

Skull Development During Anuran Metamorphosis: I. Early Development of the First Three Bones to Form—The Exoccipital, the Parasphenoid, and the Frontoparietal

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ABSTRACT In anuran amphibians, cranial bones typically first form at metamorphosis when they rapidly invest or replace the cartilaginous larval skull. We describe early development of the first three bones to form in the Oriental fire-bellied toad, *Bombina orientalis*—the parasphenoid, the frontoparietal, and the exoccipital—based on examination of serial sections. Each of these bones is fully differentiated by Gosner stage 31 (hindlimb in paddle stage) during premetamorphosis. This is at least six Gosner developmental stages before they are first visible in whole-mount preparations at the beginning of prometamorphosis. Thus, developmental events that precede and mediate the initial differentiation of these cranial osteogenic sites occur very early in metamorphosis—a period generally considered to lack significant morphological change. Subsequent development of these centers at later stages primarily reflects cell proliferation and calcified matrix deposition, possibly in response to increased circulating levels of thyroid hormone which are characteristic of later metamorphic stages. Interspecific differences in the timing of cranial ossification may reflect one or both of these phases of bone development. These results may qualify the use of whole-mount preparations for inferring the sequence and absolute timing of cranial ossification in amphibians.

Amphibian metamorphosis, particularly in anurans, entails a tremendous morphological restructuring of virtually every organ system in the body. This restructuring is nowhere more conspicuous than in the skull, where the cartilaginous larval cranium is rapidly converted into the primarily bony skull of the postmetamorphic froglet. Here, as is typical for osteocranial development in vertebrates generally, the bones form in a stereotyped, species-specific sequence that is correlated closely in time with other developmental events.

These broad features of cranial metamorphosis have been known for some time, yet detailed knowledge of the development of the osteocranium in amphibians is available for surprisingly few taxa. Indeed, descriptions of the complete sequence and timing of cranial ossification are known for only 18 of the more than 3,400 extant species of anurans (Duell-

man and Trueb, '86; Trueb, '85). Information is particularly sparse concerning the embryonic and larval precursors of osteogenic cells and the nature and timing of the developmental processes that underlie their differentiation into discrete bony elements.

In an earlier study, we described the sequence and timing of cranial ossification in the Oriental fire-bellied toad, *Bombina orientalis*, as a prelude to experimental investigations of the developmental mechanisms that underlie cranial osteogenesis in this species (Hanken and Hall, '84). Based on examination of cleared, whole-mount preparations differentially stained for bone and cartilage, we found that the first cranial bones to form are the parasphenoid and the paired frontoparietals and exoccipitals—bones which were visible no earlier than Gosner stage 37 (prometamorphosis—Etkin, '35). Subsequently, we gathered additional descriptive data, pre-

sented below, concerning the timing of ossification of these early forming bones based on serial sections. These data revealed that ossification centers corresponding to each of these bones form much earlier in metamorphosis than we had inferred from cleared-and-stained specimens. Thus, it is most appropriate to interpret the first appearance of these ossification centers in cleared-and-stained preparations as a proliferation of preexisting foci of bone formation and the deposition of calcified extracellular matrix. The latter processes may represent a response to the characteristic surge in endocrine factors, particularly thyroid hormone, during later metamorphic stages. We further conclude that important developmental events that mediate the initial differentiation of these likely neural-crest or paraxial-mesoderm derivatives occur during premetamorphosis—a phase generally considered to involve little morphological change—and much earlier than might be inferred from study of whole-mount preparations.

MATERIALS AND METHODS

All specimens of *Bombina orientalis*, a species native to Korea, were derived from matings among several adults maintained as a breeding colony for that purpose. Methods for breeding and husbandry followed established procedures (Carlson and Ellinger, '80; Frost, '82), with the following exceptions. Embryos and early stage tadpoles (\leq Gosner stage 26) were reared in 10% Holtfreter's solution; later-stage tadpoles were reared in 20% modified Holtfreter's solution buffered to pH 7.2 with Trizma-7.2 (Sigma Chemical Co., No. T-8508), instead of sodium bicarbonate. All specimens were reared in an environmental chamber at $18 \pm 1^\circ\text{C}$ with an alternating photoperiod of 12L:12D.

Specimens used in this study composed the control group from an experiment analyzing the effects of exogenous thyroid-hormone application on cranial metamorphosis. From 1 to 8 d before preservation, a plastic (Elvax) micropellet was implanted within the dermis medial to the right eye in each specimen. The micropellet, which did not contain any hormone, caused no noticeable effect on any aspect of cranial development; in all respects, development in this control group was identical to that of normal, nonimplanted tadpoles (Hanken and Hall, unpublished data).

Specimens were killed in 30% aqueous chloretone (1, 1, 1-trichloro-2-methyl-2-propanol; Sigma Chemical Co., No. T-5138) and

fixed and preserved in 10% neutral-buffered formalin. Then they were dehydrated in ethanol, embedded in Paraplast, and prepared as 6- μm serial sections; lenses were removed from the eyes before embedding to prevent tearing of adjacent tissues during sectioning. The sections were stained with a quadruple connective tissue stain (Mayer's haematoxylin, celestine blue, alcian blue 8GX, and direct red 81) that is particularly effective in distinguishing skeletogenic tissues (Hall, '86).

Development was staged according to the scheme of Gosner ('60). This scheme defines a total of 46 stages from fertilization through metamorphosis; a fully formed tadpole is stage 25. The stages which we discuss below (29–35) are distinguished by the relative development of the hindlimb; all are within the period of premetamorphosis, as defined by Etkin ('35). Descriptions are based on a total of 18 specimens.

RESULTS

Results consist of descriptions of the progressive differentiation of the exoccipital, parasphenoid, and frontoparietal bones between Gosner stages 29 and 35. These stages include development prior to visibility of ossification in serial sections to a later stage at which ossification centers are fully differentiated. Subsequent development primarily entails synthesis of calcified extracellular matrix in the ossification centers, until and beyond the time when they are first visible in cleared-and-stained preparations at stage 37 or 38.

Exoccipital

The exoccipital is a paired bone at the rear of the skull (Figs. 1B, 2). Initially, it forms via endochondral ossification within the occipital arch that connects the otic capsule at its posteromedial edge to the ventral portion of the braincase on either side of the notochord (Figs. 1A, 3B). In the adult it composes the posterior portion of the neurocranium, including the occipital condyles and the bone surrounding the foramen magnum, but usually it is fused to the prootic (Trueb, '73). In cleared-and-stained preparations, the exoccipital is first visible at stage 37 as a tiny ossification center located on the surface of the anterior, concave bend of the occipital arch (Fig. 2B).

Stage 29 (Fig. 4A)

No ossification is present where the exoccipital will form. Occipital-arch cartilage is

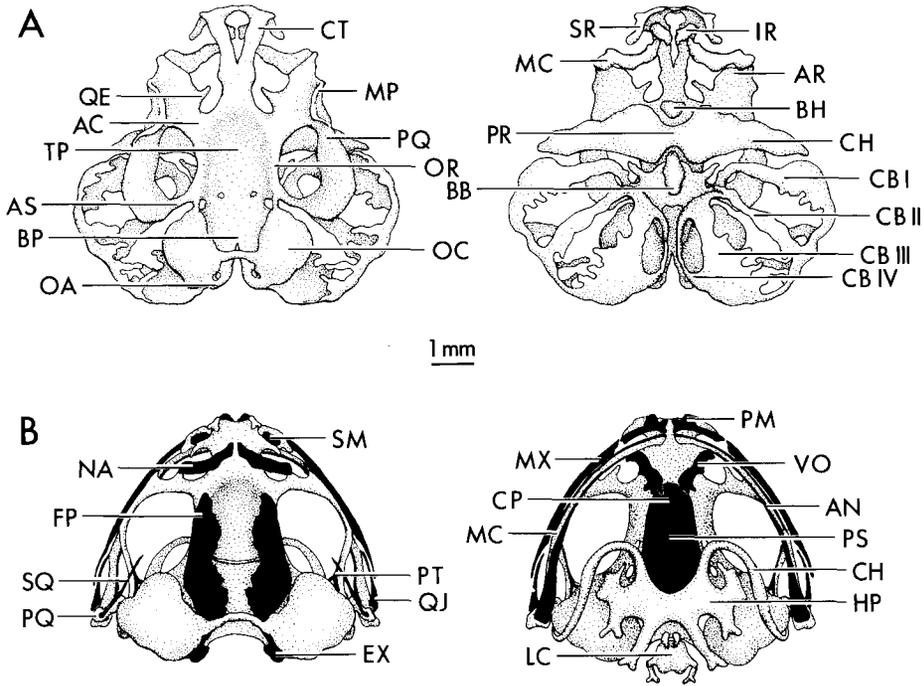


Fig. 1. Pre- and postmetamorphic skulls of *B. orientalis*, drawn from cleared-and-stained whole mounts: A, larva, Gosner stage 36; B, postmetamorphic froglet, stage 46. Left, dorsal views; right, ventral views. Cartilage is stippled; bone is solid black. The postmetamorphic specimen does not depict the full, adult complement of cranial bones, many of which are yet to form. Similarly, bones already present will become more extensive during subsequent growth and development. Abbreviations: AC, anterior commissure; AN, angulosphenial; AR, articulating process; AS, ascending process; BB, basibranchial cartilage; BH, basihyal cartilage; BP, basal plate;

CB I-IV, ceratobranchial cartilages I-IV; CH, ceratohyal cartilage; CP, cultriform process; CT, cornu trabeculae; EX, exoccipital; FP, frontoparietal; HP, hypobranchial plate; IR, infraorbital cartilage; LC, laryngeal cartilage; MC, Meckel's cartilage; MP, muscular process; MX, maxilla; NA, nasal; OA, occipital arch; OC, otic capsule; OR, orbital cartilage; PM, premaxilla; PQ, palatoquadrate cartilage; PR, pars reuniens; PS, parasphenoid; PT, pterygoid; QE, quadratoethmoid process; QJ, quadratojugal; SM, septomaxilla; SQ, squamosal; SR, suprarorbital cartilage; TP, trabecular plate; VO, vomer.

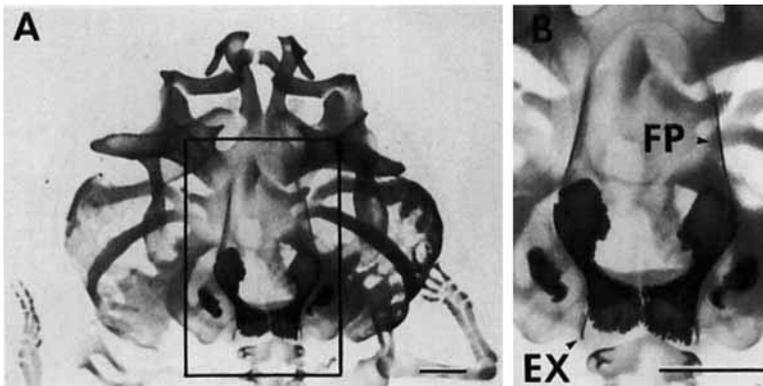


Fig. 2. A: Skull of *B. orientalis*, Gosner stage 38, cleared and differentially stained for bone and cartilage; B: Braincase region from A (rectangle) under higher magnification. Ossification centers corresponding to the exoccipital and frontoparietal bones are clearly visible; the parasphenoid is also present in this specimen, but it

is difficult to see in dorsal view. Calcified tissues appear slightly darker than adjacent cartilage, including the prominent, calcified endolymphatic sacs within the otic capsules and the braincase. Abbreviations as in Figure 1. Scale bars equal 1 mm.

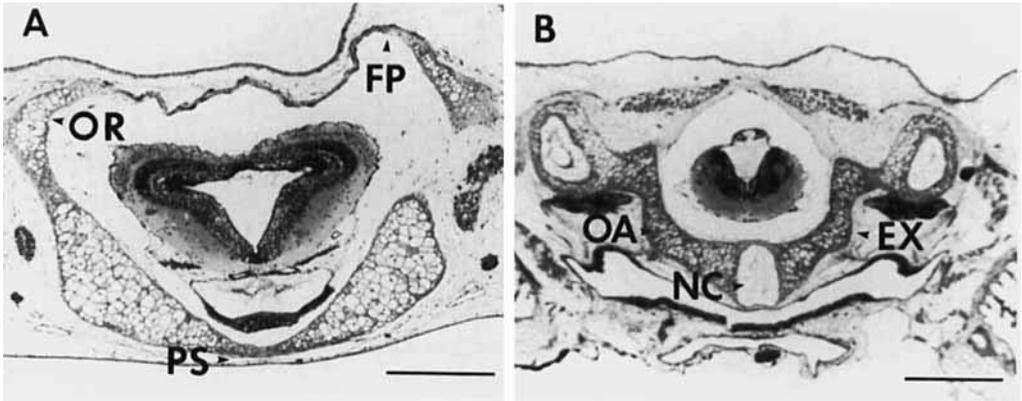


Fig. 3. Transverse sections through a metamorphosing tadpole (Gosner stage 35) indicating the sites of formation of the exoccipital, parasphenoid, and frontoparietal bones. A, anterior, between the otic capsules

and the eyes; B, posterior, at the level of the occipital arch. Additional abbreviation: NC, notochord. Scale bars equal 0.5 mm.

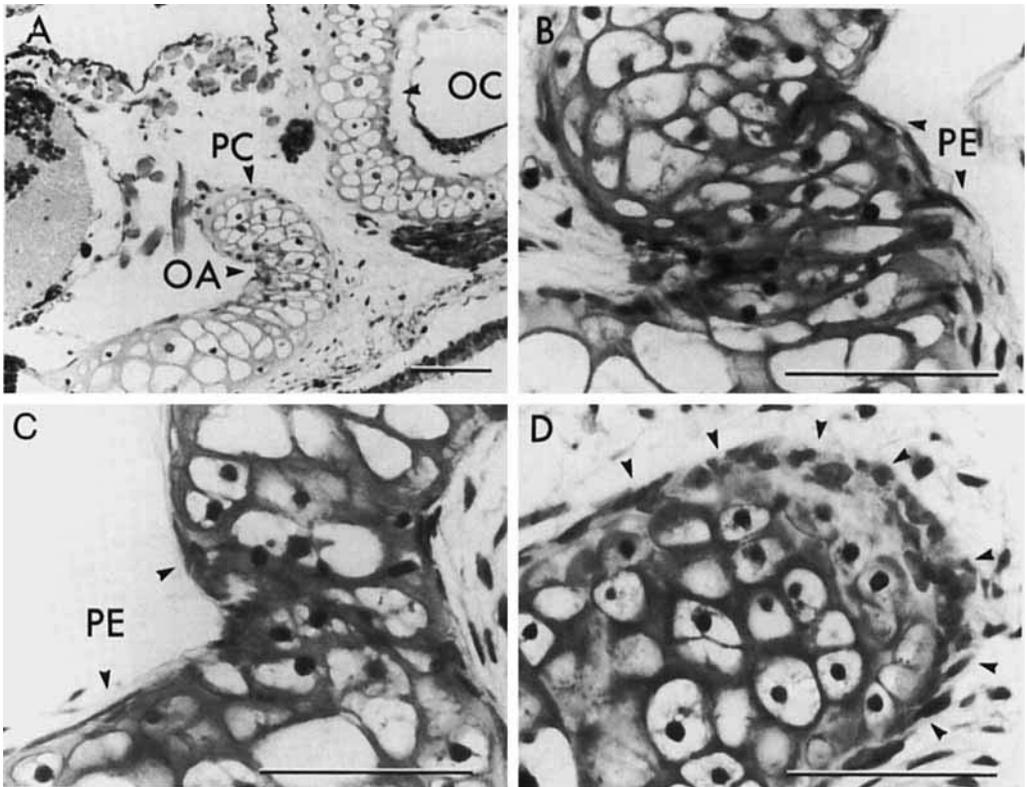


Fig. 4. Progressive stages in the development of the exoccipital: A, stage 29; B, stage 31; C, stage 33; D, stage 35. Later stages are characterized by increased development of the periosteum and associated extracellular matrix which initially invest the cartilage occipital

arch; by stage 35 (D), the bone forms a thin cap over adjacent occipital cartilage (arrowheads). Transverse sections; all are at the level of the otic capsule. Additional abbreviations: PC, perichondrium; PE, periosteum. Scale bars equal 100 μ .

surrounded by a thin perichondrium and there is no investing red-stained matrix.

Stage 31 (Fig. 4B)

Beginning stages of ossification of the exoccipital are now present. A thin, yet distinct, red matrix invests the occipital-arch cartilage on either side of the notochord. A few flattened cells that presumably compose the developing periosteum are embedded within this matrix, which does not extend beyond the arch along either the adjoining otic-capsular cartilage or the notochord.

Stage 33 (Fig. 4C)

This specimen is slightly advanced beyond the one described for stage 32. The thin, red matrix now extends along a greater extent of the occipital-arch cartilage in either direction.

Stage 34

Exoccipital development in this specimen is somewhat intermediate between that in stages 29 and 31 described above. No distinct red matrix is visible, but a dense layer of flattened cells, which presumably corresponds to the developing periosteum, is present where the exoccipital first forms in more advanced stages.

Stage 35 (Fig. 4D)

The exoccipital is extensive on the surface of the occipital arch; red matrix extends continuously from the posterior edge of the occipital arch anteriorly nearly onto the surface of the otic capsule. Even at this stage, however, there is virtually no sign of degeneration and subsequent endochondral replacement of the cartilaginous core of the occipital arch or of adjacent elements.

Parasphenoid

The parasphenoid is a single, median bone that forms the ventral layer of the braincase (Fig. 1B). In the adult, it is a dorsoventrally flattened, triradiate element; a single, median cultriform process extends anteriorly, overlapping the sphenethmoid, while paired wings, or alae, subtend the otic capsules posteriorly and laterally. It forms by intramembranous ossification immediately ventral to the cartilaginous basal and trabecular plates (Figs. 1A, 3A). In cleared-and-stained preparations, the parasphenoid is first visible (stage 37 or 38) as a small number of flat, alizarin-stained deposits lying just anterior to the otic capsules.

Stage 29 (Fig. 5A)

The parasphenoid is not present at this stage. The area between the basal plate and the one-cell-thick oral epithelium ventral to it, where the parasphenoid will first form, is sparsely populated by alcian-blue-positive connective tissue. A thin, transverse, membranelike layer of flattened cells is present and distinct in places from the adjacent perichondrium. This layer may represent presumptive osteogenic cells, but no red-stained matrix is present.

Stage 31 (Fig. 5B)

The parasphenoid is present in a series of only eight to ten sections immediately anterior to the otic capsules. A narrow band of red-stained matrix and associated periosteum straddles the midline of the head, adjacent to the thin medial region of the cartilaginous basal plate. The bone is only one cell thick, yet it is distinct from the adjacent perichondrium.

Stage 33 (Fig. 5C)

The parasphenoid is extensive, forming a thin sheath below the neurocranium from the otic capsules to the eyes. Posteriorly, it occupies approximately two-thirds of the width of the basal plate; in addition, thin sheets of flattened cells representing presumptive alae continue laterally beneath the otic capsules, but they lack distinct, red-stained matrix. The cultriform process is relatively thick and more distinct immediately anterior to the otic capsules. It extends anteriorly to a position ventral to the eyes where it may be seen as a thin layer occupying the narrow channel between the oral epithelium and cartilaginous trabecular plate.

Stage 35 (Fig. 5D)

With continued growth, the parasphenoid increases in width, length, and breadth. Posteriorly, it extends the full width of the basal plate between the otic capsules. The cultriform process extends forward anterior to the eyes and ventral to the telencephalon. In all sections, the parasphenoid is markedly thicker than in earlier stages and possesses a distinct periosteum that is visible on both dorsal and ventral surfaces.

Frontoparietal

The frontoparietal is a paired bone that forms the roof over the posterior portion of the neurocranium medial to the eyes (Figs. 1B, 2). In the adult it extends posteriorly

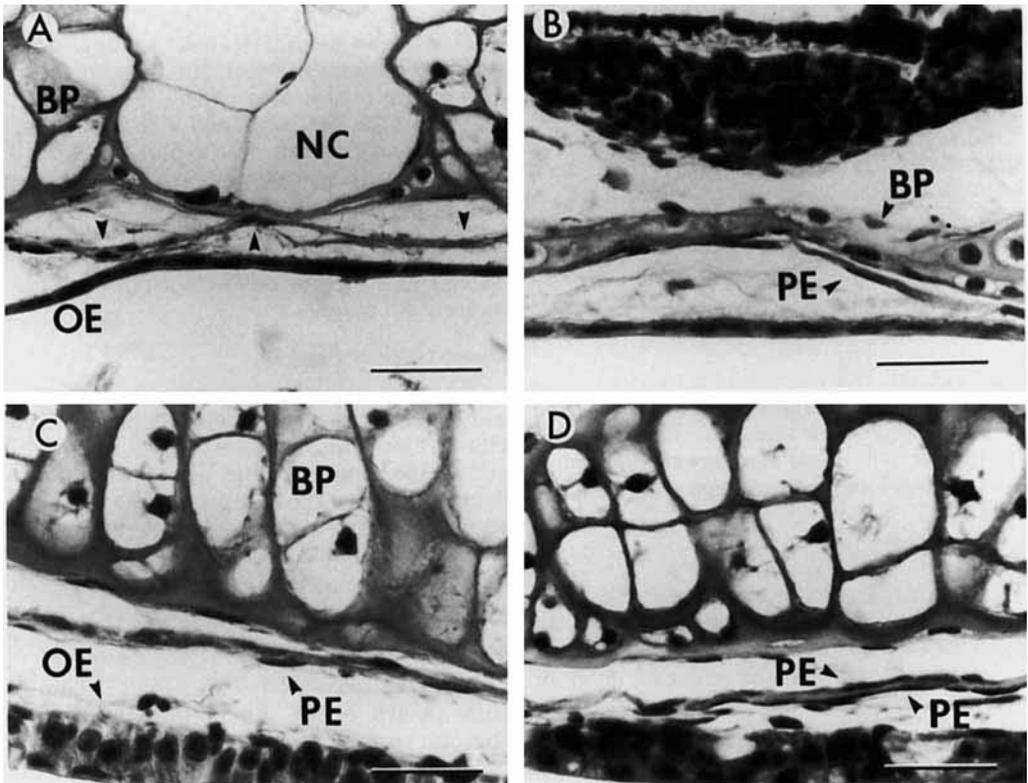


Fig. 5. Progressive stages in the development of the parasphenoid: A, stage 29; B, stage 31; C, stage 33; D, stage 35. Arrowheads in A denote presumptive ossification center ventral to the cartilaginous basal plate and median notochord; by stage 35, distinct periosteal are

visible on both dorsal and ventral surfaces. Transverse sections; A is at the level of the occipital arch; B-D are taken immediately anterior to the otic capsules. Additional abbreviation: OE, oral epithelium. Scale bars equal 50 μ m.

from the medial sphenethmoid and interfrontal to the fused prootic + exoccipital; paired frontoparietals broadly articulate in the midline. The bone forms by intramembranous ossification and is first visible as a longitudinal splint of bone anterior to the otic capsule and dorsal to the orbital cartilage, which forms the dorsolateral wall of the neurocranium (Figs. 1A, 2B, 3A). The frontoparietal, as its name implies, is the presumed homologue of the frontal and parietal which are distinct in most vertebrates other than anurans (Griffiths, '54). In *Bombina*, however, and as is typical for discoglossid frogs, it forms from a single ossification center on either side (Trueb, '73).

Stage 29 (Fig. 6A)

The frontoparietal is not present at this stage. Its imminent development, however,

is suggested by a small, dense condensation of cells at the juncture of the dorsal edge of the orbital cartilage with the cranial meninges that extend medially to invest the brain. The frontoparietal will form later at this site.

Stage 31 (Fig. 6B)

The frontoparietal is present on only one side in this specimen. It composes a narrow, thin splint of matrix that is barely thicker than the nuclei of osteocytes within the adjacent periosteum and that lies within the cranial meninges (medial to the outer, pigmented layer) at their juncture with the orbital cartilage.

Stage 33 (Fig. 6C)

The frontoparietal is present on both sides, extending longitudinally between the eye

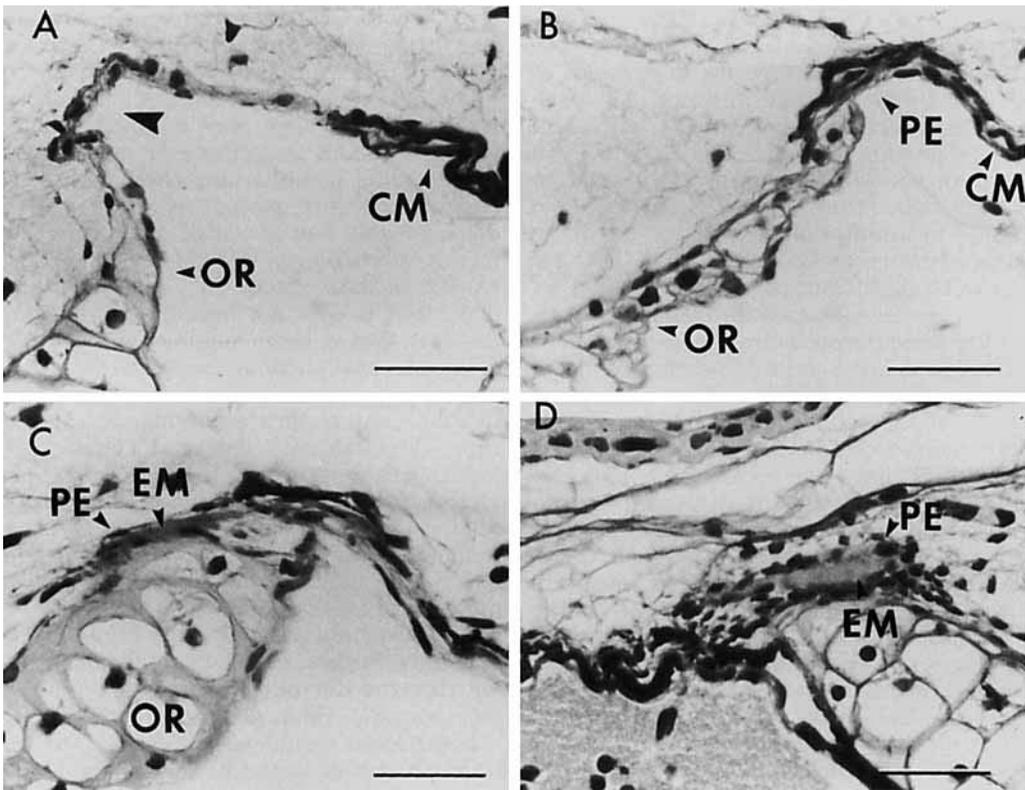


Fig. 6. Progressive stages in the development of the frontoparietal: A, stage 29; B, stage 31; C, stage 33; D, stage 35. Imminent formation of the bone is presaged at stage 29 by a small, dense condensation of cells (large arrowhead) at the junction of the orbital cartilage with the pigmented cranial meninges which extend medially and dorsal to the brain. By stage 35, the bone is rela-

tively thick and composes distinct periosteum surrounding a diffuse extracellular matrix. Transverse sections; all are at a level between the eyes and otic capsules (A–C, left side; D, right side). Additional abbreviations: CM, cranial meninges; EM, extracellular matrix. Scale bars equal 50 μm .

and the otic capsule. Along its entire length it invests the dorsolateral margin of the orbital cartilage, but it still barely extends medially onto the cranial meninges.

Stage 35 (Fig. 6D)

The frontoparietal is both wider and longer. It is most prominent between the eye and otic capsule, where it extends farther onto the orbital cartilage than previously; a thin band of compressed cells also extends lateral to the red-stained extracellular matrix, presumably portending further growth. The frontoparietal appears to extend further onto the cranial meninges, although it is difficult to delineate its medial boundary within these heavily pigmented layers. The anterior margin of each frontoparietal lies in front of the eyes and adjacent to the telencephalon. At this level, however, the bone is very narrow,

essentially forming a cap on the dorsal edge of the orbital cartilage.

DISCUSSION

Our primary aims in this study were, first, to describe from serial sections the initial morphological differentiation of the earliest bones to form in the skull of *B. orientalis*, and, second, to compare the timing and nature of these events with information previously gathered from cleared-and-stained preparations (Hanken and Hall, '84). Our results clearly show that ossification centers corresponding to the frontoparietal, exoccipital, and parasphenoid bones are present and fully differentiated well before they are first visible in cleared-and-stained preparations. This observation is, in itself, not surprising; it is to be expected that small foci of bone would be detected earlier in serial sections

than in whole mounts. What is surprising, however, is how early these ossification centers are present and how far in advance of their visibility in whole mounts. All three bones have recognizable extracellular matrix and periosteal by Gosner stage 31. This is no less than six developmental stages prior to the stage when they are first visible in whole mounts, or an interval of 7–10 d at a rearing temperature of 18°C. We consider these results to be significant in several respects.

First, the developmental events that precipitate the differentiation of osteogenic cells to form bone must occur exceedingly early in anuran metamorphosis. The embryonic origins of the cells contributing to the amphibian osteocranium are poorly known. The few studies that have approached this problem have identified a likely cranial neural-crest origin for at least some bony elements (Andres, '46, '49; de Beer, '47; Wagner, '49, '59; reviewed in Noden, '82), as has been unequivocally demonstrated for the larval, cartilaginous skull (Chibon, '67; earlier references reviewed by de Beer, '47; and Hörstadius, '50). Analogous studies of amniotes, especially the chick, have revealed a dual origin of the osteocranium from both neural crest and paraxial mesoderm—the former contributing to bones of the facial region, the latter to much of the neurocranium and cranial vault (Noden, '82, '84, '86). Regardless of origin, however, such cells presumably would require an interaction with adjacent epithelium in order to complete their differentiation into bone, as is characteristic of cranioskeletal tissues generally (Hall, '82, '84). In theory, this interaction could occur any time between when embryonic neural crest and mesoderm-derived mesenchyme may be first recognized and the beginning of metamorphosis. At the very least, these cells apparently are triggered to complete their differentiation and to form distinct ossification centers by some signal that is provided earlier than stage 31 in *B. orientalis*.

Second, important developmental events that affect the differentiation of a prominent portion of the cranial skeleton and precede both intramembranous and endochondral ossification occur during premetamorphosis (Gosner stages 26–35). This phase was formally recognized by Etkin ('35) as the first of three metamorphic periods which is followed by premetamorphosis (stages 36–41) and metamorphic climax (stages 42–46). As reviewed by Etkin, premetamorphosis is "a

growth phase characterized by rapid growth and little morphological change" ('68: 320), a view that is shared, at least implicitly, by many developmental biologists and endocrinologists. This view may accurately depict the nature and magnitude of gross, externally visible metamorphic changes that occur during this period, as well as the demonstrably low levels of circulating hormones (particularly thyroid hormone) with respect to later stages. We, maintain, however, that it does not readily accommodate the fact that a large number of important developmental phenomena, which culminate in the differentiation of several distinct ossification centers, are occurring during this time. At least with respect to these early forming centers, we prefer to consider premetamorphosis as a period of differentiation, distinct from subsequent cell proliferation, matrix synthesis, and growth of the bone foci, which occur during prometamorphosis and metamorphic climax. The degree to which these early events are regulated by endocrine factors, as has been demonstrated for changes during prometamorphosis and metamorphic climax, requires further study.

Third, these results reveal at least two distinct phases of skeletal development that may be perturbed to achieve evolutionary diversity in cranial form: differentiation, and subsequent proliferation, matrix deposition, and growth. Differences in the relative time of appearance of cranial bones characterize interspecific comparisons among amphibian taxa; such phenomena compose the evidence used to implicate heterochrony, or change in the timing of developmental events, as an important developmental process that underlies cranial evolution in amphibians (Alberch, '83; Alberch and Alberch, '81; Hanken, '84; Reilly, '86; Trueb, '85; Wake and Hanken, '82). For example, cranial development in *Rana pipiens* is characterized by precocious appearance of parasphenoid and exoccipital bones relative to their timing in *Bombina*; these bones are first visible in cleared and stained preparations at stages 29 and 32, respectively, vs. stage 37 in *Bombina* (Fig. 7) (Hanken and Hall, '84; Kemp and Hoyt, '69). Clearly, timing of the processes of cell proliferation, matrix deposition, and growth that underlie the appearance of these ossification centers in cleared-and-stained preparations differs between the two taxa. In the absence of additional information describing the timing of osteogenic dif-

ferentiation in *Rana*, however, it is not possible to say whether timing of the initial differentiation of homologous elements in the two taxa also differs, or by how much. In theory, either or both of the two phases could be altered and contribute to the evolution of cranial diversity.

Finally, these results qualify the use of cleared-and-stained whole mounts for inferring the exact timing of initial bone formation. Whole mounts now are used widely to describe the sequence and timing of cranial ossification in a wide variety of vertebrates, but especially amphibians (e.g., Reilly, '86; Trueb, '85; Wake and Hanken, '82; Wake et al., '83). With respect to *Bombina orientalis*, however, our earlier description of the time of formation of the exoccipital, parasphenoid, and frontoparietal bones, which was based on cleared-and-stained preparations, yielded estimates that were six or seven developmental stages too late compared to the data we present above. Should our finding—the presence of a significant lag between the times when a given bone is first visible in serial sections vs. whole mounts—be a characteristic feature of amphibian cranial development in this and other species, then this would restrict the use of whole mounts to establishing only the relative time of formation of ossification centers both within and among species. Data necessary to establish this phenomenon as a general pattern in amphibians are, however, with the exception of the observations we present above, not available at this time. Obtaining such information is an important goal of future research.

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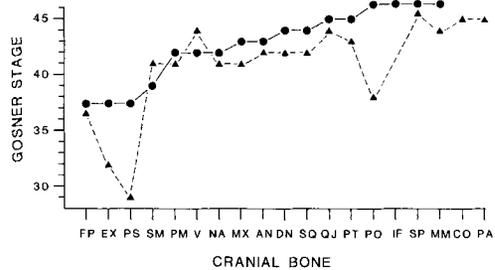


Fig. 7. Cranial ossification sequences of *B. orientalis* (solid circles) and *R. pipiens* (triangles), based on the stage of earliest appearance of each bone as determined from cleared-and-stained whole mounts. Timing is generally similar in the two species, except for the precocious appearance of the exoccipital, parasphenoid, and prootic bones in *Rana*. Data for *Rana* are from Kemp and Hoyt ('69); figure is redrawn from Hanken and Hall ('84). Additional abbreviations: CO, columella; IF, interfrontal; MM, mentomeckelian; PA, palatine; PO, prootic; SP, sphenethmoid.

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