Expression patterns of the homeobox gene, *Hox-8*, in the mouse embryo suggest a role in specifying tooth initiation and shape

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**Summary**

We have studied the expression patterns of the newly isolated homeobox gene, *Hox-8* by in situ hybridisation to sections of the developing heads of mouse embryos between E9 and E17.5, and compared them to *Hox-7* expression patterns in adjacent sections. This paper concentrates on the interesting expression patterns of *Hox-8* during initiation and development of the molar and incisor teeth.

*Hox-8* expression domains are present in the neural crest-derived mesenchyme beneath sites of future tooth formation, in a proximo-distal gradient. Tooth development is initiated in the oral epithelium which subsequently thickens in discrete sites and invaginates to form the dental lamina. *Hox-8* expression in mouse oral epithelium is first evident at the sites of the dental placodes, suggesting a role in the specification of tooth position. Subsequently, in molar teeth, this patch of *Hox-8* expressing epithelium becomes incorporated within the buccal aspect of the invaginating dental lamina to form part of the external enamel epithelium of the cap stage tooth germ. This locus of *Hox-8* expression becomes continuous with new sites of *Hox-8* expression in the enamel navel, septum, knot and internal enamel epithelium. The transitory enamel knot, septum and navel were postulated, long ago, to be involved in specifying tooth shape, causing the inflection of the first buccal cusp, but this theory has been largely ignored. Interestingly, in the conical incisor teeth, the enamel navel, septum and knot are absent, and *Hox-8* has a symmetrical expression pattern. Our demonstration of the precise expression patterns of *Hox-8* in the early dental placodes and their subsequent association with the enamel knot, septum and navel provide the first molecular clues to the basis of patterning in the dentition and the association of tooth position with tooth shape: an association all the more intriguing in view of the evolutionary robustness of the patterning mechanism, and the known role of homeobox genes in *Drosophila* pattern formation.

At the bell stage of tooth development, *Hox-8* expression switches tissue layers, being absent from the differentiating epithelial ameloblasts and turned on in the differentiating mesenchymal odontoblasts. *Hox-7* is expressed in the mesenchyme of the dental papilla and follicle at all stages. This reciprocity of expression suggests an interactive role between *Hox-7*, *Hox-8* and other genes in regulating epithelial mesenchymal interactions during dental differentiation. *Hox-8* is also expressed in the distal mesenchyme and epithelia of the lateral nasal, medial nasal and maxillary processes (in a more spatially restricted domain than *Hox-7*), Jacobson’s organs, the developing skull bones, meninges, ear, eye, whisker and hair follicles, choroid plexus, cardiac cushions and limb buds. The patterns of expression in the facial processes resemble those of the progress zone of the limb, suggesting a similar patterning mechanism in these embryonic outgrowths.

**Key words:** tooth development, craniofacial development, msh-like genes, mouse development, tooth shape, homeobox genes *Hox-7* and *Hox-8*, in situ hybridisation.

**Introduction**

Teeth are phylogenetically ancient structures, well preserved in the fossil record where their shape and position in jaw fragments play a pivotal role in the reconstruction of the anatomy, dietary habits and lineage relationships of vertebrates (Romer, 1966). Moreover, in mammals, their shape and position in the jaws are tightly linked: there are no known mutants which, for example, develop molars at the front and incisors at the rear of the mouth (Miles and Grigson, 1990). Development of the mammalian dentition therefore involves both regional (incisors, canines, premolars and molars) and temporal (differing development times of deciduous and permanent teeth) patterning of the individual tooth anlage. Development of an
individual tooth commences with an oral thickening of the jaw epithelium, its invagination into the mesenchyme to form a dental lamina, distal enlargement of the lamina to form epithelial swellings (enamel organs) associated with condensing neural crest-derived jaw mesenchyme (dental papilla and follicle) which collectively (tooth germ) progress through the well characterised morphological and differentiation stages of bud, cap and bell to form the adult tooth (Ruch, 1987; Thesleff et al., 1989; Ferguson, 1990).

Despite the importance of tooth shape and position for developmental, phylogenetic and clinical dental studies, almost nothing is known about the molecular basis of this exquisitely precise patterning. Early theories (Osborn, 1978, 1984) suggested that the developmental information required for tooth initiation was carried into the jaws by clones of neural crest cells. Some credence for this patterning idea comes from neural crest transplantation studies in the chicken embryo, whereby duplicate first branchial arch structures can be induced to form by grafting future first arch premigratory neural crest cells to the region destined to form the second arch (Noden, 1983); unfortunately, birds do not develop teeth! Extensive heterotypic or heterochronic epithelial-mesenchymal recombination experiments demonstrate that initiation and regional localisation of the early dental anlage (incisors, molars) are absolutely dependent on the rostral (i.e. oral) epithelium of the mandibular arch between E9 (mouse embryonic day 9) and E10: no other epithelium can elicit tooth development from mandibular mesenchyme (Mina and Kollar, 1987; Lumsden, 1988). Equally, the E9-E10 mandibular epithelium can only specify tooth development in neural crest (either cranial or trunk) related mesenchyme; not, for example, in limb mesenchyme (Lumsden, 1988). One effect of this interaction between mandibular arch mesenchyme and E9/E10 mandibular epithelium is the acquisition by the former of the ability to instruct competent epithelium to participate in regional specific enamel organ morphogenesis and subsequent ameloblast differentiation and enamel matrix synthesis: an ability which persists from E11 to E16 (Kollar and Baird, 1969, 1970a, 1970b; Heritier and Deminatti, 1970; Kollar, 1972, 1981; Ruch et al., 1983; Ruch 1984; Lumsden, 1988). In vivo therefore, epithelial signalling to mandibular mesenchyme at E9/E10 is reciprocated by mesenchymal signalling to the epithelium at E13, and these numerous reciprocal interactions (summarised by Lumsden, 1988) characterised by changes in extra-cellular matrix molecules (Thesleff et al., 1979, 1981, 1987, 1988, 1989, 1990; Andujar et al., 1991), growth factors (Partanen and Thesleff, 1985, 1987, 1989; Cam et al., 1990; D'Souja et al., 1990; Hata et al., 1990; Kronmiller et al., 1991) and their receptors continue throughout tooth morphogenesis and differentiation.

The discovery of the highly conserved DNA-binding motif, the homeobox, in many genes playing key roles in Drosophila embryonic development has provided a route for identifying genes with similar motifs, and possibly similar functions, in vertebrates (Gehring, 1987). In the region of 50 different homeobox-containing genes have been identified and cloned in the mouse. These genes are classified according to their homeobox sequence and chromosomal location (Martin et al., 1987). The majority of the mammalian Hox genes (38) constitute a family with homeobox sequences most similar to the Drosophila antennapedia homeobox sequence. These genes (class 1) are found in clusters on 4 separate chromosomal locations: Hox 1 (chromosome 6), Hox 2 (chromosome 11), Hox 3 (chromosome 15) and Hox 4 (chromosome 2) (Hart et al., 1985, Bucan et al., 1986; Breier et al, 1988; Featherstone, et al., 1988). Based on the collinearity of their expression patterns in embryos, their chromosomal positions and homeobox sequences, these genes probably represent evolutionary relatives of the Drosophila homeotic genes and as such, are postulated as having an important regulatory role in the control of axial specification in early embryogenesis (Graham et al., 1989; Duboule and Dolle, 1989).

The other mammalian homeobox genes not found in these clusters form different smaller classes depending on their sequences or association with other conserved motifs (Pax genes, Oct genes, etc) (Herr et al., 1988). One class of these genes is the mammalian msh-like genes. This small family of three unlinked homeobox genes, Hox-7 (mouse chromosome 5 Hill et al., 1989; Robert et al., 1989), Hox-8 (Monaghan et al., 1991) and Hox-9 (Holland, 1991) have homeobox sequences very similar to each other and to the Drosophila muscle segment homeobox gene (msh) (Hill et al., 1989; Robert et al., 1989; MacKenzie et al., 1991a,b). The embryonic expression patterns of the class 1 genes are very different to Hox-7 and Hox-8, which show a closely associated, multi-phasic, interactive pattern of expression throughout early embryonic development (MacKenzie et al., 1991a, 1991b; Monaghan et al., 1991). Earliest expression of both genes is detectable in primitive streak mesoderm, followed by expression in neural crest cells and their derivatives. In later embryos Hox-7 expression occurs in a number of discrete areas (skull, heart, limb) around the time of epithelial mesenchymal interactions in such tissues (Hill et al., 1989; Robert et al., 1989; MacKenzie et al., 1991a,b; Takahashi and Le Douarin, 1990; Takahashi et al., 1991). In the developing teeth, Hox-7 is expressed exclusively in the condensing mesenchymal cells of the dental papilla and follicle from the onset of dental development, with no expression in the dental epithelia (MacKenzie et al., 1991a).

We have investigated the patterns of expression of the Hox-8 homeobox gene during mouse craniofacial development (E9-E17) concentrating on its role in dental development. The results indicate a reciprocity of expression with Hox-7 (which we investigated in adjacent sections for comparison). In particular, the expression of Hox-8 in the epithelial dental placode and enamel knot, septum and navel, suggest a role for this gene in determining the sites of tooth initiation and the shape of the final teeth: a postulated function in keeping
with the known role of homeobox genes in patterning of the *Drosophila* embryo.

**Materials and methods**

*Preparation of tissues*

This was as previously described (MacKenzie et al., 1991a,b). Briefly, pregnant female mice (MFI strain) were killed by ether overdose, on embryonic days 9-17 (day of finding vaginal plug, day 0). Embryos were aseptically removed and fixed overnight at 4°C in 4% paraformaldehyde dissolved in phosphate-buffered saline. E16.5 and E17.5 embryo heads were demineralised in 0.5M EDTA for 4 days. Embryo heads were then dehydrated through ascending grades of ethanol and cleared in chloroform prior to embedding in Fibrowax (Raymond A. Lamb, London). 7 μm serial sections were cut and adhered to alternate glass slides previously treated with 3-aminopropyltriethoxysilane. Sections were dewaxed and proteinase K treated prior to hybridisation with riboprobes.

*Preparation of probes*

We isolated the *Hox-7* gene from an E8.5 mouse embryo cDNA library at low stringency using the *Drosophila* bicoid homeobox as probe. A 900 bp fragment of this clone 5' to the homeobox region was then subcloned into the transcription vector pSP72 and used to derive antisense and sense riboprobes as previously described (MacKenzie et al., 1991a).

A 850 bp fragment of the *Hox-8* gene was received as a kind gift from Dr Robert Hill of the MRC Human Cytogenetics Unit, Edinburgh, Scotland. This fragment has been previously described and riboprobes characterised for use by in situ hybridisation to mouse embryos (Monaghan et al., 1991).

Complementary (anti-sense) RNA probes were transcribed in vitro in the presence of 35S labelled-UTP using SP6 RNA polymerase from the 850 bp fragment derived from the *Hox-8* cDNA sub-cloned into the plasmid vector pSP72. Control probes were produced by transcription from the same plasmid with T7 RNA polymerase. Riboprobes were hybridised to mouse embryo head sections at high stringency using established methods (Sharpe et al., 1988). Since no hybridisation was detected on any section with control probes these are not shown.

**Results**

The expression patterns of *Hox-7* in a variety of developing mouse craniofacial structures, especially the teeth, has been described previously (MacKenzie et al., 1991a,b). This paper does not repeat such descriptions, but includes new details of *Hox-7* expression in order to compare the expression patterns with *Hox-8* in adjacent sections. The expression of *Hox-8* in the structures of the developing mouse eye has been described (Monaghan et al., 1991). *Hox-8* is expressed in many other mouse embryonic tissues e.g. the heart, limb and ear, but this report restricts itself to the developing facial tissues, especially the teeth.

*Branchial arches and facial processes*

At E9 and E10, *Hox-8* is expressed in the neural crest-derived mesenchyme of all four branchial arches (Fig. 1A,B). Its expression domain is slightly narrower and

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**Fig. 1.** (A) Bright field and (B, C) dark field photomicrographs of *Hox-8* (B) and *Hox-7* (C) expression in sagittal sections of an E10.5 mouse embryo. I, II, III, IV, branchial arches; fb; forebrain, hb hind brain; ov otic vesicle. Bar, 1 mm.
Fig. 2. (A) Bright field and (B,C) dark field photomicrographs of Hox-8 (B) and Hox-7 (C) expression in the mesenchyme of the lateral nasal (ln) medial nasal (mn) maxillary (mx) and mandibular (md) processes in frontal sections of an E10.5 mouse head. Note the concentration of Hox-8 expression in the maxillary process below the area of future 1st molar tooth initiation (arrowed). dl, dental lamina; nc, nasal cavity. Bar 0.5 mm.

more restricted domain compared to Hox-7 (Fig. 1C). In frontal sections, the narrower and lighter areas of labelling with the Hox-8 probe (Fig. 2D), compared to the Hox-7 probe - (Fig. 2C) in the distal mesenchyme of

the medial nasal, lateral nasal and maxillary processes are evident. With fusion and merging of the lateral nasal, medial nasal and maxillary processes, Hox-8 is expressed in the mesenchyme (particularly that closest to the oral cavity) of the closure zone. Hox-8 is also expressed in a narrow band of mesenchyme in the oral half of the mandibular process (Fig. 2B).

The domain of Hox-8 labelling in the mesenchyme of an E10.5 maxillary or mandibular process becomes progressively narrower and more focused, moving proximally into more posterior regions of the head (Fig. 3A,B,C,D). Interestingly, this focused domain of Hox-8 mesenchyme labelling (Fig. 3A,B,C,D) corresponds precisely to the area where the first molar will develop at a later date.

Development of the first molar tooth

The first marker of first molar development is the expression of Hox-8 in the mesenchyme beneath the site of dental development at E10.5 (Figs 2B, 3A,B,C). At this stage, Hox-8 is not expressed in the epithelium. However, at E11.5, Hox-8 is expressed in a small area of epithelial cells, a few microns buccal to the thickening dental epithelium immediately above its previous area of mesenchymal expression (Fig. 4A,B). This is the dental placode, a prominent structure in amphibian and reptilian dental development, where it is implicated in initiating tooth formation (Westergaard and Ferguson, 1986). The existence of the dental placode in mammals has, until now, been dubious, owing to the lack of suitable markers. The dental placode is progressively (from E10 to E12) incorporated into the buccal aspect of the invaginating dental lamina (Fig. 4A,B), so that a discrete area of the buccal external enamel epithelium expresses Hox-8 at the bud stage of tooth development (Figs 5A,B,C, 6A,B). The mesenchyme of the dental papilla and follicle immediately surrounding the invaginating dental epithelium, at the dental lamina and bud stages, shows weak Hox-8 expression: much weaker and more discretely adjacent to the dental epithelium than Hox-7 labelling (Fig. 5A,B,C). Hox-7 does not label the epithelial dental placode, dental lamina or bud stage enamel organ (Fig. 5C).

At the early cap stage (E13.5) the dense epithelium of the enamel knot, the immediately adjacent internal enamel epithelium and sub-adjacent layer of dental papilla mesenchyme cells express Hox-8 as well as the region of the buccal external enamel epithelium derived from the dental placode (Fig. 6A,B). At the mid to late cap stage (E14) the Hox-8 expressing enamel knot (and adjacent internal enamel epithelium and dental papilla mesenchyme) is physically connected to the Hox-8 expressing buccal external enamel epithelium by the enamel septum, which also expresses Hox-8 (Fig. 6A,B). Expression of Hox-8 in the enamel knot, septum and associated external enamel epithelium (which is now indented to form the enamel navel) is maximal at the late cap stage (Fig. 6A,B) when the associated (Hox-8 expressing) internal enamel epithelium inflects to form the anlage of the first (buccal)
cusp. Throughout the cap stage, Hox-8 is lightly expressed in the mesenchyme of the dental papilla and follicle, and only then in a narrow strip of 2-4 cells immediately surrounding the epithelial enamel organ (Fig. 6A,B). By contrast, Hox-7 is expressed exclusively in a broad band of mesenchymal cells of the dental papilla and follicle and is completely absent from the epithelial enamel organ (Fig. 6C).

At the early bell stage (E15), when the transitory enamel knot, septum and navel disappear, Hox-8 is expressed throughout the internal enamel epithelium, but is prominent at the points of inflection of the future
Fig. 4. (A) Bright field and (B) dark field photomicrographs of Hox-8 expression in the early dental tissues of the lower jaw in an E11.5 mouse head cut in the frontal plane. Note the early expression of Hox-8 in the dental placode (dp) and adjacent mesenchyme (m) buccal to the site of invagination of the dental lamina (dl). b, buccal; l, lingual. Bar, 40 µm.

Fig. 5. (A) Bright field and (B,C) dark field photomicrographs of Hox-8 (B) and Hox-7 (C) expression in frontal sections of an E11.5 mouse head. Note the incorporation of the Hox-8 expressing dental placode epithelia into the buccal aspect of the external enamel epithelia of the invaginating bud stage tooth germ (arrowed). Hox-7 expression is confined to the mesenchyme of the dental papilla (dp) and dental follicle (df). i, invaginating bud stage tooth germ; b, buccal; l, lingual. Bar, 100 µm.
Fig. 6. (A) Bright field and (B,C) dark field photomicrographs of *Hox-8* (B) and *Hox-7* (C) expression in a lower first molar tooth germ at the cap stage of development, a frontal section from an E14.5 embryo head. Note the expression of *Hox-8* in the enamel knot (k), septum (s), navel (n), (dimpled area of external enamel epithelium corresponding to the previous dental placode), internal enamel epithelial (iee) at the site of inflection of the future buccal cusp. *Hox-7* expression is restricted to the mesenchyme of the dental papilla (dp) and dental follicle (df). b, buccal; l, lingual. Bar, 100 μm.

*Hox-8* continues to be expressed in the stratum intermedium layer and in the external enamel epithelia and condensed stellate reticulum of the cervical crown (Fig. 8A,B). *Hox-7* is expressed throughout the dental papilla mesenchyme, but is particularly intense in the odontoblasts and sub-odontoblastic cells at the tips of the cusps, as well as in the dental follicle (Fig. 8C).

A summary in diagrammatic form, of the principal labelling patterns of *Hox-8* during molar development is given in Fig. 9.

**Development of the incisor tooth**

At E10 and E11, when the anterior oral epithelia, destined to form the incisor teeth, thicken and invaginate, *Hox-8* is expressed in the dental placode, dental lamina and adjacent stretch of oral epithelia buccal to the dental invagination site (Fig. 10A,B). By contrast, *Hox-7* is absent from the epithelia, but expressed widely in the surrounding mesenchyme, particularly in cells destined to form the premaxillary bones and mesenchyme of the upper lip (Fig 10C). In general, the anterior expression domain of *Hox-8* (Fig. 10) is much wider in both the epithelium and mesenchyme compared to that for the first molar (Fig. 2).

By E12.5, the incisor tooth germ is at the bud stage (Fig. 11A). *Hox-8* is expressed in the enamel organ, throughout the entire length of the external enamel epithelia on the buccal and cervical surfaces and in the cervical half of the lingual external enamel epithelia (Fig. 11A,B). Epithelial cells in the centre of the cervical half of the enamel organ also label intensely (Fig. 11A,B). *Hox-8* expression also extends slightly down the invaginating epithelia of the adjacent buccal gingival furrow on its oral aspect (Fig. 11A,B). This epithelial ingrowth serves subsequently to separate the lips and cheeks from the gums. The pattern of *Hox-8* labelling in the incisors is in contrast to the discrete buccal labelling of the external enamel epithelia in the region of the incorporated dental placode epithelia observed in molar teeth at the bud stage of development (Fig. 5A,B). Expression of *Hox-7* in the mesenchyme of the incisor dental papilla and follicle (Fig. 11C) is similar to that seen in the molar buds (Fig. 5C).

At E15.5 (cap stage) *Hox-8* is expressed throughout the enamel organ epithelia and the dental papilla mesenchyme of the developing incisor teeth (Fig. 12A). The oral epithelia overlying the incisor dental lamina label strongly for *Hox-8*, especially buccal to the lamiinae (Fig. 12A). *Hox-7* continues to be expressed in the mesenchyme of the dental papilla and follicle, but is absent from the epithelial enamel organ (Fig. 12B).

Examination of histological sections cut transversely through the developing incisors reveals an extraordinary symmetrical pattern of expression of both *Hox-7* and *Hox-8* during morphogenesis of incisor shape (Fig. 13A,B,C). This is in marked contrast to the asymmetrical pattern, particularly of *Hox-8* expression during molar morphogenesis (Fig. 5A,B, 6A,B, 7A,B). *Hox-8* is expressed uniformly in the dental papilla mesenchyme, stellate reticulum and external enamel epithelia of the incisor tooth germ on all sides, but is absent from the internal enamel epithelia (Fig. 13A,B). By contrast, *Hox-7* is expressed only in the mesenchyme of the dental papilla and follicle and absent in the external
enamel epithelia, stellate reticulum and internal enamel epithelia (Fig. 13C).

By E17.5, odontoblasts have differentiated from the dental papilla mesenchyme cells and ameloblasts from the internal enamel epithelial cells on the aboral side of the developing incisor teeth (Fig. 14A). Hox-8 is expressed in the odontoblasts and dental papilla mesenchyme cells and the narrow epithelia of the stratum intermedium, stellate reticulum and external enamel epithelia (Fig. 14A,B). Hox-7 expression is restricted, as before, to the mesenchyme of the dental papilla (including the odontoblasts) and follicle (Fig. 14C).

Other craniofacial structures
Both Hox-7 and Hox-8 are expressed in similar temporal and spatial patterns during formation of the skull bones and meninges and in the neural epithelium of the lateral choroid plexus, prior to and during its formation (Fig. 15A,B). Hox-8 is expressed more intensely in the areas of the future meninges and skull bones than is Hox-7, but in the formation of the choroid plexus, the opposite is the case (Fig. 15A-C). We have previously studied and discussed the expression patterns of the Hox-7 gene in the choroid plexus, the meninges and the skull bones (MacKenzie et al., 1991b). Jacobson's (or the vomeronasal) organ forms as an invagination of the nasal cavity epithelium into the mesenchyme of the nasal septum and in frontal section, takes on a kidney shape, its epithelium thinning on the side facing the nasal cavity and thickening on the opposite side (Fig. 16A,D,G). Between E13.5 and E17.5, Hox-8 is consistently expressed in the thinning epithelia and adjacent mesenchymal condensation on the nasal side of Jacobson's organs (Fig. 16B,E,H), whereas Hox-7 is restricted to the mesenchymal condensation on the nasal side, with only light labelling of the mesenchyme adjacent to the thickened abnasal side (Fig. 16C,F,I). Hox-7 and Hox-8 are also expressed in the developing whisker and hair follicles (Fig. 12A,B). However, Hox-8 is not expressed in the anterior pituitary, as is Hox-7, but is expressed in the endolymphatic ducts of the otic cyst at E11.5, where Hox-7 is not.

Discussion
Patternning of the dentition: tooth initiation and shape specification
Despite the precise regulation of the timing and sites of tooth development and the correlation of tooth shape with position in the dentition, almost nothing is known about the molecular mechanisms regulating dental
Hox-8 and tooth shape

Fig. 8. (A) Bright field and (B,C) dark field photomicrographs of Hox-8 (B) and Hox-7 (C) expression in a lower first molar tooth germ at the bell stage of development in a frontally sectioned E17.5 lower jaw. Hox-8 is now strongly expressed in the odontoblastic (o) and sub-odontoblastic (s) layer of the dental papilla (dp) mesenchyme. Hox-8 is weakly expressed in the stratum intermedium (si) and condensed external enamel epithelia and stellate reticulum in the cervical loops (c). It is absent from the differentiated ameloblast (a) layer of the internal enamel epithelia. Hox-7 is expressed exclusively in the mesenchyme of the dental papilla (dp) and dental follicle (df). 1, lingual; b, buccal. Bar 150 μm.

initiation or shape. Neural crest cells have long been thought to be the source of the dental patterning information. Andres (1946, 1949) and Wagner (1949, 1959) transplanted cephalic neural crest cells from salamander into frog embryos. In the resulting chim- aeras, the grafted cells developed donor specific features, including teeth, in an anuran host. Likewise, transplants of fore- and midbrain neural crest precur- sors in the place of hindbrain neural crest and vice versa indicated that the transplanted populations were unable to form skeletal structures appropriate to their new location: rather structures appropriate for their original donor site, including ectopic teeth, developed (Hor- stadius and Sellman, 1946; Sellman, 1946). Similar experiments in chicken embryos where future second arch crest cells were replaced with those that normally form the first arch resulted in duplicate first arch structures (Noden, 1983): but birds do not possess teeth! Collectively, these experiments indicate that the neural crest cells (perhaps a specific subpopulation) contain developmental information to initiate tooth formation. This is in keeping with the progressively more restricted expression domains of Hox-8 in the maxillary and mandibular processes, which proximally focus on the mesenchyme immediately beneath the site where the first molar will form.

Subsequently, the developmental information for tooth initiation appears to be present in discrete areas of the oral epithelium, as recombination experiments indicate that this epithelium can elicit tooth formation from any neural crest-derived mesenchyme (Mina and Kollar, 1987; Lumsden, 1988). ‘Transfer’ of this tooth initiating information to the epithelium coincides with the onset of Hox-8 expression in the dental placode epithelium and its marked reduction in the underlying mesenchyme. Reciprocal (with time) signalling between epithelium and mesenchyme characterises all subsequent stages of tooth development (Lumsden, 1988).

Histologically, the first signs of dental initiation are: increased mitotic activity of a discrete part of the jaw epithelium, its thickening with accumulation of squamous cells upon the basal layer and the commence- ment of condensation of the underlying mesenchyme (Tonge, 1966, 1969, 1989). Further growth results in the formation of a continuous epithelial band around the future dental arches - usually broader anteriorly than posteriorly: precisely, the expression domain of Hox-8 (Fig. 3D). An area of this thickened epithelium then invaginates the jaw mesenchyme as the dental lamina: EGF may be important for downgrowth of the lamina (Kronmiller et al., 1991).

How the different morphological elements of the dentition (incisors, canines, premolars, molars) form is completely unknown. Histological data of discrete initiation sites seem to favour a clone model rather than a field theory of a diffusible morphogen (Osborn, 1978, 1984; Lumsden, 1979). However, Westergaard and Ferguson (1986, 1987, 1990) have proposed a hybrid ‘progress zone model’: interestingly, the progressive disto-proximal restriction of Hox-8 expression in the
**Fig. 9.** Diagrammatic representation of Hox-8 expression (in black) during the formation of the lower first molar tooth at the times of (A) initiation, (B) dental lamina invagination, (C) bud, (D) cap, (E) early bell, (F) late bell stages of development. a, ameloblasts; c, cervical loop; df, dental follicle; dpi, dental placode; dl, dental lamina; dp, dental papilla; e, jaw epithelium; eee, external enamel epithelium; ek, enamel knot; en, enamel navel; eo, enamel organ; es, enamel septum; iee, internal enamel epithelium; m, mesenchyme; o, odontoblasts; si, stratum intermedium; sr, stellate reticulum.

epithelium and mesenchyme (Fig. 3D) is in keeping with the predictions of this model.

Hox-8 expression, therefore represents the earliest known marker for sites of dental initiation and, a gene whose product may be important in causing dental initiation and patterning. Furthermore, the expression of Hox-8 in the oral epithelium confirms the existence of the dental placode in mammals (Westergaard, 1989): previously it was best known in reptiles (Westergaard and Ferguson, 1986, 1987, 1990). Moreover we also show for the first time, that the epithelium of the Hox-8 expressing dental placode is progressively drawn into the buccal aspect of the invaginating external enamel epithelium as development progresses to the bud stage. Surprisingly, in the cap stage tooth germ, this site of placodal incorporation corresponds precisely to the site of the enamel navel which is connected by the enamel septum and knot to the internal enamel epithelium. In view of the proposed role of the enamel navel, septum and knot in tooth shape (see below) this association of Hox-8 expressing tissues provides the first molecular link between the site of tooth initiation and subsequent tooth shape: features that are known to be tightly associated in the mammalian dentition.

The enamel navel, septum and knot have been observed in the molar cap stage tooth germs of every species of eutherian or marsupial mammal examined (Bolk, 1920b; Churchill, 1935; Widdowson, 1946; Gaunt, 1955) but their brief transitory appearance has often led to them being overlooked, described as of inconsistent appearance and generally dismissed as morphological minutiae of no functional or developmental importance (Lefkovitz et al., 1953; Gaunt and Miles, 1967; Gaunt et al., 1971). Historically, this was not always the case. Thus, Bolk (1920a,b,c, 1921, 1922) attached considerable phylogenetic significance to these structures and believed, erroneously, that they represented the sites of fusion (dimerisation) between two conical reptilian tooth germs in the evolution of a mammalian molar. This false 'dimerisation theory' was soon replaced by a hypothesis that the transitory enamel knot, septum and navel were important in determining the shape of the molar tooth (Orban, 1928a,b; Butler, 1956). Collectively, this cellular condensation connecting the external and internal enamel epithelia was postulated to act as a local restraint, causing the postmitotic internal enamel epithelium to inflect at the site of the future first (buccal) cusp and the external enamel epithelium to dimple (to form the
enamel navel) as the swelling pressure (due to the secretion and hydration of glycosaminoglycans) of the developing stellate reticulum, separated the external and internal enamel epithelia everywhere else in the tooth germ. The centre of the enamel knot is mitotically active and is postulated to be the source of cells which both concentrate in the cavities of the cervical loops and form the stratum intermedium at subsequent stages (Provenza, 1964): all areas that express Hox-8.

It is significant that the Hox-8 expressing tissues of the enamel navel, septum and knot are located over the site of the future buccal cusp, which is the first to form developmentally (Gaunt, 1955; Butler, 1956, 1979).

Fig. 10. (A) Bright field and (B,C) dark field photomicrographs of Hox-8 (B) and Hox-7 (C) expression in the anterior incisor region of an E11.5 mouse head sectioned in the frontal plane. Note Hox-8 labelling of the invaginating dental epithelium (d) and adjacent oral epithelium, particularly on the buccal side (o). Hox-7 expression is widespread in the mesenchyme surrounding the invaginating dental epithelium, in the distal mesenchyme of the lip (l) and the forming pre-maxillary bones (p). Bar, 500 μm.

Fig. 11. (A) Bright field and (B,C) dark field photomicrographs of Hox-8 (B) and (C) Hox-7 expression in a frontal section of the anterior lower jaw of an E13 mouse head. The incisor tooth germ is at the bud stage of development. eo, enamel organ; b, buccal external enamel epithelium; c, cervical external enamel epithelium; l, lingual external enamel epithelium; bg, buccal gingival groove; mc, meckel's cartilage; i, central incisor tooth germ; mx, maxillary process; dp, dental papilla; df, dental follicle. Bar, 100 μm.

This buccal cusp is also the first to appear (as the paracone) during evolution of the mammalian heterodont dentition (Gregory, 1934; Patterson, 1956; Crompton, 1971; Kuhne, 1973; Osborn and Crompton, 1973; Butler and Joysey, 1978; Lillegraven et al., 1979).
Fig. 12. Dark field photomicrographs of Hox-8 (A) and Hox-7 (B) expression in a frontal section of an E15.5 mouse head (half head only shown), eo, enamel organ; dp, dental papilla mesenchyme; mn, mandible; ns, nasal septum; oe, oral epithelium; t, tongue; wf, whisker follicle. Bar, 500 \( \mu m \).

Hox-8 expression patterns provide the first molecular evidence for the involvement of the enamel knot, septum and navel in the specification of molar shape, particularly the creation of the first buccal cusp. Once the first cusp forms from a previously uniform sheet of internal enamel epithelium, the remaining cuspal patterns can be generated by e.g. differential cell division, using the first cuspal inflection as a point of reference (Osborn and Ten Cate, 1983), although it is intriguing that Hox-8 expression patterns (in the external and internal enamel epithelia, condensed stellate reticulum and dental papilla mesenchyme) also correlate with the formation of the second lingual cusp. This evidence for involvement of Hox-8 in the specification of tooth shape is strengthened by the symmetrical expression patterns of Hox-8 in the symmetrical cuspless incisor teeth which lack an enamel navel, septum and knot. Furthermore, the antero-posterior domains of Hox-8 expression in the jaw epithelia and mesenchyme become progressively restricted from a broad anterior region to a narrow posterior region, giving the appearance of an elongated diamond with its wide central base on the jaw midline, and tapering posteriorly in each jaw half (Fig. 3G). This expression domain is in keeping with postulated models of dentition initiation and patterning (Osborn, 1984; Westergaard and Ferguson, 1987).

This postulated role for Hox-8 in specifying and linking the initiation sites and shape of teeth and thereby determining the pattern of the mammalian dentition is in keeping with the developmental role of homeobox genes in pattern formation in Drosophila embryos. Hox-8 appears to be an excellent marker for: homologising tooth position/shape in phylogenetic studies of extant animals (embryos) (Butler and Joysey, 1978), analysing experimental investigations of tooth initiation/shape (Mina and Kollar, 1987; Lumsden, 1988) and experimentally elucidating the role of mammalian Hox genes in late embryonic pattern formation.

Tooth differentiation
Up until the cap stage of tooth development, Hox-8 is
expressed predominantly in the epithelial tissues of the enamel organ, with an extremely limited expression domain in the dental papilla mesenchyme. By contrast, Hox-7 is expressed exclusively in the mesenchyme of the dental papilla and follicle and is absent from the epithelial enamel organ. This pattern suggests an
interacting role for these two genes, perhaps in signalling reciprocal epithelial mesenchymal interactions. Such a postulate is given some support by the experimental observations that the requirement for epithelia to induce cranial bones is correlated with an epithelial induction/maintenance of Quox-7 expression in bone-forming mesenchyme of the quail embryonic cranium (Takahashi et al., 1991).

The expression domains of Hox-8 alter in a significant way at the late cap/bell stage: an important time for epithelial mesenchymal interactions, specifying ameloblast and odontoblast differentiation (Thesleff and Hurmerinta, 1981; Lumsden, 1988). Up to the cap stage, expression of Hox-8 in the dental papilla mesenchyme is restricted to a few cells immediately adjacent to the internal epithelial enamel. However, with odontoblast differentiation at the bell stage, Hox-8 is strongly expressed in a band of odontoblastic and sub-odontoblastic cells of the dental papilla. Contrarywise, up to the bell stage, Hox-8 is strongly expressed in the internal enamel epithelial cells, but as the latter differentiate into ameloblasts, they no longer express

\[ \text{Hox-8. Hox-8 expression therefore switches germ layers with differentiation: being strongly expressed in undifferentiated internal enamel epithelia, but absent in differentiated ameloblasts; weakly expressed/absent in undifferentiated dental papilla mesenchyme, but strongly expressed in odontoblasts and differentiated dental papilla cells. At all times, the stratum intermedium expresses Hox-8 in keeping with its postulated origin from the enamel knot (see earlier). By contrast, Hox-7 is expressed at all times, only in the mesenchymal cells of the dental papilla and follicle. Our previous assertion that Hox-7 expression ceased at the bell stage (MacKenzie et al., 1991a) is incorrect and probably due to differences in the demineralisation regimes used to process the earlier teeth, resulting in their loss of hybridisation signal. Hox-7, but not Hox-8, is expressed in the cells of the dental follicle, which is surprising, given the apparent origin of these from the dental papilla mesenchyme (Palmer and Lumsden, 1987).}

This dental tissue-specific expression of Hox-7 and Hox-8 is shared by other genes. Thus, the homeobox gene S8 is expressed in the mesenchyme of the dental papilla and follicle-like Hox-7 (Opstelten et al., 1991). N-myc is expressed in the dental papilla and follicle-like Hox-7, whilst C-myc is expressed in the enamel organ epithelia-like Hox-8 (Hirning et al., 1991). Moreover, as cartilage differentiates, Hox-7 expression decreases (MacKenzie et al., 1991a,b; Takahashi et al., 1991), whilst cartilage expresses C-myc, but not N-myc (Hirning et al., 1991). BMP-2A, a growth factor of the TGF-β family, is first expressed in the epithelial cells of the bud stage enamel organ, but by the bell stage, following odontoblast and ameloblast differentiation, the transcripts are present in the odontoblast and dental papilla layer (Lyons et al., 1990) just like Hox-8. BMP-2A is also expressed in a similar pattern to Hox-8 in other tissues: distal limb bud ectoderm and mesoderm, developing heart valves, hair follicles etc (Lyons et al., 1990) suggesting a possible interaction between the two.

Hox-7 and Hox-8 and evolution of the vertebrate head

The most cephalic expression boundary of the class 1 homeobox genes is the second branchial arch (Hunt and Krumlauf, 1991; Hunt et al., 1991a,b,c). As in Drosophila, therefore, the mouse head probably has its pattern established by a different set of genes from those that form the trunk, these might include Pax genes (Goulding et al., 1991; Krauss et al., 1991), distalless (Price et al., 1991) and msh-like genes (MacKenzie et al., 1991a,b).

msh-like genes, including Hox-7 and Hox-8, have been identified in mouse, quail, zebrafish and ascidian embryos (Takahashi and Le Douarin, 1990; Holland, 1991). It is significant that these diverged, homeobox genes are all expressed in the developing cranial sense organs: eyes, ears, nose and mouth, skull bones and teeth (MacKenzie et al., 1991a,b; Monaghan et al., 1991). These are the important structures to emerge with the evolution of the vertebrate head and its specialised sensory and feeding structures. Given the importance of these sensory and feeding structures for
nutrition and defence, it is not surprising that control of their development should be conserved and tightly regulated. Thus, for example, changes in the patterning of the dentition may have dramatic consequences in terms of feeding or defence ability and hence, survival of the adult organism. Despite the postulated importance of Hox-7 and Hox-8 in epithelial-mesenchymal interactions regulating development of the eyes, ears, facial processes (nose and mouth) teeth and skull bones, it is perhaps surprising that they are not expressed at other sites of epithelial-mesenchymal interaction in the developing head e.g. the secondary palate (Ferguson, 1988). The secondary palate is a relatively new cranial structure in evolutionary terms, being absent in fish, amphibians, most reptiles, present in a rudimentary form in birds, and fully developed in mammals (Ferguson, 1988). One possible interpretation of these data is that the newer the structure in evolutionary terms, the more diverged the homeobox genes controlling its development may be. A prediction from this hypothesis is that the Hox genes regulating palate development may be even more highly diverged than Hox-7 and Hox-8.

Facial processes and developing limbs: molecular and patterning similarities

Hox-8 and Hox-7 are expressed in the distal epithelium (Hox-8 only) and mesenchyme (Hox-8 more distally restricted than Hox-7) of the medial nasal, lateral nasal and maxillary processes with proximo-distal and cranio-caudal gradients of expression; reminiscent of the progress zone of developing limb buds (Dolle et al., 1989; Yokouchi et al., 1991). This progress zone-like pattern of expression of Hox-7 and Hox-8 in the facial processes is shared by a number of other genes, including the transcription factors, AP-2, (Mitchell et al., 1991), paired box gene Pax-3 (Goulding et al., 1991) distal-less (Price et al., 1991), M-twist, (Wolf et al., 1990), the insulin-like growth factor binding protein IGF-BP-2 (Wood et al., 1990), the retinoic acid/receptor and cellular retinoic acid binding proteins (Ruberte et al., 1990) Dolle et al., 1989, 1990). Frequently, these genes are expressed in a similar pattern in the developing limbs and often in other tissues e.g. choroid plexus, cardiac cushions, teeth etc (Mitchell et al., 1991; Gavin et al., 1990; Bondy et al., 1990; Wood et al., 1990; Dolle et al., 1989, 1990; Ruberte et al., 1990) to Hox-7/8, suggesting possible interactions in their transcriptional regulation. That such an interaction may occur is given further credence by the fact that the cranial epithelium regulates Hox-7 expression by the mesenchyme cells of the developing quail skull (Takahashi et al., 1991), presumably by the release of a soluble factor. Retinoic acid is known to regulate the expression of many homeobox genes (Simeone et al., 1990) and AP2 (Luscher et al., 1989) whilst exogenous application of retinoids results in characteristic defects of the limbs and facial processes (Wedden, 1987; Wilde et al., 1987; Satre and Kochha, 1989). We have previously (MacKenzie et al., 1991b) discussed the similarities between the target tissues in retinoic acid embryopathy (Lammer et al., 1985) and the expression of Hox-7. Identical arguments apply to Hox-8. In addition, it should be noted that retinoid application (both experimentally and in man) can lead to premature fusion of the cranial sutures (craniosynososis) and to missing or supernumerary teeth (Kudusdon, 194a,b; Avery et al., 1991; Beyodoun et al., 1991): all structures that developmentally express Hox-7 and Hox-8.

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