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The developmental specification of the vertebrate skull

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Summary

The initial form of the embryonic bony skull is determined in two ways; cranially, by the relative growth of the developing brain, and facially, by the chondrocranium. Both are essentially acting as structural templates around which the bony components of the skull are assembled. Assuming, therefore, that the specification of form and pattern in the facial skeleton occurs at the formation of the chondrocranium, this paper will focus on precisely how the chondrocranium forms. Any acceptable explanation of chondrocranial morphogenesis must satisfy at least two prerequisites. First, given the constancy of chondrocranial form in vertebrates, any model proposed should be equally applicable to all vertebrates. Second, it should enable us to answer questions of homology concerning the skull and, in particular, provide explanation for those instances where 'homologous' structures have a different (lineage) composition.

From studies limited to a small number of amphibian, avian and mammalian species, it is apparent that chondrogenesis in the vertebrate skull is largely, if not entirely, elicited by epitheliomesenchymal tissue interactions. Analysis of such interactions (and of those promoting osteogenesis) reveals that these are matrix-mediated and, recently, the expression of certain 'relevant' matrix components has been shown to be developmentally regulated in a fashion that correlates with the location and timing of these interactions. From these, and related, observations a morphogenetic model, the so-called 'Flypaper Model', has been proposed to explain the specification of chondrocranial form. A number of predictions arising from that model are currently being tested experimentally and the current status of the model is reviewed. Finally, the ability of this model to satisfy the prerequisites defined above is assessed.

Key words: morphogenesis, skull, chondrogenesis, neural crest, epitheliomesenchymal interactions.

Introduction

Almost two hundred years ago, in 1790, while strolling in a Venetian cemetery, Johannes Wolfgang Goethe and his manservant came across a sheep's skull. We are told (de Beer, 1937) that the skull was picked up by the manservant and handed to Goethe who studied it reflectively. Undoubtedly, Goethe had studied skulls of various types before but it was this particular incident that led him to propose that the vertebrate skull represented a series of modified vertebrae, thus making it serially homologous with the backbone. In fact, Goethe's 'Vertebral theory of the skull' (Goethe, 1820) was wrong but the arguments for and against this interpretation continued for some seventy years before T. H. Huxley delivered the final *coup de grâce* in his Croonian Lecture presented to the Royal Society in 1858. Ironically, one legacy of this idea but one not originally envisaged by Goethe, was that the entire head might be segmented (Goodrich, 1930). Recently the possibility that head mesenchyme, or at least mesodermally derived mesenchyme, might be segmented has been resurrected in the context of somitomeres (e.g. Meier, 1981) and as a result of the claim that somitomeric organization exists transiently in the embryonic vertebrate head (Jacobsen & Meier, 1987) even though recognizable head somites apparently never subsequently form. However, this is currently the topic of much debate since an alternative view, propounded recently, is that the head is largely an evolutionary novelty, unsegmented and 'added' onto the anterior end of a primitive segmented ancestor (Gans & Northcutt, 1983; Northcutt & Gans, 1983; and see Alberch & Kollar, this volume).

Nowadays we do not believe the skull to represent

a series of modified vertebrae or to be a segmented structure (other than in the occipital region and in the gill arches of the viscerocranium) but, nevertheless, we must still ask ourselves how this topographically complex form is specified in development. In fact, the initial form of the vertebrate bony skull is determined embryonically in two major ways; *cranially*, by the relative growth of the developing brain and, *facially*, by the chondrocranium, a solid unsegmented cartilaginous structure which presages the appearance of bone in the developing head (strictly speaking, the chondrocranium consists of 'viscerocranial' and 'neurocranial' components; although the single term 'chondrocranium' will be used throughout this review, most of what follows pertains to the neurocranium). Both cranial and facial factors are essentially operating epigenetically in that they act as structural templates around which, and on which, the bony components are subsequently formed (membrane and endochondral bone, respectively). Thus, specification of primary form of the facial bony skeleton occurs with the formation of the chondrocranium (Bosma, 1976). As is characteristically the case in morphogenesis, 'order builds upon (pre-existing) order' and, therefore, if the shape of the chondrocranial template is altered, then corresponding changes in the form of the skull will result.

The extent to which the basic chondrocranial pattern has been conserved during the course of evolution is striking. This pattern comprises two paired elements, the trabeculae anteriorly and the parachordals posteriorly, which grow and fuse together along mutually contacting edges to form a plate-like cartilage around the margin of which three pairs of capsular cartilages form. These cartilaginous capsules, olfactory, optic and otic, protect and support the organs of smell, sight and hearing, respectively (see right half of Fig. 3). Phylogenetic differences in chondrocranial form may be seen but arise simply as consequences of allometric growth (especially determined by the expansion rate of the enclosed brain and sense organs, e.g. Hanken, 1983), heterochronic shifts in the rate and timing of developmental events (Alberch & Alberch, 1981; Wake, 1980), and sometimes functional adaptations to the environment and circumstances of embryonic development (see Gans, this volume). However, allowing for some limited variation, chrondrocranial pattern is essentially conserved across the vertebrate taxa (de Beer, 1937).

The mesenchyme cells from which the chondrocranium forms have a dual origin, being derived from both mesoderm and from the neural crest ('ectomesenchyme'). Whether or not factors specifying a particular differentiative fate, or eliciting differentiation, are identical for cells derived from both of these two, broadly defined, lineages is not known at this stage (see later), but undoubtedly both sources contribute chondrogenic cells to the chondrocranium. The precise contributions of the two lineages to the bony and cartilaginous elements of the avian skull have been analysed independently in two laboratories (Le Lievre, 1978; Noden, 1978; and see Le Douarin & Couly, this volume), both using the heterospecific quail/chick grafting system and, broadly speaking, there is reasonable concordance between the two sets of data (minor disparities may relate simply to procedural differences in grafting technique). Individual skeletal elements may be entirely mesodermal in origin, or entirely ectomesenchymal or even of mixed composition with contributions from both mesoderm and neural crest. In the last of these three possibilities, there is no mixing, at the level of individual cells, and the cells from each source comprise definable regions of the element in question. However, there is no detectable border or interface between such regions other than those that are revealed by techniques that identify cell origin in heterospecific grafting experiments (Noden, 1983; and see Noden, this volume).

In addition to this 'cataloguing' of lineage composition of the craniofacial skeleton it has also been possible, by analysis of the fate of cells in orthotopic grafts before their differentiation and at progressively earlier intervals postgrafting, to trace migration routes of neural-crest-derived cells as they move around within the developing head prior to reaching the site at which they will differentiate. Thus, in the avian embryo maps of migration route are known for cranial crest cells at all axial levels along the anterior neural tube (Noden, 1975).

The chondrocranium is, therefore, a topographically complex shape, composed of cartilage differentiating from mesenchyme cells of two lineage sources. At this point, it will be evident that elucidating mechanisms determining skeletal pattern in the head is (perhaps) a unique problem. Where skeletal tissues form in the trunk or limb, no large-scale rearrangements of cells occur as they do in the head. Thus, skeletogenic cells at limb and trunk sites might be thought of as differentiating *in situ* whereas in the head a further dimension exists i.e. that of extensive rearrangements of cells (principally neural-crest-derived) both actively, by migration, and passively, by displacement due to growth.

The requirements of a model

I suggest that the question of how skull form is specified in the embryo might be better restated as 'how is chondrocranial form specified...?'. Further-

more, any causal model proposed to explain morphogenesis of the chondrocranium must satisfy several prerequisites. First, given the relative constancy of chondrocranial pattern in the vertebrates, any model put forward should be equally applicable to all vertebrates from Agnatha to Man. It should **not** be unique or specific to any particular experimental system that might have been used to generate the underlying data. Second, the model should enable us to answer questions concerning homology of parts of the skull and, in particular, provide an explanation for those instances where 'homologous' structures in two or more species have a different (lineage) composition. Variation in lineage composition of individual elements clearly creates interpretational problems when homologizing structures, where embryonic origin rather than function or morphology is the chief criterion (Presley & Steele, 1976; Bellairs & Gans, 1983; and discussed at length in de Beer, 1937). A similar challenge is presented by those situations where experimental perturbation, usually created by heterotopic grafting, can shift the lineage composition of those 'mixed' elements to which both NC and mesoderm-derived cells normally contribute. For example, grafts of first branchial arch NC to more posterior myelencephalic levels in the avian embryo not only create ectopic mandibular elements but, of relevance to this question, considerably increase the crest contribution to the otic capsular cartilage which was nevertheless described as 'normal in both shape and size, despite the substitution of crest-derived mesenchyme for some mesoderm cells' (Noden, 1983). Such changes in composition of individual elements, although created experimentally, parallel changes arising phylogenetically (i.e. during the course of evolution). Morphogenetic models must encompass both classes of phenomena.

A third point, but one which is an ancillary question rather than a prerequisite of any model, is to ask if mechanisms initiating and controlling chondrogenesis in the head are in any sense qualitatively different from those mechanisms underlying cartilage formation in the rest of the axial skeleton, i.e. along the length of the vertebral column. It has been suggested elsewhere that 'induction' of cartilage at three different sites, head, trunk and limb, probably reflects three different mechanisms (Kratochwil, 1983). Yet, in the absence of a clear and comprehensive molecular understanding of the events leading to chondrogenesis in any one of these three systems, such an assumption seems unjustified and the possibility of a common mechanism should not be dismissed too readily (discussed in Hall, 1987).

Skeletogenic differentiation in the head

Although currently we know virtually nothing of the commitment state, potency or precise lineage of the cells fated to give rise to the chondrocranium or indeed any of the head skeleton, it is clear that epitheliomesenchymal tissue interactions, operating locally, have some fundamental role in causing head mesenchyme to differentiate into cartilage and bone. For instance, isolated premigratory cranial neural crest, from amphibian or avian embryos, lacks the ability to form cartilage or bone *in vitro* and yet in association with certain epithelia, *in vitro* or *in vivo*, cranial crest will differentiate chondrogenically and osteogenically (Drews, Kocher-Becker & Drews, 1972; Epperlein & Lehmann, 1975; Tyler & Hall, 1977; Bee & Thorogood, 1980; Graveson & Armstrong, 1987). Thus, factors extrinsic to the cells are causally implicated but, given our current ignorance over the commitment state of the cells concerned, these factors may simply constitute a permissive environment or alternatively be providing an instructive signal (Wessells, 1979). It has been argued elsewhere that consequently such interactions are best described in more neutral terms as 'chondrogenesis-promoting' or 'osteogenesis-promoting' until we acquire evidence of something more than just a simple elicitation of differentiation (Thorogood, Bee & von der Mark, 1986; Thorogood, 1987). Various experimental strategies exploited over a number of years have provided a limited understanding of the mechanisms involved. Thus, removal of ectoderm from the presumptive cranial aspect of the avian head or ablation of underlying neuroepithelium results in an absence of calvarial and/or frontal membrane bones (Schowing, 1968; Tyler, 1983). Such epitheliomesenchymal interactions underlying membrane bone formation have been investigated in an extensive range of tissue dissociation/recombination experiments carried out *in vitro* by Hall and colleagues, working primarily on avian mandibular development (reviewed Hall, 1982, 1984). One of several important conclusions to emerge from this programme of work was that osteogenesis-promoting interactions are matrix-mediated, possibly effected by a collagenous component of the mandibular ectodermal basal lamina (Hall, van Exan & Brunt, 1983). However, mandibular mesenchyme is capable of mounting an osteogenic response to epithelia from sites other than in the head (e.g. limb bud and dorsal trunk ectoderms) so clearly this interaction is not specific in the strictly instructive sense. Furthermore, although the presence of an epithelium is necessary for osteogenesis to ensue, the form or morphology of the developing bony element is clearly a property of the mesenchymal partner in the recombination and is not

determined by the epithelium (Hall, 1981).

Parallel studies on the avian embryo, in my own laboratory, examining the developmentally earlier, chondrogenesis-promoting, tissue interactions (using a comparable range of transfilter culture techniques and ultrastructural assessment of the *in vivo* interface at which the interaction takes place), reached similar conclusions; that is, chondrogenesis also is elicited by a matrix-mediated interaction (Smith & Thorogood, 1983; Thorogood & Smith, 1984). Immunocytochemical analysis of extracellular matrix at the interface between the two cell populations in one such chondrogenesis-promoting interaction (between presumptive pigmented retina and mesencephalic-crest-derived periocular mesenchyme) revealed a compositional change correlated with the duration of the interaction. More precisely, type II collagen, the predominant collagen species of cartilage matrix and therefore regarded as a specific marker for chondrogenic differentiation, is transiently expressed at the interface, i.e. at the basal aspect of the epithelial tissue. Further investigation revealed that type II collagen is expressed elsewhere in the head, in addition to previously reported ocular locations within the corneal stroma and vitreous (see Figs 1, 2). Principally these locations are around the basolateral aspects of the diencephalon, mesencephalon and rhombencephalon, around optic and otic vesicles (Thorogood *et al.* 1986) and around the olfactory conchi (Croucher & Tickle, personal communication). In fact, distribution of this collagen species maps precisely with the sites of those interactions known to generate the constituent parts of the chondrocranium (see Fig. 3). Not only is the spatial pattern of expression coincident but so too is the timing of transient type II expression which correlates with the duration of the interactions concerned (Thorogood *et al.* 1986). Thus, there is a strong spatial and temporal correlation between the transient expression of type II collagen and the sites and duration of matrix-mediated interactions which generate the component parts of the chondrocranium.

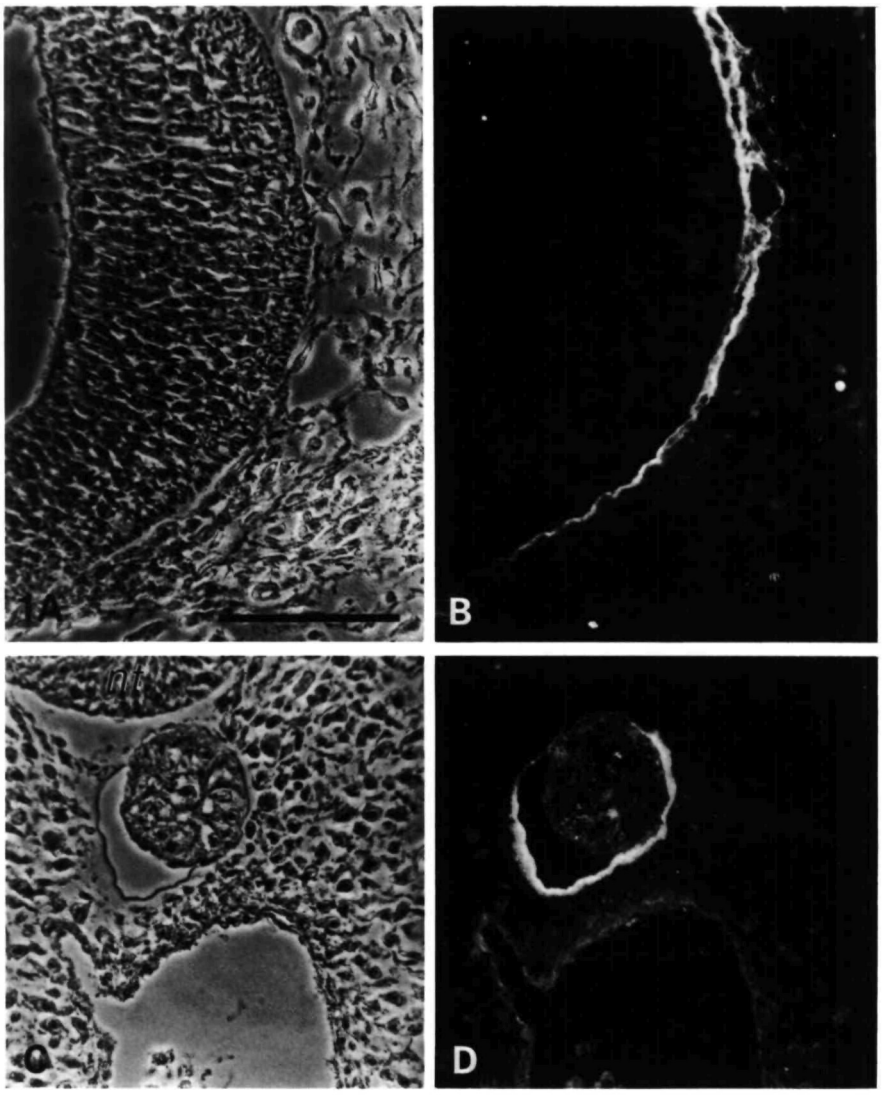

Fig. 1. Phase-contrast (A,C) and immunofluorescent (B,D) micrographs prepared using affinity-purified, polyclonal rabbit anti-chick type II collagen, followed by incubation in an FITC-conjugated goat anti-rabbit. (A,B) T.S.rhombencephalon of stage-16 quail embryo. Note staining only around basal surface of neuroepithelium. (C,D) T.S.notochord at cervical level of stage-24 quail embryo. Staining, predictably, in perinotochordal sheath (artefactually detached on one side), plus a very small amount of staining within the notochord itself. Bar, 30 μm.

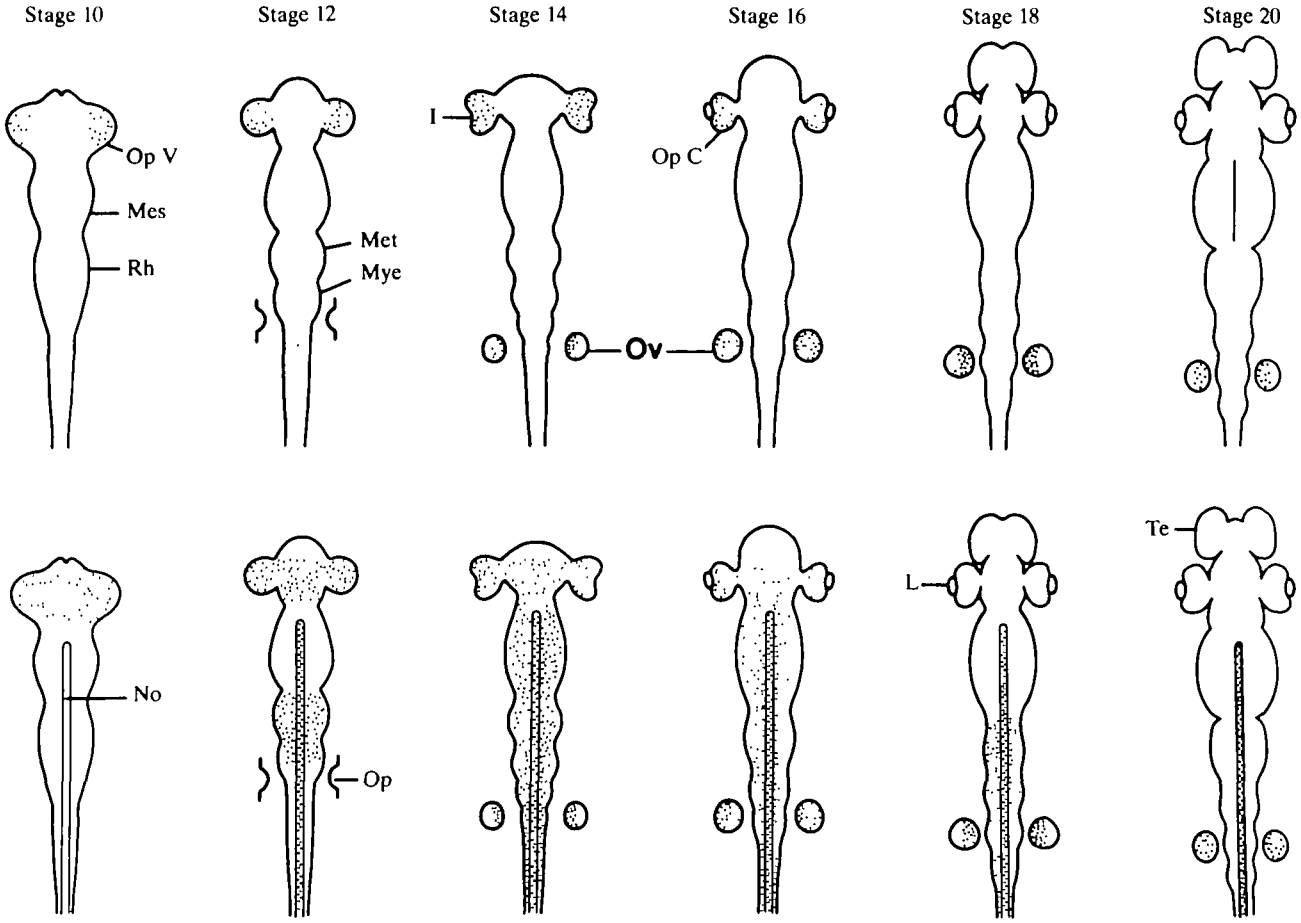

Fig. 2. Diagram summarizing the distribution of type II collagen around the brain and notochord of the early avian embryo. The upper row illustrates the dorsal aspect, the lower row, the ventral aspect, for a series of developmental stages. OpV, optic vesicle; Mes, mesencephalon; Rh, rhombencephalon; No, notochord; Met, metencephalon; Mye, myelencephalon; Op, invaginating otic pit; I, invaginating optic vesicle; Ov, otic vesicle; OpC, optic cup; L, lens; Te, telencephalon. Not drawn to scale (taken from Thorogood *et al.* 1986).

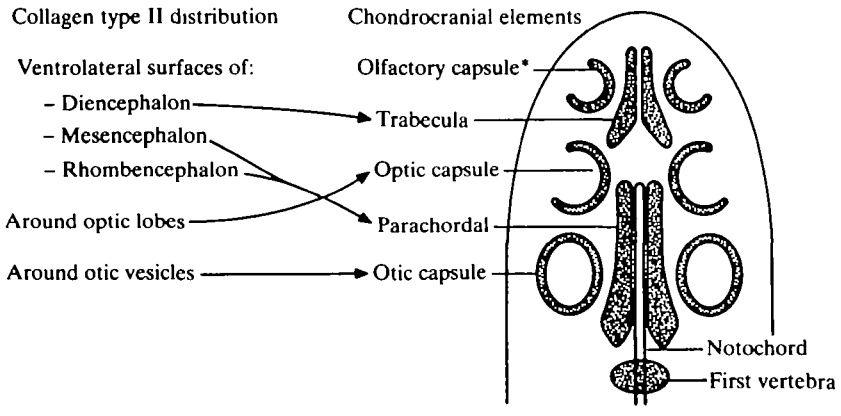

Fig. 3. Diagram relating the distribution of collagen type II (as depicted in Fig. 2) to the component parts of the cartilaginous neurocranium which form as a result of tissue interactions at these sites (the viscerocranial part of the chondrocranium is not shown; the neurocranial part has been idealized to a general vertebrate pattern. Taken from Thorogood *et al.* 1986). ★ Although not shown in this diagram there is a prior, transient, expression of type II collagen relating to the olfactory capsules; it has recently been located around the basal surface of the forming olfactory conchi but at stages somewhat later than the range illustrated in Fig. 2 (Croucher & Tickle, personal communication).

This result is reminiscent of the notochord–sclerotome interaction in which sclerotome cells, derived from the medial aspect of the somite, migrate towards the midline, undergo a matrix-mediated interaction with the notochord and differentiate chondrogenically to form the cartilaginous primordia of the vertebral bodies. The perinotochordal matrix or 'sheath' contains, in addition to typical basal lamina components, two components characteristic of cartilage, i.e. type II collagen and cartilage-specific proteoglycan, synthesized briefly by the notochord itself. *In vitro* exposure of sclerotome cells to perinotochordal sheath, or to either of these two components, elicits a sclerotomal synthesis of the same two molecules, i.e. the collagen and the proteoglycan (Kosher & Church, 1975; Lash & Vasan, 1978), and in a functionally stable form (Vasan, 1987) as a chondrocytic phenotype becomes established.

The origin of the transient type II collagen in the present system is crucially important if we are to understand the causal sequence of events at the cellular level. Is it produced by the mesenchyme cells at these interfaces, destined to differentiate into cartilage and, if so, why is it only briefly expressed? At some sites, completion of the interaction (i.e. the establishment of chondrogenic commitment in the mesenchyme) and disappearance of the type II are followed by a period of up to several days before overt differentiation commences within the mesenchyme. Currently we believe that the type II collagen is epithelially derived and, as might be deduced from Fig. 2, largely derived from neuroepithelium. Although type II is typically a stromal collagen and characteristically made by chondrocytes, there are three precedents for epithelial synthesis – the notochord (see earlier), the corneal epithelium (Linsenmayer, Smith & Hay, 1977) and lastly, a true neuroepithelium – the neural retina (Smith, Linsenmayer & Newsome, 1976). These synthesize the type II collagen found in the perinotochordal sheath, primary corneal stroma and the vitreous, respectively. Evidence in support of a neuroepithelial origin in the present system comes from two sources. First, the precise ultrastructural location of the type II at the tissue interfaces concerned has been defined using TEM immunogold techniques and reveals that, during the interactions, transiently expressed type II is closely associated with the basal lamina of the epithelium and is not around adjacent mesenchyme cells (see Fig. 4). If synthesized by the mesenchyme, a general distribution in the extracellular compartment might be anticipated but this is not seen (although a polarized secretion by those mesenchyme cells at the interface cannot be excluded at this stage). Second, the most unequivocal evidence comes, perhaps predictably, from *in situ* hybridization experiments using a cDNA probe specific to chick type II collagen mRNA. Work in progress indicates that type II message is detectable in cells of the notochord, otic vesicle, neuroepithelium and neural tube 'long before' message is detectable in the responding mesenchyme (Masando Hayashi – personal communication). Whereas the previously described immunocytochemical evidence simply defines the spatiotemporal distribution of the accumulated gene product, *in situ* hybridization data, when complete, will permit a definition of that window in developmental time during which the type II genes are transcriptionally active. It is likely that such data will be esssential for a full understanding of the mechanism of chondrocranial pattern specification.

How chondrocranial pattern might be specified – the 'Flypaper Model'

The previous section deals with identification of a possible signalling molecule which, it has been speculated, is produced by an epithelium and which directs, or at least influences, in some unspecified way, the differentiative fate of responding mesenchyme. Given that in craniofacial development we are dealing with a system in which there is extensive mesenchymal cell rearrangement, by both active cell migration and by passive displacement, then the mechanism that ensures precise localization of those mesenchyme cells capable of mounting a response to such a signal is crucially important. Thus, control of cell migration, particularly as it applies to neural crest, becomes fundamental in skull morphogenesis and for two reasons. First, it is the event of cell migration that creates opportunities for interactions to take place between epithelia and migrating neural crest cells or their progeny. Second, it is the time and position of arrest of migration, within the developing head, that determine precisely where ensuing skeletogenic differentiation will be expressed (Thorogood, 1981). Through closer study of arrest of migration we have found that matrices derived from certain regions of epithelium in the embryonic head can cause arrest of NC cell motility in culture. Thus, mesencephalic crest cell behaviour is dramatically changed on plastic surfaces 'conditioned' by pigmented retina extracellular matrix (Fig. 5); the cells cease directional movement, become spindle-shaped or round-up and may even aggregate in small groups (Thorogood & Smith, 1984; Yallup, Smith-Thomas & Thorogood, 1988). This behavioural response closely parallels events occurring *in vivo* when mesencephalic NC cells, having arrived in the periocular region, cease migrating and condense around the basal aspect of the optic vesicle/optic cup as a prelude to differentiating into (scleral) cartilage several days later.

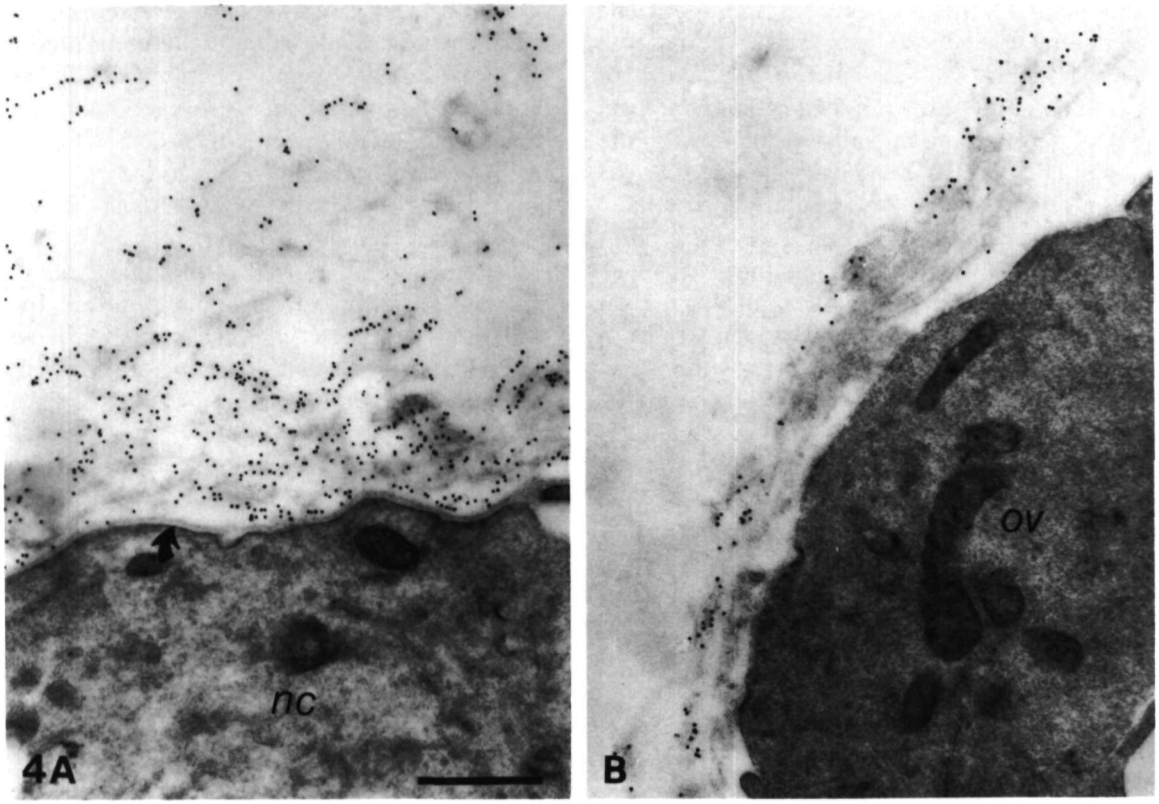

Fig. 4. Transmission electron micrographs illustrating pre-embedding immunogold staining. Tissue was lightly fixed in 4 % paraformaldehyde/0·25 % glutaraldehyde, incubated initially in affinity-purified, rabbit polyclonal anti-chick type II collagen, and subsequently in Staphylococcal protein A conjugated with 15 nm gold particles, before a standard 2·5 % glutataraldehyde fixation, post-osmication and resin embedding. The sections shown here are of sections left unstained (by standard techniques) in order that the gold particles are clearly visible. (A) Perinotochordal sheath of stage-14 chick embryo; *nc*, basal cytoplasm of single notochordal cell; arrow, basal surface of cell overlaid by basal lamina. Note abundance of type II staining in the reticulate lamina; conventional staining of adjacent sections confirms that the gold particles coincide with fibrous components of the extracellular matrix. (B) Matrix surrounding a partially isolated otic vesicle from a stage-16 chick embryo. Here, even in the unstained specimen, the location of the gold particles on the fibrous components of the matrix is evident; *ov*, a single epithelial cell of the otic vesicle wall; arrow, basal surface. Bar, 200 nm.

These studies on factors which might position NC cells by causing localized arrest of migration, together with the previously described studies on tissue interactions affecting differentiative fate, have been used as the basis for formulating a morphogenetic model – the so-called 'Flypaper Model' (Thorogood, 1987). This is best explained stepwise and sequentially but, bearing in mind that this is, in all probability, a continuous and stochastic process, the following apply.

(1) The early developing head is best viewed as a convoluted bag of folded epithelium (undergoing its own morphogenesis) enclosing an extensive extracellular compartment which is only sparsely populated by mesodermally derived cells.

(2) Into the matrix-filled spaces, NC cells migrate autonomously and opportunistically, migrating wherever there is matrix capable of supporting their locomotion.

(3) Thus, the migratory behaviour of these cells is, to some extent, controlled by extracellular matrix and, in particular, by epithelially derived matrices. Certain matrices will serve a 'trapping' function causing localized arrest of cell migration (as the pigmented retina does *in vitro*).

(4) At such sites, cells will accumulate and undergo a matrix-mediated interaction with those epithelia and differentiate into cartilage. Thus, the pattern of cell trapping will epigenetically determine the form of the chondrocranium (certain matrix molecules may serve a dual role, functioning in both steps 3 and 4). This possibility emerges from observations that type II collagen, shown to be present at the tissue interfaces concerned, is very potent in arresting NC cells in culture, whereas fibronectin and laminin, widely distributed matrix glycoproteins, apparently promote migration *in vitro* (B. Yallup, unpublished observations).

(5) The spatial pattern of these 'trapping' events and the ensuing tissue interactions are a consequence of epithelial folding (i.e. of epiblast and, later, of ectoderm and neuroepithelium). Thus, topographical differences in invagination and evagination of the various epithelia in the head will create different patterns of cell trapping. Precisely how epithelially associated matrix in the developing head becomes regionally different in composition is not clear. The uniform absence of immunocytochemically detectable type II, prior to stages 9–10, suggests that it arises regionally during epithelial morphogenesis and is not present in the 'unfolded' blastoderm (see Fig. 6).

The model therefore reduces control of chondrocranial form to simply two parameters. First, the manner in which the various epithelia undergo their own morphogenesis and, second, the rate and timing of neural crest migration (passive displacement of mesenchyme cells is undoubtedly implicated too but has not been dealt with separately at this stage of model development). It is generally recognized that minor quantitative changes in particular developmental programmes can have profound morphological consequences (Gould, 1977; Alberch, 1982) and I propose that minor quantitative shifts in these two parameters underlie much of the phylogenetic diversity of skull form and congenital abnormality of the skull. Different patterns of cell trapping will generate different, and sometimes new, chondrocranial forms. Because the chondrocranium serves as a structural template for deposition of the bony elements of the skull (at least facially), then one further epigenetic consequence will be a change in overall skull form. Genetic control of skull form will therefore be mediated indirectly through gene loci affecting these two

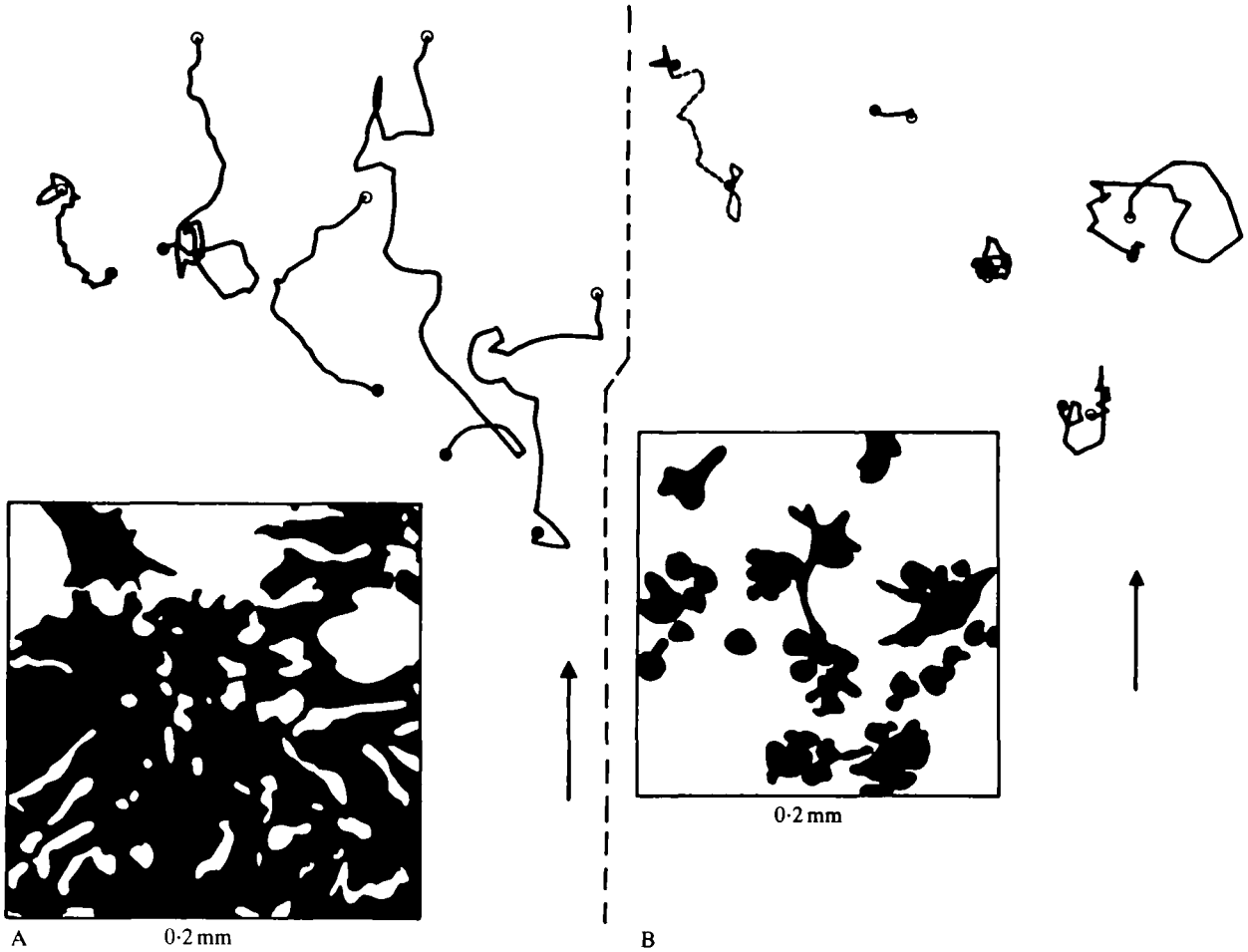

Fig. 5. Traces of pathways of cell migration, over a 4 h period, at the margin of outgrowth from explanted, stage-9, chick mesencephalic neural crest (arrows indicate radial direction away from centre of explant). (A) Control cells grown on plastic. (B) Cells grown on plastic previously conditioned with matrix deposited by stage-16 pigmented retina; the epithelial sheet is stripped off prior to explantation of crest cells. Note the extensive and directional migration of the control cells and the reduced path length and apparent lack of directionality of those cells on matrix. The inset boxes represent sample areas at the outgrowth margin from which such traces were made; black, represents the cell sheet; white, the intercellular areas. See text for further details (taken from Yallup *et al.* 1988).

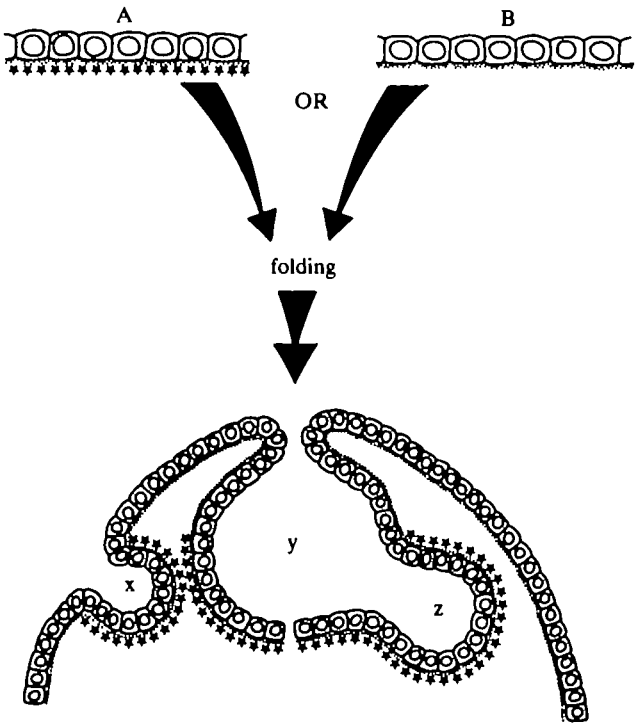

Fig. 6. Diagram depicting the possible distributions of a trapping component in matrix (depicted as ★; e.g. type II collagen) prior to epithelial morphogenesis in the head. (A) Uniform distribution of trapping component; (B) Trapping component uniformally absent. As a result of folding, the distribution of trapping component becomes expressed regionally around areas of high radius of curvature, either because it disappears locally (subsequent to A) or because it appears locally (subsequent to B). The lower part of the diagram is a composite to indicate the broad range of possible epithelial morphogenesis and known distribution of type II collagen. x, Invagination from surface ectoderm, as in the otic pit/vesicle; y, neural tube; z, evagination from the neural tube as in the optic vesicle. Analysis indicates that B is correct in that type II collagen is not detectable before stage 10 and its subsequent appearance is concomitant with, or immediately follows, major events of epithelial morphogenesis in the head. However, the time of first appearance of type II mRNA is not currently known.

parameters, such as regulation of synthesis and turnover of matrix components, cell surface receptors for matrix components and cytoskeletal organization and function. It could be argued that skull form is not so much 'specified' in a strict genetic sense but determined by local interactions between cells and their environment, operating within what has been called the 'epigenetic domain' (Alberch, 1982).

Testing the model

A number of predictions can be made which we are currently testing experimentally and which will be briefly reviewed here:

(1) If 'trapping' underlies the arrest of cell migration, as suggested, then migrating neural crest cells should express a receptor or binding protein of some sort to mediate attachment to those matrix components ostensibly responsible for trapping. Current work reveals that premigratory cells synthesize, and migrating cells express at their cell surface, a binding protein for type II collagen – Anchorin CII (Thorogood, Hoffmann & von der Mark, in preparation). Anchorin CII is a 34K membrane protein typically expressed by chondrocytes and which has a high-binding affinity for the abundant type II collagen normally found in the chondrocytes' matricial environment (von der Mark, Mollenhauer, Pfaffle, van Menxel & Muller, 1986). Immunocytochemical study of cultured mesencephalic crest has shown that Anchorin CII is expressed by only a subpopulation of cells (which raises interesting questions about neural crest population composition) and it is for these cells that we propose Anchorin CII mediates trapping as they encounter type II during their migration in the developing head (see Fig. 7).

(2) Supplementation of the endogenous trapping matrix, by introducing exogenous type II collagen ectopically somewhere along the migration route, should cause precocious arrest of migration and may even result in ectopic cartilages being formed. Experiments using implanted microbeads as a support medium for a variety of matrix molecules are currently in progress.

(3) The parallel between chondrogenesis-promoting interactions in the head and in the rest of the axial skeleton was briefly mentioned earlier. From data on the notochord/somit interaction, it can be predicted that other matrix components, thought or known to have a role in this interaction (Vasan, 1987), will show similar patterns of distribution to that displayed by collagen type II during craniofacial development. Cartilage-specific proteoglycan is found not only in cartilage but also in perinotochordal sheath along with type II. One matrix marker that has been used for locating this, is the monoclonal antibody MZ15, which binds keratan sulphate and therefore recognizes the keratan sulphate-rich domain on the proteoglycan in both cartilage (Zanetti, Ratcliffe & Watt, 1985) and perinotochordal sheath (Smith & Watt, 1985). Mapping of MZ15-staining patterns during craniofacial development in the avian embryo reveals a large degree of codistribution with the previously described patterns for type II collagen. For instance, both molecules are present at the same stages around the otic vesicle (Heath & Thorogood, in preparation). However, the lack of codistribution at some other sites (e.g. no MZ15 staining around the optic

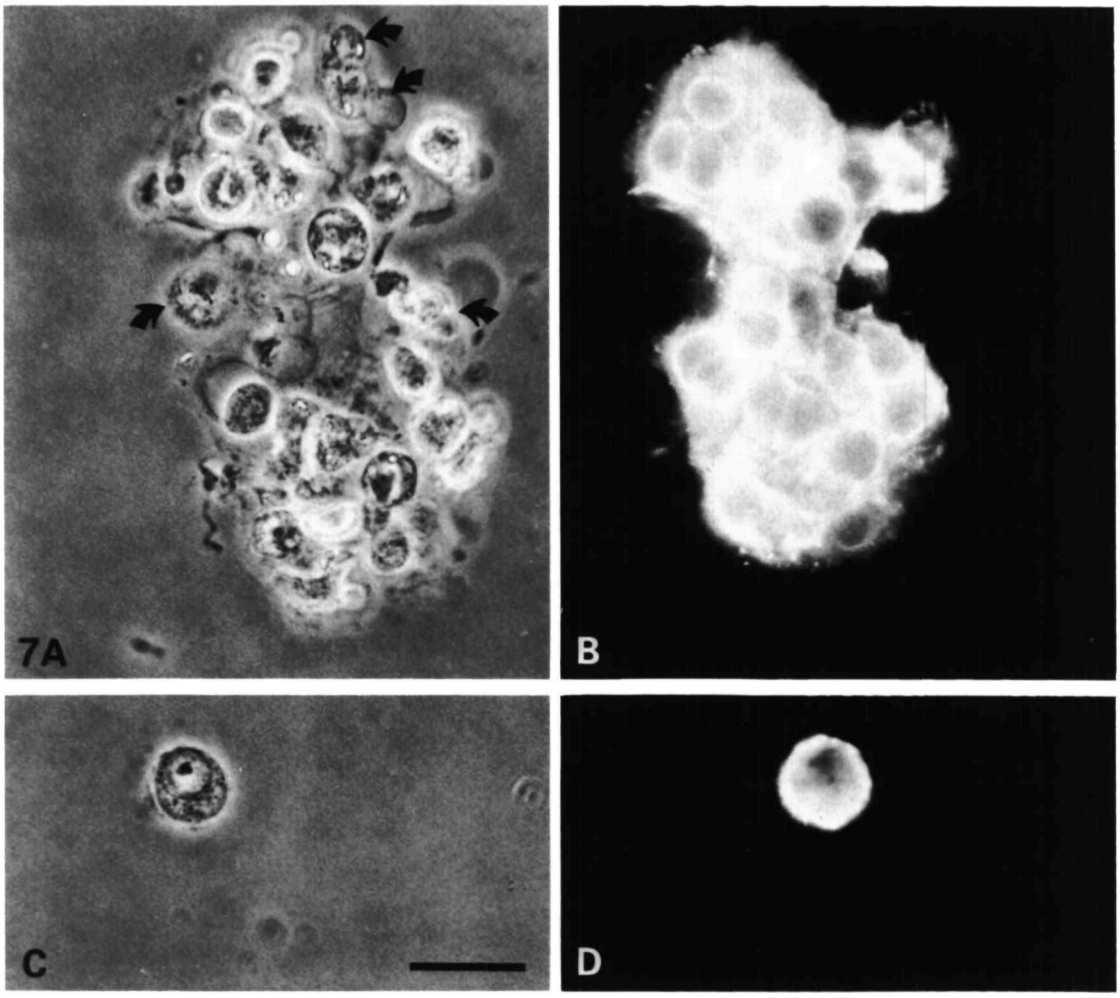

Fig. 7. Phase-contrast (A,C) and immunofluorescent (B,D) micrographs of living mesencephalic neural crest cells in culture, prepared using a polyclonal rabbit anti-chick chondrocyte Anchorin CII, followed by incubation in an FITC-conjugated goat anti-rabbit. (A,B) A cluster of crest cells; arrows indicate cells which do not express Anchorin CII in B. (C,D) An isolated crest cell expressing Anchorin CII (N.B. although initially flattened at the outset of staining, as the cells are unfixed, they tend to round up during the process). Bar, 10 μm.

vesicle) indicates that this will not be a simple story of complete codistribution. Further work using more specific markers, such as monoclonals against the core protein of cartilage-specific proteoglycan, might be especially valuable in this context.

(4) The results described earlier arise entirely from work on avian embryos. It is anticipated that the Flypaper model will be applicable generally to vertebrates to describe an evolutionarily conserved developmental mechanism underlying the phylogenetically constant chondrocranial pattern. If that is so, then comparable data should emerge from investigation of other vertebrate taxa. We have started such a comparative study and it has already emerged, from our current work on mammalian craniofacial development, that type II collagen is found on the basal aspect of some neuroepithelia and anlagen of the sense organs of the mouse embryo (Wood & Thorogood, in preparation). A pattern of transient expression is found which is similar although not quite identical to that described for the bird. Equally important will be investigations of type II distribution and associated cell–matrix interactions in the 'lower' vertebrate taxa, in particular the agnathans, teleosts and amphibians.

Counter evidence?

The theme of the Flypaper model is that during early craniofacial development, local interactions between epithelia and mesenchyme will determine not only differentiative fate of the mesenchyme cells but also specify skeletal pattern (ectomesenchyme has been dealt with principally but mesodermally derived mesenchyme can be incorporated into the model). This is unlike our current understanding of events in the developing limb where primary specification of skel-

etal pattern is normally regarded as residing in the mesoderm (Saunders & Reuss, 1974; and see later). However, the model also is in contradiction to Noden's hypothesis of 'regional morphogenetic specification' which postulates that much of the skeletal pattern in the head is 'programmed' in neural crest cells prior to their migration, possibly even in crest precursors within the neuroepithelium (Noden, 1983, 1986). This proposal is based on experiments in which heterotopic grafts were made, between quail and chick, of first arch crest into sites formerly occupied by the host's own second and third arch crest (anterior- and mid-myelencephalic levels, respectively). Graft cells migrated along routes normal for second or third arch crest cells but proceeded to give rise to a second, i.e. duplicate, set of first arch skeletal structures. Local interaction, of the sort proposed earlier, would have little, if any, role in pattern specification of this type. These experiments are clearly important and the result is probably telling us something fundamental about skeletal patterning, but is 'regional morphogenetic specification' a feasible interpretation? More specifically, do these results constitute an unanswerable challenge to the Flypaper model?

It is important to remember that although morphogenesis is 'driven' at the level of individual cells (by changes in cell shape, adhesivity, contractility, etc.) it is the collective activity of those cells that generates tissue form and pattern; thus morphogenesis is a population phenomenon. It is difficult to think of a molecular or cellular mechanism maintaining pattern specification within a cell population unless the cells maintain their spatial relationships to each other. However, maintenance of neighbour-to-neighbour contact between cells seems unlikely during the large-scale migration that crest cells undergo. One further problem is that not only does first arch crest produce ectopic first arch derivatives but so too does more rostral crest. Crest that normally gives rise to frontonasal, maxillary and trabecula skeleton will give rise to a duplicate first arch skeleton when grafted to myelencephalic levels (Noden, 1983). This last observation seems incompatible with the idea of an early (premigratory) specification of pattern appropriate for each axial level. In fact, the result, arguably, might be used as evidence for a local specification of pattern occurring during or after migration. What may be important is whether or not crest cells encounter type II collagen, and similarly active matrix molecules, during their migration and, if so, *where* and *when*? (in fact, a correlation has been made between lack of chondrogenic commitment at the end of migration and the absence of type II collagen along that particular migration route; see p. 508, Thorogood *et al.* 1986). Local interactions occurring between cranial crest cells, derived from perhaps any axial level, and an appropriate epithelial environment might elicit the formation of an ectopic, duplicate mandibular skeleton if the formation of a mandible normally is the result of epigenetic phenomena.

Given the extensive rearrangements undergone by cell populations of both mesenchymal lineages during craniofacial development, it makes functional sense that another tissue might provide a frame of positional reference for subsequent differentiation of mesenchymal cells. The epithelia of the head, although undergoing extensive invaginations and evaginations, display little cell mixing and therefore provide just such a planar reference system. It is perhaps significant that similar conclusions have been reached independently in experimental studies of tooth development (Lumsden, 1987), the patterning of sensory neurites (Lumsden & Davies, 1984) and from a paleontological assessment of the evolution of the vertebrate dermal skeleton (Thomson, 1987). The apparent absence of such a role for ectoderm in the limb may simply reflect an intrinsic difference between axial and appendicular skeletons or relate to the fact that limb mesenchyme does not display cell rearrangements on the scale seen in the developing head.

Does the model satisfy the prerequisites?

Two prerequisites were stipulated at the outset. The first was that any acceptable model should be generally applicable to all vertebrates. With the Flypaper model, although it arises from work on avian embryos, there is no inherent reason why it should not be applied to the embryos of other vertebrate taxa. Preliminary observations on mammalian embryos suggest that this is indeed possible. Quantitative variation of the parameters of the model could generate a variety of chondrocranial patterns and, therefore, of skull form. It also makes functional sense that the developing brain and sense organs should, themselves, determine precisely where their protective and supportive tissue will be formed. The model also lends itself to interpretation, and prediction, of congenital abnormality by permitting a definition of 'sensitive periods' during the specification of skull form.

The second prerequisite was that the model should help to explain different lineage composition of otherwise homologous cartilages and, similarly, to explain the experimentally created alterations in proportional contributions from the two mesenchymal lineages, to 'mixed composition' skeletal elements which are otherwise normal in shape and size. Although in this review less emphasis has been placed

on craniofacial mesoderm, there is no evidence that mesoderm cells cannot respond to matrix signals or cues in a fashion identical to ectomesenchyme cells (and in the sclerotome, they clearly do). In fact, the Flypaper model does not distinguish where mesenchyme cells come from. The (neuro)epithelium simply specifies, to a responsive mesenchyme, where and when cartilage should form. Viewed in this way, differential lineage composition of 'homologous' skeletal elements, in terms of ectomesenchymal or mesodermal contributions, becomes largely irrelevant.

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