Somitogenesis in the marsupial frog *Gastrotheca riobambae*

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ABSTRACT This paper reports the first description of somitogenesis in a non-aquatic-developing amphibian, the Andean marsupial frog, *Gastrotheca riobambae*. This frog develops from an embryonic disk located on top of a large yolky egg, bearing some resemblance to the embryo of the chick. Besides the histological characterization of somite formation, we quantified cell number in the developing somites of *Gastrotheca*, *Xenopus laevis* and the chick. *Gastrotheca* was found to have a mode of somitogenesis which has previously been encountered in the aquatic-developing toad *Bombina*. Somitic cell number was found to be an order of magnitude higher than that of *Xenopus*, and approximately double that of the chick. We discuss the possible relation between mode of somitogenesis, somitic cell number, speed of development and egg size.

KEY WORDS: segmentation, frog development, Anura, mesoderm, myotome

Introduction

The process of somitogenesis has been studied most intensively in the embryo of the chick (reviewed by Carlson, 1990), in which the somites begin to form from the dorsal mesoderm, from about 22 h onwards at an approximate rate of one pair every 100 min (Keynes and Stern, 1988). In transverse section the cells of the somite are arranged in a roughly radial manner, and a cavity, the myocoel, soon develops in the center. This arrangement has been termed the "rosette" (Bellaïrs, 1979). As the somite develops, differentiation occurs into sclerotome, the partial progenitor of the vertebral column, and dermomyotome, which gives rise to the myotome and dermatoche, progenitors of axial skeletal muscle and the dermis respectively (reviewed by Keynes and Stern, 1988).

Initially, after separation from the pre-somite mesoderm, the somite of the African clawed toad, *Xenopus laevis*, is similar to that of the chick, with a radial arrangement of pre-myotomal cells from the pre-myocoel, although the rosette configuration is less obvious (Hamilton, 1969). However, as the somite is formed, it undergoes myotomal re-orientation (Hamilton, 1969; Kielbowna, 1981), in which the radially arranged cells rotate ninety degrees to lie in the longitudinal axis of the embryo. The re-oriented cells span the length of the somite, and in transverse section appear as small circles.

Comparison of somite formation of the anurans *Pelobates fuscus*, *Bombina variegata* and *Rana sphenochepala* with *Xenopus*, indicates that the pattern of somitogenesis is essentially different in these four species (Kielbowna, 1981; Youn and Malacinski, 1981). In *R. sphenochepala*, there is neither myocoel nor rosette configuration, and myotomal re-orientation is only through forty-five degrees. This is followed by cell fusion (Youn and Malacinski, 1981). Somites of *B. variegata* are initially disorganized. The cells then undergo a process of elongation and interdigitation, and the resulting elongated myotomal cells span the length of the somite. There is no myotomal rotation. In *P. fuscus* there are further contrasts. The cells of the disorganized early somite fuse to produce long multinucleate myoblasts at a very early stage. There is nothing resembling the cellular interdigitation of *Bombina*, nor the myotomal rotation of *Xenopus* (Kielbowna, 1981).

In the urodeles, *Ambystoma mexicanum* and *Pleurodeles waltl*, the rosette configuration of the early somite is particularly pronounced. There is also myotomal re-orientation through ninety degrees, but it is not identical to *Xenopus* since there are changes in cell shape. Unlike *Xenopus*, cell fusion occurs to produce multinucleate myotomal cells. There is also persistence of the myocoel for a longer period (Youn and Malacinski, 1981).

The Andean marsupial frog *Gastrotheca riobambae* produces large eggs of 3 mm in diameter, which undergo a very long period of maternal incubation, with a correspondingly slow rate of early development, and an unusual form of gastrulation, developing from an embryonic disk (del Pino and Elinson, 1983; reviewed by del Pino, 1989). In view of these interesting features, we analyzed the pattern of somitogenesis, and the cell number of the developing somites in *Gastrotheca*, in comparison with *Xenopus* and other vertebrates.

Any consideration of somitogenesis in the Amphibia must take into account the diversity of developmental processes within this group. Besides the several different patterns of somite formation (reviewed by Radice et al., 1989), other aspects of early development also show considerable variability within the Amphibia. These include mode of fertilization, germ cell determination, mesoderm induction (reviewed by Nieuwkoop and Sutasa, 1979) and gastrulation (reviewed by Keller, 1986). A comparative approach to...
the embryology of amphibians is therefore justified, in that it might lead to the discovery of new developmental mechanisms, or to fresh insights into those already being studied. This paper demonstrates further diversity in the pattern of cellular re-arrangement during somite formation, and also shows that there may be a considerable variation in the number of cells per somite between species.

Results

Development of the somites in G. riobambae

In G. riobambae, the embryo develops from a disk of cells on top of a mass of yolky endoderm (reviewed by del Pino, 1989), its appearance being slightly reminiscent of the avian embryo (Fig. 1). The first somites can be observed on the surface of the embryo, with the aid of the dissecting microscope, in the lateral plate mesoderm of whole-mounted embryos at stage 12 (staging according to del Pino and Escobar, 1981). This is the stage at which head formation begins around the anterior neuropore (Fig. 2A). These somites are larger than those observable in whole-mounts of chick embryos (Figs. 1, 2B).

The first indications of segmentation, which are not visible on the surface, occur in stage-11 embryos, the stage of neural fold formation (17 days after fertilization at 18°C). Some embryos at stage 11 appeared to be unsegmented (Fig. 3A,B). However, sections of some other embryos at this stage show that the mesoderm contains fissures which may be the incipient boundaries of the first somites (Fig. 3C,D). This indicates that segmentation begins during stage 11.
The stage 12 (Fig. 2A) or 12-13 embryo (18 to 22 days old) has definite somitic masses. The cells may show some polarization, there may be an epithelial layer peripherally and some loose rounder cells in the center, giving rise to a structure which approximates to a rosette (Fig. 4A,B), but does not have the highly organized structure of rosettes in chick and Ambystoma (Bellairs, 1979; Youn and Malacinski, 1981). In later stages, the cells in the caudal somites are small and numerous and no obvious arrangement can be seen (Fig. 5A,B,C,D). As in Xenopus, the presence of a large number of yolk granules in the somites at early stages tends to make the visualization of cell nuclei difficult. The yolk granules also frequently tend to become dislodged during the processing of the material, leaving irregular spaces of varying sizes within the somite. These spaces are not seen in adjacent sections, suggesting that they are not myocoels. We have found no convincing evidence of a myocoel in any of the stages studied.

Stages 14, 15 (Figs. 1, 2B) and 16 (23 to 30 days old) are highly important in organogenesis, with the beginnings of development of the bell gills and cardiovascular system. However, in sections, the somites of these three stages have a generally similar appearance.

**Fig. 3. Early stages of somite formation in Gastrotheca.** Parasagittal sections through the triaminal germ disk of a stage-11 embryo. In (A,B), there is little sign of segmentation, but the beginnings of somite formation, with some sub-division of the mesoderm, can be seen in (C,D). ec: ectoderm. s: somite. m: mesoderm. en: endoderm. Scale bar, 50 μm.
Fig. 4. Intermediate stages of somite formation in *Gastrotheca*. (A,B) Sagittal section through an early rostral somite of a stage-12 embryo. The cellular organization approximates to a rosette. This is not seen in young somites at later stages. (C,D) Horizontal section through a mid-body somite of a stage-16 embryo. The cells are extending and interdigitating and a mitotic figure can be observed (mc: ectoderm. s: somite. en: endoderm. n: notochord. my: myotome. sc: sclerotome. mc: mitotic cell. Scale bars, 50 μm.

Immediately rostral to the pre-somitic, paraxial mesoderm there are early, disorganized somites. Progressive elongation and interdigitation of cells occurs in the middle and more rostral somites. Although elongating, the cells do not stretch the entire length of the somite and they are still mononucleated. These cells are still dividing, as indicated by the presence of mitotic figures (Figs. 4C,D, 6A,B). The division between myotome, dermatome and sclerotome is visible in rostral somites at this stage (Fig. 6A,B), the dermatome appearing as a layer of cells lateral to the more densely packed, elongating cells of the myotome, and the sclerotome as a loose, medial group of cells with rounder nuclei.

By stage 18 (40 days old), the embryo has lost its resemblance to that of the chick. There is eye pigmentation, mouth formation, and the embryo begins to acquire a more recognizable amphibian tadpole shape, sitting on top of an enlarged yolk intestine (Fig. 7A,B). The embryos have some differentiated skeletal muscle, enabling them to move inside the jelly capsule of the egg. Rostrally, it is possible to observe differentiated myocytes, which are multinucleated (Fig. 7C,D). In the mid-body region of the embryo there are some somites containing interdigitating cells and a small percentage of multinucleated myocytes (not shown). In the extreme end of the tail, there are still four or five somites in the disorganized state (somites n and n-1 to n-4 in Fig. 7E,F; compare Figs. 7E,F and 4A,B), and a small quantity of pre-somatic mesoderm (Fig. 7E,F). Since multinucleated myocytes are not visible in the rostral somites of stage-16 embryos, we conclude that myoblast fusion first occurs in rostral somites at stage 17, at the earliest.

Comparison with other species

The histological appearance of the most caudal somites in *Gastrotheca* (somites n and n-1 to n-4 in Fig. 7E,F and somite n in Fig. 8A,B) shows no obvious differences to that of the pre-somatic mesoderm (Figs. 7E,F, 8A,B), and there is no process of re-orientation as observed in *Xenopus*. Unlike the chick, *Xenopus* and
the urodeles, in transverse section, the caudal somites have no obvious rosette structure, and appear as mere aggregations of mesodermal cells (Fig. 5A, B, C, D), similar to the early somites of mammals (reviewed by Milaire, 1974). The transition from somites which are internally disorganized, to the type exhibiting elongated interdigitating cells, occurs gradually without any sudden boundary between different types. Unlike Bombina variegata and Xenopus, but like Pelobates fuscus, the cells of both disorganized and more advanced somites continue to divide, as evidenced by the presence of mitotic figures (Figs. 4C, D, 6A, B).

**Cell number in developing somites**

The large size of the somites in whole-mounts (Figs. 1, 2B), and the quantity of cells seen in sections suggested an analysis of somitic cell numbers in comparison with other species. We counted the total number of nuclei in the somites of a stage-15 Gastrotheca (Fig. 2B), a stage-25-26 Xenopus (16-somite) and a 13-somite chick embryo. Although most of the somite mass consisted of myotomal cells, the nuclei in the dermatomal and sclerotomal portions were counted as well where visible. Embryonic stages were chosen according to the following criteria: early stages of Gastrotheca contain many yolk platelets, thus making the cells difficult to visualize. Later stages begin to demonstrate cell fusion in the rostral somites. Therefore we selected the stage-15 embryo, which is free from these problems. We then chose stages of Xenopus and chick which corresponded roughly in external morphology and the number of somites, with the Gastrotheca embryo. The Gastrotheca embryo had 15 somites at stage 15, but only the most caudal nine could be counted in serial horizontal sections, due to the curvature of the embryo at the head end. Despite this deficiency, it can be seen that the Gastrotheca embryo has a number of cells which is an order of magnitude greater, in all somites, than Xenopus (Fig. 9). The chick embryo falls somewhere between the other two.

In all three species, the more rostral somites, which are the earliest somites to be formed, have a larger number of cells than the more caudal, later, somites. This has previously been observed in Xenopus (Cooke, 1988). This difference is greater in the chick (Fig. 9), and may depend on the number of cells originally allocated to each somite. Additionally, in those species in which there is cell division within the somite, for example Gastrotheca but not Xenopus, this factor may also be important.

In order to place these cell counts in a wider perspective, we reviewed previous studies on somitogenesis (Hamilton, 1969; Bellairs, 1975; Kielbowna, 1981; Youn and Malacinski, 1981;
Fig. 6. Differentiation of somite in Gastrotheca at the stage of cell elongation. (A) Horizontal section through rostral somites of stage-16 embryo. (B) Diagram showing separation of myotome (my), dermatome (d) and sclerotome (sc), and segmentation of all three portions of the somite. The cells are extending and interdigitating and a mitotic figure can be observed (m). Scale bar, 50 μm.

Boudjelida and Muntz, 1987; Tam and Beddington, 1987; Cooke, 1988; Jaffredo et al., 1988; Ott et al., 1991. Our observations suggest that the somites of Gastrotheca and B. variegata contain many more cells than those of Xenopus and Ambystoma. Somite cell number in the chick has been quantified as approximately half that of Gastrotheca (Fig. 9). The murine embryo has somites in which the number of cells and internal organization are similar to Gastrotheca (see illustrations in Tam and Beddington, 1987 and Ott et al., 1991).

Discussion

Gastrotheca develops very slowly in comparison with Xenopus and other amphibians (Table 1). Stages 11 to 18 of Gastrotheca, considered here, take some 23 days (del Pino and Escobar, 1981). In contrast, Xenopus passes through the comparable embryonic stages in about 24 hours (Nieuwkoop and Faber, 1967). It has been suggested that the general rapidity of embryonic development in Xenopus is a strategy for predator avoidance. Somitic re-orientation may have evolved as a part of this (Radice et al., 1989). To clarify this issue, it would be interesting to study somitogenesis in amphibians with extreme developmental rates. The following candidate organisms may be suggested. The genus Eleutherodactylus contains some of the fastest developing amphibians, which emerge from the egg as froglets in 12 to 15 days (reviewed by Elgin et al., 1990). At the other extreme, G. richambae is one of the most slowly developing anurans, but among the aquatic urodeles there are several even more slowly developing species. The Pacific giant salamander, Dicamptodon ensatus, for instance, requires 275 days to hatchling of the larvae, which then grow for a further 700 days to metamorphosis (Duellman and Trueb, 1986).

We believe that in Gastrotheca, unlike Pelobates fuscus, Rana sphenocephala, and the urodeles, there is no cell fusion in the somites until complete elongation of the somitic cells has occurred, since there is no evidence of multinucularity in somites with interdigitating cells (Figs. 4C, D, 6A, B), and cell division is still occurring (Figs. 4C, D, 6A, B). However, without electron microscopy, this possibility cannot be definitely excluded. Like R. sphenocephala, the somites of Gastrotheca do not exhibit a myocoel. Neither is there any clear division into two epithelial sheets, as observed in the dogfish, Scylliorhinus canicula (reviewed by Mialaire, 1974), although at stage 12, the earliest somites approximate to a rosette, as already described (Fig. 4A, B).

Cooke (1988) observed that, in sibling pairs of Xenopus embryos where one egg was double the diameter of the other, the first somites to be formed in the smaller embryo contained significantly fewer cells than the corresponding somites of the larger sibling. In Xenopus, therefore, it seems that there is a mechanism scaling somitic size to the size of the embryo as a whole. From this we infer that there may be some mechanism relating egg size, or embryonic size, to somitic cell number, at least in the early stages of somitogenesis. This in turn may place constraints on the mechanism of somite formation.

Our comparison of somite cell number in G. richambae and Xenopus suggests that egg size, embryonic size and somitic cell number may be related across species (Fig. 9). Elgin (1987) has argued convincingly that egg size is relevant to the control of several developmental processes. Further comparative studies of a wide range of species with extreme egg sizes is desirable. These might include Gastrotheca ceratophrys, G. cornuta, G. weinlandii, and Hemiphractus scutatus and some other egg-brooding hylid frogs with egg diameters of about 10 mm (del Pino and Escobar, 1981). At the other extreme, the viviparous Nectophrynoides occidentalis has eggs of 0.6 mm in diameter (Duellman and Trueb, 1986).

Although we do not claim to have reached any final conclusions regarding the relationship between egg size, developmental rate, number of cells per somite and mechanisms of somitogenesis, it seems that patterns of somitogenesis which involve the rotation of elongated cells, as in X. laevis, may require a relatively low somitic cell number. Egg diameter, or total embryonic cell number, may be
Fig. 7. Latter stages of somitogenesis in Gastrotheca. (A,B) Whole-mount preparation of a stage-8 embryo from which the large yolky intestine and the bell gills have been removed, indicating the regions of the embryo from which the following views were prepared. (C,D) Oblique para-sagittal section through the trunk, showing differentiated muscle (m). (E,F) Para-sagittal section through the tail, showing the continued production of new somites at this stage. vc: vertebral column. m: muscle. ec: ectoderm. nt: notochord. psm: pre-somitic mesoderm. Somites are numbered from the most caudal and recently formed (n) to the most rostral in the figure (n-4). Scale bars. (A,B), 1 mm; (C-F), 50 μm.
an important factor in controlling the number of cells per somite, and thus may have an indirect influence on the pattern of somite formation.

This idea may also be applicable in urodeles. Although the egg diameter of *Ambystoma mexicanum* is approximately double that of *Xenopus* (Table 1), developing somites of this urodele have relatively small numbers of cells. However, *Ambystoma* has far larger cells than most anurans. Nuclear DNA value, and thus the nuclear and cellular size, are generally larger in urodeles than in the anurans (Fankhauser, 1945; Brown and Dawid, 1968; Sommerville, 1977; Horner and Macgregor, 1983; del Pino, 1989). Therefore, despite its relatively large egg size, the embryonic cell number of *Ambystoma*, at any given stage, may be smaller than that of any frog. It is notable that somitogenesis in *Ambystoma* does involve a mechanism of cell rotation, comparable, although not identical, to the somitic re-orientation of *Xenopus* (Table 1), which suggests that low somitic cell number may be required for this mechanism.

*Gastrotheca riobambae* is able to provide maternal protection for its embryos. This may have resulted in selection pressure for large eggs, since embryos in the pouch need to be nurtured by considerable reserves of yolk. Large eggs with holoblastic cleavage may allocate many cells to each somite, and somitogenesis is carried out by a process of elongation of large numbers of interdigitating cells. In *Bombina*, selection pressure favored large eggs (Table 1) and a small clutch size of only up to 100 eggs, comparable to that of *G. riobambae* (Sussman and Betz, 1978; del Pino and Escobar, 1981). Somitogenesis of the large *Bombina* eggs follows the pattern seen in *Gastrotheca*.

Finally, it may be stated that the variety of patterns of somitogenesis in amphibians, at the morphological level, is reflected in a corresponding variation in the molecular aspects of the process, as has been demonstrated for actin and myosin expression in anuran versus urodele somites (Neff et al., 1989). In this context, *G. riobambae* presents an interesting new system for molecular studies of morphogenesis.

**Materials and Methods**

Methods for the handling of *Gastrotheca riobambae* embryos are according to Elinson et al. (1990). *Gastrotheca* embryos from stages 11 to 18 were removed from the dorsal pouch of a gravid female. Embryos were staged according to del Pino and Escobar (1981), fixed in Smith's fixative at room temperature for 24 h, then washed several times in distilled water and stored at room temperature in 4% formalin. After fixation, the embryonic disks were dissected free of the yolk masses and processed for histological sections as described below. *Xenopus* embryos were staged according to Nieuwkoop and Faber (1967), fixed in buffered 4% formaldehyde and stored in methanol until processing.

Embedding was in JB-4 plastic resin (Polysciences) and sectioning at 5 μm using a Reichert-Jung 2050 microtome. For best visualization of the somites, sections were cut sagittally, transversely and horizontally (i.e., coronally). Staining was in Lee's methylene blue-basic fuchsin stain (Bennett et al., 1976) and observation on a Zeiss Axiophot. Photographs were taken under direct light and phase contrast. Serial sections of a 33-h chick embryo were obtained from Gen. Biol. Inc., Chicago.

In *Gastrotheca*, *Xenopus* and the chick, the number of cells per somite was determined by the counting of all nuclei in the somite in serial sections using a manually-operated counting meter. In the cases of *Gastrotheca* and *Xenopus*, it was first established by examination of serial sections to be counted that the thickness of the sections was great enough to prevent the

**Fig. 8. Absence of somitic re-orientation in *Gastrotheca*. (A,B) Horizontal section through stage-16 embryo of *Gastrotheca*, showing absence of somitic re-orientation and similarity of histological structure of pre-somatic mesoderm (psm) and somite (numbered n). nt: notochord. ec: ectoderm. Scale bars, 50 μm.**

**Fig. 9. Comparison of somite cell number.** Total number of cells per somite in *Gastrotheca* (triangles), *Xenopus* (filled squares) and the chick (circles). Somites are numbered consecutively beginning from the most caudal in, as explained in Fig. 7. Total somitic cell number was determined by adding counts of nuclei from serial sections. Nuclei in all parts of the somite were counted.
TABLE 1

DEVELOPMENTAL SPEED, EGG SIZE AND MODE OF SOMITOGENESIS

<table>
<thead>
<tr>
<th>Species</th>
<th>Egg size (mm)</th>
<th>Time to gill circulation (hours)</th>
<th>Somitogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>X. laevis</em></td>
<td>1.2</td>
<td>66</td>
<td>Cell rotation</td>
</tr>
<tr>
<td><em>A. mexicanum</em></td>
<td>2</td>
<td>190</td>
<td>Cell rotation/fusion</td>
</tr>
<tr>
<td><em>R. sphenophoca</em></td>
<td>1.75</td>
<td>110</td>
<td>Partial cell rotation</td>
</tr>
<tr>
<td><em>B. variegata</em></td>
<td>3</td>
<td>108</td>
<td>Cell interdigitiation</td>
</tr>
<tr>
<td><em>G. riobambae</em></td>
<td>3</td>
<td>650</td>
<td>Cell interdigitiation</td>
</tr>
<tr>
<td><em>P. fuscus</em></td>
<td>1.5</td>
<td>132</td>
<td>Cell fusion</td>
</tr>
</tbody>
</table>

1 References: X. laevis (Nieuwkoop and Faber, 1967); A. mexicanum (Malacinski, 1978; Duellman and Trueb, 1986); R. sphenophoca (average for genus Rana from Duellman and Trueb, 1966; Youn and Malacinski, 1981); B. variegata (for B. orientalis from Sussman and Betz, 1978; Horner and Macgregor, 1983); G. riobambae (del Pino and Escobar, 1981); P. fuscus (Duellman and Trueb, 1988).


appearance of any one nucleus in two consecutive sections. In the chick, the thickness of the sections was greater, therefore nuclei were counted by focusing through the section. Once again, adjacent sections were carefully compared to ensure that no nucleus appeared in two consecutive sections. Whole-mount preparations of Gastrotheca were kindly provided by H. Steinbeisser, from a study of gene expression in whole-mount in situ hybridization (unpublished data).

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