The triple origin of skull in higher vertebrates: a study in quail-chick chimeras

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SUMMARY

We have used the quail-chick chimera technique to study the origin of the bones of the skull in the avian embryo. Although the contribution of the neural crest to the facial and visceral skeleton had been established previously, the origin of the vault of the skull (i.e. frontal and parietal bones) remained uncertain. Moreover formation of the occipito-otic region from either the somitic or the cephalic paraxial mesoderm had not been experimentally investigated. The data obtained in the present and previous works now allow us to assign a precise embryonic origin from either the mesoderm, the paraxial cephalic mesoderm or the five first somites, to all the bones forming the avian skull. We distinguish a skull located in front of the extreme tip of the notochord which reaches the sella turcica and a skull located caudally to this boundary. The former (‘prechordal skull’) is derived entirely from the neural crest, the latter from the mesoderm (cephalic or somitic) in its ventromedial part (‘chordal skull’) and from the crest

for the parietal bone and for part of the otic region. An important point enlighten in this work concerns the double origin of the corpus of the sphenoid in which basipresphenoid is of neural crest origin and the basipostsphenoid is formed by the cephalic mesoderm. Formation of the occipito-otic region of the skeleton is particularly complex and involves the cooperation of the five first somites and the paraxial mesoderm at the hindbrain level. The morphogenetic movements leading to the initial puzzle assembly could be visualized in a reproducible way by means of small grafts of quail mesodermal areas into chick embryos.

The data reported here are discussed in the evolutionary context of the ‘New Head’ hypothesis of Gans and Northcutt (1983, Science, 220, 268-274).

Key words: neural crest, somites, cephalic mesenchyme, facial skeleton, origins of frontal, parietal, occipital bones, sphenoid bones

INTRODUCTION

The skull of vertebrates consists of bones of membranous and cartilaginous origin, which have the double role of protecting the brain and sense organs and of providing the animal with a masticatory apparatus, which can also function as a prehensile system for capturing preys.

The participation of the ectomesenchyme (also called mesectoderm), derived from the neural crest, in the facial and visceral skeleton was first recognized at the end of the last century by the pioneer works of Kastschenko (1888), Goronowitsch (1892, 1893) and Platt (1893, 1897) and was later well documented in lower vertebrates (see Hördadius, 1950 for a review). The quail-chick marker system in the avian model eventually allowed the demonstration that in higher vertebrates the skeleton is entirely of neural crest origin (reviewed in Le Douarin, 1982).

Two points, however, remained unclear about vertebrate head skeletogenesis. One concerned the origin of the vault of the skull, composed of the parietal and frontal bones, which were considered by Le Lièvre (1978, see also Le Douarin, 1982) as essentially derived from the cephalic mesoderm. The second is the extent of the participation of the vertebrae to the occipital region of the skull.

The first problem clearly rose when, in one of our earlier works on the embryology of the vertebrate head (Couly and Le Douarin, 1985, 1987, 1988), we scrutinized the fate of the cephalic paraxial mesoderm (Couly et al., 1992) from the early somitic stages onward. We realized that the paraxial mesoderm essentially yielded muscle structures whereas its contribution to the skull was reduced to the sphenoidal and otic regions.

No cells of paraxial cephalic mesoderm were found to participate in the skull vault i.e. to the frontal and parietal bones. We then decided to reinvestigate this question by using the classical substitution technique of defined embryonic territories between quail and chick embryos (Le Douarin, 1969, 1973). The idea was to study the fate of the cephalic neural folds from an earlier stage of development than envisaged in previous works and to follow carefully the development of the dermis and membrane bones forming the vault of the skull. The experiments were done at the late neurula stage (3-somite-stage) and showed an early migration of the cephalic neural crest cells, which had so
far remained unnoticed and which yields the dermis and membrane bones of the entire vault of the skull including the frontal and the parietal bones and their sutures.

The remaining question of the participation of somites in head morphogenesis is an ancient one. This idea was first expressed in the XIXth century by Goethe (1791) and Oken (1807) (see Jeffs and Keynes (1990) and references therein) and was formulated in an extreme form in the vertebrate theory of the skull, according to which the skull is made up of one (or three) somehow modified vertebrae. Progress in embryology and comparative anatomy however, showed that only the occipital region of the skull could be considered of vertebral origin (reviewed in Augier, 1931). Moreover, it is thought that no incorporation of vertebrae into the skull exists in the skull of the agnathes and that, during evolution of vertebrae, the enlargement of the head involved the contribution of an increasing number of cephalic vertebrae to form the occipital bone of the gnathostomes. No experimental analysis, however, had so far been conducted to investigate the embryonic origin of the occipito-otic region of the skull.

This old problem was rejuvenated by the discovery in vertebrates of genes characterized by a highly conserved sequence of nucleotides, the homeobox (Hox genes), encoding proteins that regulate the expression of other genes (see Gehring, 1987; De Robertis et al., 1990; Kessel and Gruss, 1991 for reviews and references therein).

The possible involvement of Hox genes of vertebrates in patterning the segmental structures of the axial organs (paraxial mesoderm and neural tube) was suggested by their strictly regulated spatiotemporal pattern of expression in the major segmented structures, the hindbrain and the paraxial mesoderm, and in the non-segmented neural tube. The class I Hox genes are distributed in four clusters located on four chromosomes in mouse and man. They form 13 paralog groups based on the similarity of their highly conserved homeobox. The demonstration that Hox genes actually define axial level specification in the vertebral column was first brought about by Kessel and Gruss (1991) who demonstrated that retinoic acid (RA) administered to the embryos at defined developmental stages, while altering the pattern of Hox gene expression in the somites, also modifies the combinatorial arrangement of Hox gene expression at each axial level, thus generating homeotic transformations in the vertebral. Targeted mutation of the Hox-3.1 gene in the mouse also generated homeotic transformation in the vertebral column (Le Mouellic et al., 1992). Another hint pointing to the same assumption was the observation that transgenic mice expressing ectopically the Hox-1.1 gene exhibited anomalies of the cervical region of the vertebral column (Kessel et al., 1990). The anomalies observed in the genetic manipulations of the embryo cited above mostly affected the base of the skull: absence of the basisphenoid and supraoccipital, malformations and/or fusions involving the exo- and basioccipital bones. Most interestingly, in about one third of the embryos that received RA at E7, “additional ossifications between, and fused to, the occipital bones and the atlas indicated the manifestation of a partial occipital vertebra, a proatlas” (quoted from Kessel and Gruss, 1991, p. 93).

It seemed therefore timely to reinvestigate, using classical methods of experimental embryology, what is, in normal development, the participation of the somites to the skull. This was done by substituting successively the six first somites of the chick by their quail counterpart in 3- to 6-somite-stage embryos.

By combining the data obtained in previous works (Johnston, 1966; Le Lièvre and Le Douarin, 1975; Le Lièvre, 1978; Noden, 1982) and in the present investigation, we now provide a complete picture of the embryogeny of the skull in birds and we assign to each bone a precise embryonic origin from either the ectomesenchyme, the paraxial cephalic mesoderm or the somites.

We also show that the boundary between the mesoderma! and the mesectodermal parts of the skull corresponds to the middle of the sella turcica, which is the cranialmost level reached by the notochord.

**MATERIAL AND METHODS**

Quail and chick (White Leghorn strain) eggs were from a commercial source and incubated in a humidified atmosphere at 38°C.

Quail to chick chimeras were constructed by isotopic and isochronic substitution of defined regions of the neural folds (i.e. the presumptive neural crest), of the paraxial cephalic and rostral somitic mesoderm in the chick embryo by their counterparts from the quail at 3-somite-stage. In a second experimental series, the fates of somites 4, 5 and 6 were investigated in 4-, 5- and 6-somite-stage embryos. The grafts were always performed unilaterally. The details of the experiments are recorded in Table 1.

**Experimental series**

**Experiment I.** The level for the operation was a 450 μm length piece of neural fold of the presumptive diencephalon and anterior half of the mesencephalon according to the fate map of the neural primordium that we have previously established (Couly and Le Douarin, 1985, 1987, 1988). Fig. 1 shows the quail graft in situ 2 1/2 hours after implantation into the stage-matched chick embryo.

**Experiment II** involved the graft of the neural fold corresponding to the posterior half of the mesencephalon and metencephalon down to the level of the 1st somite.

**Experiment III** involved the graft of the neural fold corresponding to the level of the three first somites (i.e., the rostral myelencephalon).

**Experiment IV** involved the graft of the median paraxial mesoderm (MPM) at the mesencephalic level as defined in our previous work on the fate of the paraxial mesoderm (Couly et al., 1992).

**Experiment V** involved the caudal region of the MPM corresponding to the metencephalon.

**Experiments VI, VII, VIII, IX, X and XI** correspond, respectively, to grafts of the 1st, 2nd, the 3rd right somite in 3-somite-stage embryos, the 4th, 5th, 6th somites in, respectively, 4-, 5- and 6-somite-stage embryos.

**Chimerism analysis**

145 embryos were operated upon and 73 developed normally and were further analyzed at stages varying from 8 to 14 days of incubation (E8 to E14) (Table 1). It was necessary to keep chimeras alive until E14 since identification of certain bones of the skull (e.g. frontal, parietal, supraoccipital) can be reliably identified only during the second half of the incubation period. The head was fixed in Zenker’s fluid, embedded in wax and cut in 5-6 μm coronal sections. Identification of normal and chimera bones was done on sections that were serially examined. The shape of each bone...
Fig. 1. (A) Dorsal view in SEM of the head of a 3-somite-stage chick embryo. (B) At the same stage, transverse section at the presumptive level of the mesencephalon. No cephalic mesodermal cells are present dorsally within the neural fold. (C) 2.5 hours after the graft. SEM view of a chick embryo in which the right prosencephalo-mesencephalic neural fold (arrows) has been replaced at 3-somite-stage by its counterpart taken from a stage-matched quail embryo. Note the perfect incorporation of the transplant into the host neural fold. Scale bar, 100 µm.

Table 1. Experimental series and number of embryos examined

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and its position with respect to the different brain regions were also studied on dissected skulls, namely for identification of the roof and occipital regions. Quail and chick cells were identified by the structure of their nucleus after Feulgen-Rossenbeck’s staining. Bone and cartilage were evidenced with alcian blue and chlo- rantine fast red (Lison, 1954). Alcian blue stains the extracellular matrix of cartilage in blue and chlo rantine fast red stains the bone matrix in red.

Scanning electron microscopy (SEM)
For SEM study, the embryos were immersed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) overnight at 4°C. After rinsing in the same buffer, the specimens were postfixed in 1% osmium tetroxide in the same buffer for 2 to 3 hours at room temperature, thoroughly rinsed and incubated in thiocarbohydrazide (saturated aqueous solution) for 10 minutes (Kelley et al., 1973). After rinsing in water, they were returned into 1% osmium tetroxide in water for 1 hour. After washing in water, the specimens were dehydrated through a graded series of ethanol substituted with iso-amyl acetate and critical point dried from CO2. After mounting on stubs with silver conductive paint, the specimens were vacuum coated with gold and examined with a Jeol 1200 EX electron microscope at 20 kV accelerating voltage.

The nomenclature of the bones used in the present work has been adopted from Romanoff (1960) for the early stages of development, De Beer (1937) for the chondrocranium and from Baumel (1979) and Nickel et al. (1977) for the late embryonic stages and adult birds.

RESULTS

I - Grafts of cephalic neural crest
Grafts of the diencephalon and anterior mesencephalic neural crest (experiment I)
The results were examined in ten embryos ranging from E8 to E14 (Table 1). In the facial area, the premaxillary, maxillary, nasal and vomer (i.e. membrane bones) and interorbital septum (still cartilaginous at E14), the sclerotic and the supraorbital bone were made up of quail cells. In addition, we found that the dermis of the scalp, the meninges of the forebrain (see Couly and Le Douarin, 1987) and the totality of the frontal bone, together with the anterior region of the squamosal, the basipresphenoid, the rostrum of the paraspHENoid and the pterygoid were of graft origin. The sutural spaces between the membraneous bones (the two frontal bones particularly) were also made up of quail connective tissue (Figs 2, 3). It is noticeable that this experiment shows the caudal boundary of the participation of the neural crest in the floor of the skull. This limit lies within the sella turcica and strikingly corresponds to the anterior tip of the notochord (Fig. 4A,B).

Graft of the posterior mesencephalic, the metencephalic and the myelencephalic neural crest (experiments II and III)
Experiment II concerns the posterior mesencephalic and the metencephalic level of the crest. Chimerism analysis was performed on ten embryos from E8 to E14 (Table 1).

The quail cells were localized in the parietal bones and dermis at the same level, the squamosal, the jugal, the quadrate, the pterygoid, the palatine, the skeleton of the lower jaw (Meckel’s cartilage, dentary, angular, opercular) (see also Johnston et al., 1974 and Le Lièvre and Le Douarin, 1975) (Fig. 5). In addition, an overlap of labelling was observed in experiments I and II (compare Figs 2B and 5B). This is likely due to the mixing of crest cells in this region of the head where morphogenetic movements are active. In all cases, the cells of the sputural space, still incompletely ossified at E14, were of donor type. In addition, part of the cochlearis area of the otic capsule (pro-otic area) originated from the grafted neural crest cells. The hyoid cartilage was also entirely of donor type (Fig. 5). Fig. 4A,B show the contribution of the neural crest to the lower jaw and second branchial arch region.

Experiment III involved grafting the myelencephalic neural crest. Analysis of chimera was performed on four embryos (E8 to E14) (Table 1). Migration of crest cells from this level did not involve the cephalic area. The cells colonized the 3rd, 4th branchial arches and their derivatives in the anterior thoracic region as previously established (see Le Douarin, 1982 for a review).

II - Grafts of cephalic mesoderm
Grafts of the MPM at the mesencephalic level (experiment IV)
Chimerism analysis was performed on eight embryos (Table 1). The graft does not yield quail dermis but only that part of the chondrocranium corresponding to the sphenoid bone complex (basi-postphenoïd, alipostphenoïd also called pleurophenoïd), the postorbital bone and a small contribution to the pars cochlearis of the otic capsule, as shown in Couly et al., (1992) (illustrated in figure 9 of Couly et al., 1992 and in Fig. 4A,B herein). In addition, this graft gives rise to vascular endothelial cells of quail type.

Graft of the MPM at the metencephalic level (experiment V)
Eight embryos have been examined (Table 1). In that case, the supraoccipital bone and the margin of the medial portion of the vestibular region of the otic capsule are of quail origin (Fig. 6). Moreover, the mesoderm of the metencephalic MPM participates in the formation of the wall of the semicircular canal in the following way: quails cells are found in the lateral part and the ventral bend of the upper semicircular canal, the rest of it originates from mesodermal cells of somitic origin (see below) (Fig. 4A, B). In addition, the grafted cephalic mesoderm gives rise to vascular endothelial cells of quail type located in the laterofacial structure of the chick host.

III - Grafts of the six first somites
All these grafts give rise to occipital, dorso-cervical dermis and also to the meninx of myelencephalon and rostral medulla. Details of experimental series are given in Table 1.

Grafts of the 1st somite (experiment VI)
The first somite is smaller than the following ones, in addition, it loses its individuality by 7- to 10-somite-stage, in fusing with MPM of the metencephalic level. Then the
second somite formed becomes the most cranial of the series. Since the cells of the 1st somite disperse, their exact fate was difficult to assess with precision. They can be followed throughout development by the quail nuclear marker. They form the exoccipital part of the chondro-occipital arch (Figs 7, 8 and 9), and a narrow cartilaginous stripe in the basioccipital between the 1st and 2nd roots of the hypoglossus nerve (Fig. 8). In addition, the 1st somite yields most of the pars canalicularis of the otic capsule (Figs 7, 8 and 9). The muscles of the neck and of the larynx were also partly labelled (Fig. 9, Table 2). The dermatome of the 1st somite forms the dermis in the occipital region of the head.

Fig. 2. Results of experiment I. Graft of prosencephalic and anterior mesencephalic neural crest. (A) Transverse section of the head of a chimeric embryo at E14 and (B) the corresponding schematic drawing showing the localization of quail cells in the cephalic skeleton of this chimera. The totality of the frontal bone (C,D), the supra-orbital bone (E) and metopic (interfrontal) suture (F) are of quail origin (Fr, frontal bone; S.O, supra-orbital). Scale bar, 1 mm (A) and 10 µm (C-F).
Fig. 3. Results of experiment I. Graft of prosencephalic and anterior mesencephalic neural crest. (A) Transverse section of the same chimeric embryo as in Fig. 2 at the level of the sella turcica (d and v indicate the dorsoventral axis). The dermis of the lateral region of the skull (squamosal) (B) and the masticatory aponevrosis (C) are of quail origin. The chondrocytes of the rostral half of the sella turcica (basipresphenoid) (D), the pterygoid (E) and parasphenoid bone (F) are of quail origin. (G) High magnification of the sutural space between pterygoid and palatine bones. (H) Enlargement of the central part of A (framed) corresponding to the sella turcica. (I) Boundary between the neural crest (quail cells) and the mesodermal (chick cells top) contribution to the cartilage of the sella turcica. The boundary corresponds to the limit between the basipresphenoid (quail) and the basipostsphenoid (chick). (C, chick; Q, quail; Ah, adenohypophysis; Rh, rhombencephalon). Scale bar, 1 mm (A,H) and 10 µm (B-G and I).
Embryogeny of skull in vertebrates

(Fig. 7B), thus, showing the boundary between the dermis of neural crest and of mesodermal somitic origin in the dorsal head region.

Graft of the 2nd somite (experiment VII)
The contribution of the graft is a stripe of cartilaginous cells located between the 2nd and 3rd roots of the hypoglossus nerve caudally to the area derived from the 1st somite (Fig. 10). Tongue and cervical muscles were also partly of graft origin (Table 2). Notable is the fact that the tongue and muscles are innervated by the hypoglossus nerve (N. XII).

Fig. 4. Chondrocranium of a bird at E9 (after De Beer, 1937) showing the origin of the different capsules. (A) Lateral view including the lower jaw and hyoid complex. (B) Dorsal view without the lower jaw and hyoid complex. Note that the participation of somites (in green) to the occipital and otic capsules is not detailed here; see Fig. 8 for a more precise analysis. Red, cartilage of neural crest origin; blue, cartilage of cephalic mesoderm origin; green, cartilage of somitic origin. The black line indicates the notochord.

1 nasal capsule
2 orbital capsule
3 otic capsule (pars ampullaris)
4 otic capsule (pars cholecaris)
5 occipital arch
6 exo-occipital
7 basi-occipital
8 supra-occipital
9 interorbital septum
10 hyoid cartilage
11 Meckel’s cartilage
12 quadrato-articular cartilage
13 basisphenoid (a, basi-post sphenoid; b, basi-presphenoid).
which originates from the myelencephalon (i.e. rhombomere 8th) (Fig. 8).

Graft of the third somite (experiment VIII)
The contribution of the 3rd somite is a specific zone of the corpus occipitale located between the 3rd and 4th rostral roots of the hypoglossus nerves (Fig. 8). In this case also, tongue and cervical muscles are partly derived from the myotome of the 3rd grafted quail somite (Table 2).

Graft of the fourth somite (experiment IX)
This somite gives rise to the region of the chondro-occipital arch located caudally to the 4th hypoglossus root (Figs 8 and 11). Tongue and cervical muscles are also partly labelled by this graft (Table 2).

Graft of the fifth somite (experiment X)
The 5th somite yields the distal part of the chondro-occipital arch, corresponding to the occipital condyles, the anterior arch of atlas and the odontoid apophysis of axis (the rest of the axis is of chick host origin in this experiment, Fig. 12G). Therefore the 5th somite contributes to three different skeletal structures which form the occipito-atlanto-odontoid articulation (Fig. 8). In addition, some tongue and cervical muscles are of quail origin in this experiment (Fig 12, Table 2).

Graft of the sixth somite (experiment XI)
The dorsal arch of the atlas vertebra and the rostral half of the axis are of quail origin whereas its caudal half belong to the host. No tongue muscles were labelled. The myotome of somite 6 participates in cervical muscles (Figs 8, 13, Table 2).

CONCLUSIONS AND DISCUSSION
The question raised at the initiation of this work concerned the embryonic origin of the skull from the ectoderm via the neural crest and from the mesoderm via the somites and the cephalic paraxial mesoderm. The origin of the various bones constituting the avian skull was investigated by using the quail/chick chimera system. Chimeras in which definite territories of the chick embryo were replaced isotopically by their counterpart coming from stage-matched quails were constructed first in 3-somite-stage embryos when clo-
sure of the neural tube had not yet started. In a second experimental series (experiments IX to XI), the fate of somites 4 to 6 was investigated in older embryos. At 3-somite-stage, the head neural fold can be removed and replaced selectively while no emigration of neural crest cells has happened yet. Therefore, the paraxial mesoderm, which is accessible to surgical manipulation (see Couly et al., 1992), is not possibly contaminated by ectomesenchyme coming from the crest. This makes the experimental approach presented here different from the previous ones.

Fig. 5. Results of experiment II. Graft of the posterior mesencephalic and anterior rhombencephalic neural crest. (A) Transverse section of the head of a E14 chimera and (B) the corresponding schematic drawing showing the localization of the quail cells in the cephalic skeleton. The parietal bone (C) and membranous fronto-parietal suture (D) are of quail origin (P, parietal bone; F, frontal bone; S, squamosal). Scale bar, 1 mm (A) and 10 µm (C,D).
Fig. 6. Results of experiment V. Graft of MPM at the metencephalic level. (A) Transverse section of the head of a E-13 chimera and (B) high magnification of the supra-occipital bone where the quail marker is located (d and v indicate the dorsoventral axis). Scale bar, 1 mm (A) and 10 µm (B).

Table 3. Origin of cephalic skeleton

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<td>Bones of cephalic mesoderm origin</td>
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<td>* pars canalicularis and cochlearis</td>
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<td>* maxillar</td>
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<td>* vomer</td>
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<td>* palatine</td>
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<td>* quadratojugal</td>
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<td>* mandibular</td>
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Embryology of skull in vertebrates (e.g., Le Lièvre, 1978) that were based essentially on experiments carried out at later developmental stages. Moreover, the developmental stage of the chimeras analysed in the present work ranged from E8 to E14 i.e. when the different components of the skull and associated dermis can be clearly identified.

Fig. 7. Results of experiment VI. Graft of the 1st somite. (A,C) Transverse sections of the head of a E9 chimera respectively at the level of the occipital bone and of the laryngeal muscles. The dermis of the occipital region (B), the pars ampullaris of the otic capsule (D), the m. ceratoglossus (E), the rostral basi chondro-occipital (F) and the m. dilatator glottidis (G) contained quail cells (see Table 2). Connective cells located between the quail muscle cells (E and G) originate from the neural crest of the chick host (see Couly et al., 1992). Scale bar, 1 mm (A,C) and 10 μm (B,D-G).
Contribution of the neural crest to the skull and dermis

The results obtained are indicated in Fig. 14A, B and in Table 3. In our earlier work (Couly et al., 1992), it clearly appeared that the frontal and parietal bones were not mesodermal in origin. This is why we decided to undertake a refined study on the mapping of the anteriormost part of the neural crest by operating on embryos early and by allowing the chimeras to develop up to a stage when the skull is formed and when every bone can be clearly identified (for the parietal bone for example from E13 and for the supra-occipital from E12, see Romanoff, 1960). The results presented here show without any doubt that the parietal and frontal bones and the associated sutures are derived from the neural fold corresponding to the presumptive level of the diencephalon, mesencephalon and metencephalon. They also demonstrate that the dermis corresponding to these bones as well as the totality of the dermis of the face and ventral part of the neck (as previously stated, Le Lièvre and Le Douarin, 1975) originates from the neurectoderm.

In addition to the facial and visceral skeleton (Le Lièvre, 1978), the neural crest contributes entirely to the roof of the skull. The extent of this contribution was not perceived in previous experiments (Le Lièvre, 1978) in which the major part of the frontal and the parietal bones were considered as derived from the mesoderm. In the present work, we have analysed in a very detailed manner the origin of the floor of the skull.

Origin of the chondrocranium

1. The occipital and otic regions

The chondrocranium includes certain elements originating from mesoderm and others from neural crest (Fig. 14A,B; Tables 3, 4). The five first somites contribute to the occipital bone: the first somite yielding the exo-occipital and the second, third, fourth and rostral part of the fifth forming the basi-occipital and the condyles (Fig. 8). The paraxial mesoderm of the metencephalic level (MPM of Couly et al., 1992) forms the supra-occipital. Therefore, the occipital as a whole can be considered as a giant vertebra enlarged to form a cupula in which the brain rests and in which the neural arch is represented by the exo-occipital and the

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Table 4. Contribution of the cephalic medial paraxial mesoderm (MPM)* to the genesis of the chondrocranium

<table>
<thead>
<tr>
<th>Sphenoid</th>
<th>Latero-mesencephalic</th>
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<td>- Basipost-</td>
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<td>- Orbito-</td>
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<td>- Pleuro-</td>
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<td>Otic capsule</td>
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<td>- Pars cochlearis</td>
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<td>- Pars ampullaris</td>
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<td>Occipital</td>
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<tr>
<td>- Supra</td>
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*The respective situations of the latero-mesencephalic and latero-metencephalic medial paraxial mesoderm (MPM) are indicated in Table 1 (experiment IV and V) and in Couly et al., 1992).
supra-occipital. The corpus of the vertebra is represented by the basi-occipital. We can use these embryonic origins to extrapolate the morphogenetic movements of mesoderm. The metencephalic MPM migrates dorsally (to form the supra-occipital); the sclerotome of the 1st somite remains laterally, while the corpus of the ‘occipital vertebra’ is formed by the concentration around the notochord of sclerotomal cells belonging to somites 2, 3, 4, 5 and a very small part of the 1st somite (Fig. 8).

Notable is the fact that the region of fusion between the basi-post-sphenoid of MPM origin and the rostral part of the occipital bones of somitic origin correspond to the

**Fig. 9.** Results of experiment VI. Graft of the 1st somite. (A) Cephalic transverse section of a chimeric embryo at E13 crossing the otic region; the exo-occipital bone (B) and the cartilaginous pars canalicularis of otic capsule (C) are of quail origin. The *m. cucullaris cervicis* is also of quail origin (D). Scale bar, 1 mm (A) and 10 µm (B, C and D).
human sphenoo-occipital synchondrosis, a zone which in humans is the site of growth activity up to 20 years of age.

In this conception of the construction of the occipital area, it is interesting to notice that the paraxial cephalic mesoderm (MPM) replaces, in the 'giant occipital vertebra', the sclerotomal somitic cells which normally form the neural arch (Bagnall et al., 1988, 1989). It has been clearly established by the use of the quail-chick chimera system that, in the trunk, each vertebra arises from the joint development of the posterior and anterior halves of two consecutive somites (Bagnall et al., 1988).

Fig. 10. Results of experiment VII. Graft of the second somite. (A,D) Transverse sections of the head of a E9 chimera at two different levels of the rhombencephalon showing the quail label in the basichondro-occipital (B,C) between the 2nd and 3rd roots of the hypoglossus nerve (HN 2-3) and in m. hypoglossus obliquus (E). Scale bar, 1 mm (A,D) and 10 µm (B,C,E).
thus confirming the ancient view put forward by Remak (1855). Moreover, the rostral part of each somite is the site where the dorsal root sensory ganglia (DRG) develop and the motoneurone fibers penetrate (Keynes and Stern, 1984; Teillet et al., 1987). It is important to notice that the behaviour of the five first somites differs strikingly from that of the more posterior ones. With the exception of the caudal part of the 5th somite, they all fuse to form the basioccipital bone. The remaining evidence for the origin of this bone from the five first segments is the foramens by
which the roots of the XIth nerve (hypoglossus) exit from the myelencephalon.

No sensory ganglia arise from the five first somites (Teillet et al., 1987; Le Douarin et al., 1992) whereas a large amount of neural crest cells from this level reach the gut to form the enteric ganglia (Le Douarin and Teillet, 1973) and the parasympathetic ganglia of the heart (Kirby et al., 1983).

In contrast to the cephalic paraxial mesoderm, which does not yield dermis (Couly et al., 1992), the six first
Fig. 13. Results of experiment XI. Graft of the sixth somite. (A,F) Transverse sections of the head of a E9 chimera. The corpus of axis (B), dorsal arch of atlas (G) and m. rectus capitis (E) consist of quail cells. In contrast, the odontoid apophysis of axis (D) and the anterior arch of atlas (C) are of host origin. Scale bar, 1 mm (A,F) and 10 μm (B-E,G).
somites generate the dermis covering the occipital and dorsocervical region of the head (see Table 2). As for their myotomal components, these somites give rise to the tongue muscles innervated by the hypoglossus nerves originating from the level of rhombomere 8 (see Noden, 1983; Lumsden and Keynes, 1989; Keynes and Lumsden, 1990).

The **otic capsule** is formed by the cooperation of osteochrondrogenic cells from three different sources. The main one is the 1st somite from which most of the pars canalicularis of the otic capsule arises (Table 2). The metencephalic and mesencephalic median paraxial mesoderm (MPM) also contributes to the pars canalicularis and forms most of the pars cochlearis of the otic capsule (see Couly et al., 1992) (Table 4). Finally the neural crest participates in the formation of the dorsolateral part of the pars cochlearis (pro-otic process) close to the squamosal. The neural crest is also involved in the constitution of the columnella (see Le Lièvre, 1978; Noden, 1983). Therefore, as previously stated (Le Lièvre, 1978), the otic capsule results from the cooperation of mesodermal and mesectodermal cells.

(2) The **sphenoid bone complex** and the double origin of the sella turcica

The sphenoid bone complex is formed by the mesencephalic MPM, which generates the cartilaginous rudiment of the orbitosphenoid from which the basipostsphenoid and the pleurospheoid will arise (Fig. 4A,B) (Coul et al., 1992). The contribution of the neural crest to the sphenoid bone complex comprises the basipresphenoid (Fig. 14A,B), the rostrum of the para-otic and the pterygoid. The sella turcica is therefore of mesodermal origin caudally and of crest origin rostrally. The contribution of the neural crest to this structure had not been reported before. It is interesting to notice that the anterior tip of the notochord reaches the limit of the basipostsphenoid and therefore lies at about the midpart of the sella turcica as already described in the human embryo (see figure 121, p. 167, Augier, 1931).

The mesenchyme participating in adeno-hypophysis histogenesis (to form the connective tissue located between the glandular cords and the blood vessel walls with the exception of the endothelia) is derived from the neural crest (Le Lièvre and Le Douarin, 1975; Couly and Le Douarin, 1985, 1987). Therefore, the basiphenoid including the sella turcica are of mesodermal and ectodermal (via the neural crest) origin. Thus this bone crosses the limit between the ‘chordal’ and the ‘prechordal’ chondrocranium (Table 3). The anterior limit of the notochord then corresponds to the limit where the mesodermal skeleton ends and the neural crest derived skeleton begins. The latter will be called the ‘prechordal’ skeleton.
The extent of this contribution was not perceived in previous experiments (Le Lièvre, 1978) in which the major part of the frontal and the parietal bones were considered as derived from the mesoderm. This discrepancy comes from the fact that the experiments reported by Le Lièvre (1978) were done at later stages of development and that the host embryos were observed at early stages when these bones were not completely individualized.

**Origin of the skull and the new head hypothesis of Gans and Northcutt (1983)**

Gans and Northcutt (1983) and Northcutt and Gans (1983) put forward the hypothesis that the more anterior (or rostral) part of the head including sense organs, prosencephalon and mesencephalon together with the corresponding region of the skull, is derived from the neurectoderm. The results presented here and in our previous article (Couly et al., 1992) support this view by showing that the entire vault of the skull (including the frontal and parietal bones) and what we call the ‘prechordal’ skull are actually of neural crest origin. Moreover they also demonstrate that the contribution of the mesoderm to the ‘new head’ is reduced essentially to muscles and a limited and posterior (or caudal) part of the skull (see Fig. 14A,B).

In addition, it should be noted that the vertebrate head not only is built up from the neural crest in its more rostral region but also results from the incorporation in its caudal area of an increasing number of vertebrae from the agnathes (where no vertebra participates in the skull (Augier, 1931, page 164)) to the higher vertebrates where, as shown here, up to 5 somites participate in the occipital bone complex (Fig. 15).

In our first investigations on the ontogeny of the early neural primordium (Couly and Le Douarin, 1987, 1988), we constructed a fate map (Fig. 16) showing that the mediodorsal and anterior region of the neural plate corresponds to the territory of the hypothalamo-adenohypophysis, whereas the ectoderm of the first branchial arch is located laterally to the adenohypophyseal placode and the presumptive nasal ectoderm (Couly and Le Douarin, 1990a,b). The rostral part of the head (i.e. most of the so-called ‘new head’) is formed by the lateral regions of the anterior neural plate from which originate the prosencephalon (including the cerebral hemispheres), part of the mesencephalon and the neural crest that forms the vault of the skull, the ‘prechordal’ chondrocranium and the maxillary and mandibulary region of the face (Fig. 16).

The development of sense organs and the increase of the brain volume have therefore involved the construction of the anterior skull from the neurectoderm and also the incorporation of more and more paraxial mesoderm in the occipital and otic region of the head (Fig. 15).

**The Hox-code and the specification of the occipito-otic region of the skull**

The notion that the specification of the skull along the anteroposterior body axis is determined by the combinatorial expression of various Hox genes, thus generating a Hox-code, was clearly formulated by Kessel and Gruss (1991).
It was also supported by several experimental evidences based either on the use of retinoic acid or by altering the Hox-code by transgenes. Retinoic acid administered to the pregnant mice at defined stages of fetal development as well as hyperexpression of Hox-1.1 (Kessel et al., 1990) in transgenic mice or targeted mutation of Hox-1.6 (Lufkin et al., 1991; Chisaka et al., 1992) and Hox-1.5 genes (Chisaka and Capecchi, 1991) result in malformations of vertebral morphogenesis. It is striking to see that many of the dysgeneses so induced are localized in the occipital region. The experiments reported here reveal that the occipito-otic region of the skull is the site of very complex morphogenetic events involving fusions of embryonic structures that in the vertebral column develop independently. The Hox-code therefore seems to exert a stringent control on the movements of cells and other morphogenetic mechanisms leading to the incorporation to the skull of elements of the cervical column. Differential expression of Hox genes at the hindbrain and first somites levels has therefore been involved in the evolutionary selection process that led to the construction of the skull in higher vertebrates.

The authors are grateful to Dr F. Dieterlen for her reading of the manuscript. The authors wish to thank M. Le Thierry for her technical assistance, B. Henri, S. Gourmet and E. Bourson for help in preparing the manuscript. This work was supported by the Centre National de la Recherche Scientifique and grants from the Association pour la Recherche contre le Cancer, The Fondation pour la Recherche Médicale Française and the Ligue Nationale contre le Cancer.

REFERENCES


(Accepted 23 October 1992)