensate for loss of *Dlx1* and *Dlx2*, enabling mandibular molar tooth development to proceed normally (Thomas et al., 1997).

The function of the epithelial expression of *Dlx* genes in the maxilla and mandible has not, however, been established. In the mandible, *Dlx5* and *Dlx6* are expressed in the same epithelium as *Dlx1* and *Dlx2*, and *Dlx3* is expressed in the epithelium of the maxilla (Qiu et al., 1997). It is possible therefore, that these other *Dlx* genes compensate for loss of *Dlx1* and *Dlx2* in the epithelium in the mutant mice, therefore masking any potential role of the *Dlx1* and *Dlx2* epithelial domain.

It is clear from in situ expression studies that the boundaries of the epithelial and mesenchymal *Dlx2* expression domains abut at the early stages, prior to the initiation of tooth development. *Dlx2* expression in the epithelium overlaps with that of *Bmp4*, suggesting that this epithelial domain defines a distinct distal territory. Since the mesenchymal domain appears to be important for the patterning of the dentition the coordinated regulation of the epithelial and mesenchymal domains may function to restrict the future molar mesenchyme to the proximal domain.

It has been believed for some time that the epithelium patterns the site of tooth formation, but tooth shape is determined by the mesenchyme in which that tooth develops (Lumsden, 1988). Recent work has shown that, as predicted by the odontogenic homeobox code (Sharpe, 1995; Thomas and Sharpe, 1998), the molecular composition of the mesenchyme determines tooth shape (Tucker et al., 1999). Highly restricted expression of *Barx1* to proximal mesenchyme in presumptive molar regions was found to be regulated by antagonistic interactions between FGF8 and BMP4 secreted from overlying epithelium (Tucker et al., 1998a). Inhibition of BMP action by Noggin resulted in loss of the BMP4 repressive signal and, consequently, misexpression of *Barx1* in more distal presumptive incisor mesenchyme. The outcome of the misexpression was the transformation of incisor teeth into molars. Significantly, *Dlx2* expression in Noggin-treated mandibular arch explants was not affected, suggesting that restriction of *Dlx2* expression to proximal mesenchyme is regulated differently to *Barx1* (P. T. S., unpublished data).

To investigate the regulation of *Dlx2* expression in the first branchial arch, and to identify the relationship between the distinct epithelial and mesenchymal domains of *Dlx2*

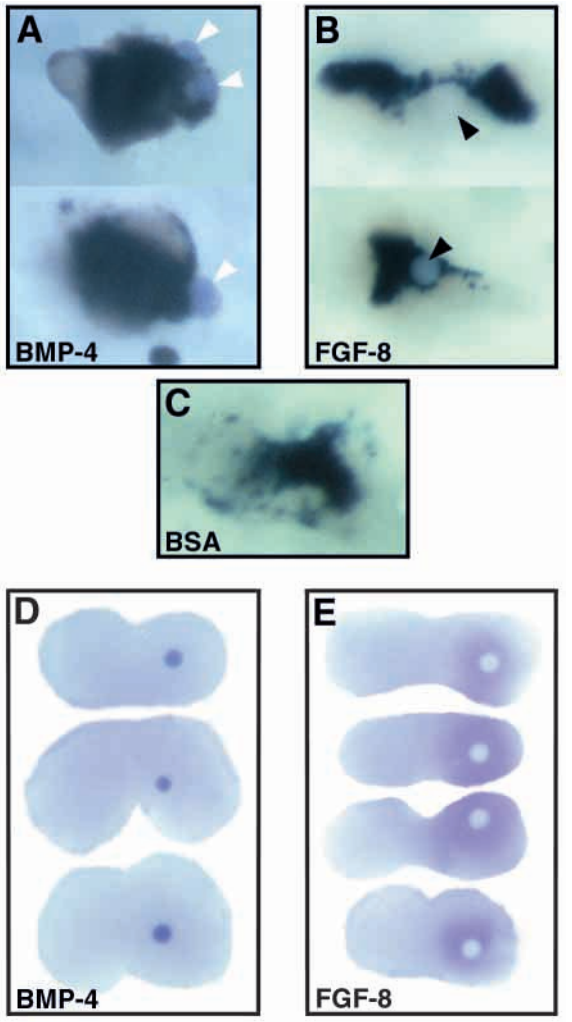

Fig. 4. BMP4 induces *Dlx2* epithelial expression by a planar signal, while FGF8 induces *Dlx2* in the mesenchyme and represses *Dlx2* epithelial expression via a mesenchymal signal. (A) β-Gal staining showing that the *Dlx2* epithelial expression is induced by BMP4 in the absence of mesenchyme. (B) β-Gal staining showing that FGF8 has no downregulatory effect on *Dlx2* epithelial expression in the absence of mesenchyme. Arrowheads indicate the positions of the beads. (C) Control showing residual expression. (D,E) In situ hybridisation using a *Dlx2* antisense probe showing that removal of epithelium from E10.5 mandibles results in downregulation of *Dlx2* expression in the mesenchyme. Mandibles cultured with BMP4 soaked beads do not induce expression (D), whereas FGF8 soaked beads induce *Dlx2* expression (E).

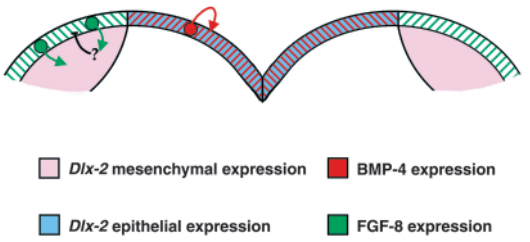

Fig. 5. Schematic diagram demonstrating the regulation of *Dlx2* by FGF8 and BMP4 in the mandible at E10.5. BMP4 (red) is expressed in the epithelium of the distal region of the mandible. It induces *Dlx2* in the epithelium (blue shading) via an intra-epithelial signal. FGF8 (green) is expressed in the epithelium in the proximal region of the mandible. FGF8 induces *Dlx2* in the underlying mesenchyme. In addition FGF8 inhibits expression of *Dlx2* in the epithelium by means of a signal that passes via the mesenchyme, and in this way restricts the epithelial domain of *Dlx2* from extending over the mesenchymal *Dlx2* domain.

expression, we mapped *Dlx2* regulatory regions in transgenic mice. Regulatory elements capable of reproducing the endogenous expression of *Dlx2* in facial epithelium and in the apical ectodermal ridge (AER) were located in a 1.47 kb region, 1 kb upstream of the gene. Sequencing of this region revealed no obvious transcription factor binding motifs, although the region did contain a large GAAA repeat. The function of this repeat is unknown, but it is possible it may be involved in approximating enhancer binding sites when activated, thus enabling the promotor to function. Significantly, no facial mesenchymal expression was produced by the region extending 3.77 kb upstream of *Dlx2*, indicating that the epithelial and mesenchymal expression of *Dlx2* in the face are regulated differently. At least some of the regions responsible for first arch mesenchymal expression appear to lie in the intergenic region between *Dlx1* and *Dlx2* (M. Ekker, personal communication).

The 1.47 kb region does not contain enhancers exclusive to epithelial cells since the region also produced expression in mesenchymal cells during limb development and also in the genital tubercle. Interestingly, while the expression of *Dlx2* in the epithelium of the first arch can be induced by BMP4 and repressed by FGF8, this is unlikely to be the case in the limb. The potential roles of BMP4 and FGF8 in inducing and repressing *Dlx2* in the epithelium of the first arch correlate with the relative spatial expression domains of these proteins, where *Bmp4* and *Dlx2* are coexpressed in epithelium at E10-E10.5 and *Fgf8* is expressed outside the *Dlx2* epithelial domain. However, in limb development *Dlx2* is coexpressed with *Fgf8* in the epithelium of the flank epithelium and subsequently in the AER, and is therefore unlikely to be repressed by this protein as in the face.

BMP4 induction of *Dlx2* expression in epithelium did not require the presence of mesenchyme, implying that this signalling pathway acts within the epithelium. Several previous studies have shown that BMP4 produced by the early distal epithelium is responsible for inducing expression of distal mesenchyme genes such as *Msx1* and repressing proximal mesenchyme genes such as *Barx1*. This is the first demonstration of oral epithelium being a target for BMP4. Similar planar signalling within early oral epithelium has, however, been noted for SHH where *Shh*, and both *Ptc* and *Ptch2* receptors are expressed in the epithelium (Hardcastle et al., 1998; Motoyama et al., 1999).

FGF8 had a striking long-range effect on *Dlx2* expression, resulting in selected repression of expression on the contralateral side of the explant to the bead within the endogenous domain of *Dlx2* epithelial expression. The restricted response to the long-range signalling suggests that oral epithelium is compartmentalised with respect to its response to FGF8 signalling. Interestingly, the epithelial cell domain of *Dlx2* expression, which was repressed by FGF8,

correlates with the presumptive incisor tooth epithelium, suggesting that odontogenic epithelium is specified very early within oral epithelium.

The repression of *Dlx2* epithelial expression by FGF8 requires the presence of mesenchyme. This is significant, since epithelially expressed splice variants of FGF8 have been shown to activate mesenchymally expressed FGF receptors rather than epithelially expressed variants (MacArthur et al., 1995). The results presented here suggest that FGF8 has dual effects on mesenchyme cells; it can act to induce *Dlx2* expression in the mesenchyme cells themselves and at the same time repress *Dlx2* expression in the overlying epithelium. It is not known whether the presence of *Dlx2* in proximal mesenchymal cells is a requirement for inhibition of *Dlx2* in the epithelium. It is equally possible that the FGF8 inhibition of epithelial *Dlx2* expression occurs via another mesenchymal signalling pathway.

Based on these results we have proposed a model to explain the proximodistal regulation of *Dlx2* in the mesenchyme and epithelium of the first branchial arch (Fig. 5). Localised expression of FGF8 in the proximal regions of the oral epithelium induces *Dlx2* expression in the underlying mesenchyme. The FGF8 epithelial to mesenchymal signal also results in the mesenchyme signalling back to the overlying epithelium, preventing *Dlx2* expression within it. Thus, in the proximal domain, *Dlx2* is expressed in the mesenchyme but not in the overlying epithelium. Distally, *Dlx2* expression is induced by BMP4 in the epithelium by a planar signal. BMP4 does not appear to play a role in regulating mesenchymal expression of *Dlx2* since treatment of explants with Noggin, thus disrupting the BMP4 signal, has no effect on *Dlx2* expression (P. T. S., unpublished data). At the most proximal limit of the *Dlx2* epithelial expression domain its boundary is set at the junction with the *Dlx2* mesenchymal domain by an inhibitory signal from the mesenchyme induced by FGF8 in the overlying epithelium.

It is not known how Dlx2 interacts with other homeoproteins. BMP2, BMP4 and FGF8 have been shown to regulate mesenchymal expression of *Pax9* in the mandibular arch mesenchyme (Neubüser et al., 1997). *Pax9* was shown to be induced by FGF8 and the expression was restricted to a small area of mesenchyme, purported to be the prospective molar mesenchyme, as a result of repression by BMP2 and BMP4. The results presented here suggest a model where FGF8 induces *Dlx2* expression in the proximal mesenchyme, and BMP4 induces *Dlx2* in the distal epithelium, with the proximal limit of the epithelial expression occurring at the boundary of the *Dlx2* mesenchymal domain. FGF8 and BMP4 are thus involved in the spatial domains of at least three homeobox genes, *Dlx2*, *Barx1* and *Pax9*, which are known to be important for correct early development. A key to understanding the basis for patterning of the dentition therefore seems to lie in understanding the spatial regulation of FGF8 and BMP4 in the first branchial arch.

We wish to thank S. M. N. N. Faruque and M. Walker for technical expertise in producing the transgenic mice. We are grateful to Randy Johnson for the kind gift of the RCAS Noggin Retrovirus. We are very grateful to M. Ekker and I. Thesleff for sharing unpublished data with us. We thank C. Ferguson for critical reading of the manuscript. This work was supported by the MRC, and B. Thomas was supported by an MRC Clinical Training Fellowship.

REFERENCES

Bei, M. and Maas, R. (1998). FGFs and BMP4 induce both Msx1-independent and Msx1-dependent signalling pathways in early tooth development. *Development* **125**, 4325-4333.

Bulfone, A., Kim, H.-J., Puelles, L., Porteus, M.H., Grippo, J.F. and Rubenstein, J.L.R. (1993). The mouse *Dlx-2* (*Tes-1*) gene is expressed in spatially restricted domains of the forebrain, face and limbs in midgestation mouse embryos. *Mech. Dev.* **40** 129-140.

Christiansen, J. H., Dern, P., C., Wicking, C. A., Monkley, S. J., Wilkinson, D. G. and Wainwright, B. J. (1995). Murine *Wnt-11* and *Wnt-12* have temporally and spatially restricted expression patterns during embryonic development. *Mech. Dev.* **51**, 341-350.

Dassule, H. R. and McMahon, A. P. (1998). Analysis of epithelial-mesenchymal interactions in the initial morphogenesis of the mammalian tooth. *Dev. Biol.* **202**, 215-227.

Ferguson, C., Tucker, A. S., Christensen, L., Lau, A. L., Matzuk, M. M. and Sharpe, P. T. (1998). Activin is an essential early mesenchymal signal in tooth development that is required for patterning of the murine dentition. *Genes Dev.* **12**, 2636-2649.

Grigoriou, M., Tucker, A. S., Sharpe, P. T. and Pachnis, V. (1998). Expression and regulation of *Lhx6* and *Lhx7*, a novel subfamily of LIM homeodomain encoding genes, suggests a role in mammalian head development. *Development* **125**, 2063-2074.

Hardcastle, Z., Mo, R., Hui, C.-C. and Sharpe, P. T. (1998). The Shh signalling pathway in tooth development: defects in *Gli2* and *Gli3* mutants. *Development* **125**, 2803-2811.

Jonk, L. J. C., Itoh, S., Heldin, C. H., ten Dijke, P. and Kruijer, W. (1998). Identification and functional characterisation of a Smad binding element (SBE) in the *JunB* promotor that acts as a transforming growth factor-β, activin, and bone morphogenetic protein-inducible enhancer. *J. Biol. Chem.* **273**, 21145-21152.

Lumsden, A. G. S. (1988). Spatial organisation of the epithelium and the role of neural crest cells in the initiation of the mammalian tooth germ. *Development* **103 Supplement**, 155-169.

MacArthur, C. A., Lawshé, A., Xavier, J., Sanicola-Ocampo, S., Heikinheimo, M., Chellaiah, A. T. and Ornitz, D. M. (1995). FGF-8 isoforms activate receptor splice forms that are expressed in mesenchymal regions of mouse development. *Development* **121**, 3603-3613.

Motoyama, J., Liu, J., Mo, R., Ding, Q., Post, M. and Hui, C. C. (1999). Essential function of *Gli2* and *Gli3* in the formation of the lung, trachea and oesophagus. *Nature Genetics* **20**, 54-57.

Neubüser, A., Peters, H., Balling, R. and Martin, G. R. (1997). Antagonistic interactions between FGF and BMP signalling pathways: A mechanism for positioning the sites of tooth formation. *Cell* **90**, 247-255.

Parr, B. A., Shea, M. J., Vassilera, G. and McMahon, A. P. (1993). Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. *Development* **119**, 247-261.

Qiu, M., Bulfone, A., Ghattas, I., Menses, J. J., Shimamura, K., Presley, R., Puelles, L., Rubenstein, J. L. R. (1997). Role of the Dlx homeobox genes in proximodistal patterning of the branchial arches: mutations of Dlx-1, Dlx-2 and Dlx-1 and -2 alter morphogenesis of proximal skeletal and soft tissue structures derived from the first and second arches. *Dev. Biol.* **185**, 165-184.

Qiu, M., Bulfone, A., Martinez, S., Menses, J. J., Shimamura, K., Pedersen, R. A. and Rubenstein, J. L. R. (1995). Null mutation of Dlx-2 results in abnormal morphogenesis of proximal first and second branchial arch derivatives and abnormal differentiation in the forebrain. *Genes Dev.* **9**, 2523-2538.

Sanes, D. R., Rubenstein, J. L. R. and Nicolas, J.-F. (1986). Use of a recombinant retrovirus to study postimplantation cell lineage in mouse embryos. *EMBO J.* **5**, 3133-3142.

Saxén, L. (1966). The effects of tetracycline on osteogenesis in vitro. *J. Exp. Zool.* **162**, 269-294.

Sharpe, P. T. (1995). Homeobox genes and orofacial development. *Conn. Tiss. Res.* **32**, 17-25.

Storey, K. G., Goodwin, A., Sargent, C. M., Brown, J. M., Burns, H. D., Abud, H. E. and Heath, J. K. (1998). Early posterior neural tissue is induced by FGF in the chick embryo. *Development* **125**, 473-484.

Thomas, B. L. and Sharpe, P. T. (1998). Patterning of the murine dentition by homeobox genes. *Eur. J. Oral Sci.* **106** (suppl. 1), 48-54.

Thomas, B. L., Tucker, A. S., Qiu, M., Ferguson, C., Hardcastle, Z., Rubenstein, J. L. R. and Sharpe, P. T. (1997). Role of *Dlx-1* and *Dlx-2* genes in patterning of the murine dentition. *Development* **124**, 4811-4818.

Tucker, A. S., Matthews, K. L. and Sharpe, P. T. (1998a). Transformation of tooth type induced by inhibition of Bmp signalling. *Science* **282**, 1136-1138.

Tucker, A. S., Al Khamis, A. and Sharpe, P. T. (1998b). Interactions between Bmp-4 and Msx-1 act to restrict gene expression to odontogenic mesenchyme. *Dev. Dyn.* **212**, 533-539.

Tucker, A. S., Yamada, G., Grigoriou, M., Pachnis, V. and Sharpe, P. T. (1999). Fgf-8 determines rostral-caudal polarity in the first branchial arch. *Development* **126**, 51-61.

Vainio, S., Karavanova, I., Jowett, A. and Thesleff, I. (1993). Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. *Cell* **75**, 45-58.

Wang, J. and Shackleford, G. M. (1996). Murine *Wnt 10a* and *Wnt 10b*: cloning and expression in developing limbs, face and skin of embryos and in adults. *Oncogene* **13**, 1537-1544.

Wilkinson, D. G. (1992). *In Situ Hybridisation: A Practical Approach.* Oxford, UK: IRL press.