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Cellular Organization and Appearance of Differentiated Structures in Developing Stages of the Parasitic Platyhelminth *Echinococcus granulosus*

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Abstract *Echinococcus granulosus* is the causative agent of hydatidosis, a major zoonoses that affects humans and herbivorous domestic animals. The disease is caused by the pressure exerted on viscera by hydatid cysts that are formed upon ingestion of *E. granulosus* eggs excreted by canine. Protoscoleces, larval forms infective to canine, develop asynchronously and clonally from the germinal layer (GL) of hydatid cysts. In this report, we describe the cellular organization and the appearance of differentiated structures both in nascent buds and developed protoscoleces attached to the GL. Early protoscolex morphogenesis is a highly complex and dynamic process starting from the constitution of a foramen in the early bud, around which nuclei are distributed mainly at the lateral and apical regions. Similarly, distribution of nuclei in mature protoscoleces is not homogenous but underlies three cellular territories: the suckers, the rostellar pad, and the body, that surrounds the foramen. Several nuclei are associated to calcareous corpuscles (Cc), differentiated structures that are absent in the earlier bud stages. The number of nuclei is similar from the grown, elongated bud stage to the mature protoscolex attached to the GL, strongly suggesting that there is no significant cellular proliferation during final protoscolex development. The amount of DNA per nucleus is in the same range to the one described for most other platyhelminthes. Our results point to a sequential series of events involving cell proliferation, spatial cell organization, and differentiation, starting in early buds at the GL of fertile hydatid cysts leading to mature protoscoleces infective to canine. J. Cell. Biochem. 94: 327–335, 2005.

Key words: cell organization; differentiation; protoscolex development

The cestode *Echinococcus granulosus* is the causative agent of hydatidosis, or hydatid disease, a major zoonoses that affects humans as well as various animals, including domestic ones [Thompson and Lymbery, 1995; Cabrera et al., 2003]. The study of this parasite has deserved important biochemical, molecular, and immunological work during the last two decades. Nevertheless, fundamental questions concerning *E. granulosus* biology which are relevant to the control and treatment of the hydatid disease are still open, in particular those related to the mechanisms controlling the development and growth of the parasite [Thompson and Lymbery, 1995; Fernández et al., 2002; Galindo et al., 2002; Chemale et al., 2003].

Hydatid disease is produced by the mechanical pressure exerted in the viscera of intermediary hosts (human and herbivorous) by hydatid cysts [Gottstein and Hemphill, 1997]. This parasitic structure is composed of three layers: an inner germinal layer (GL) that is supported externally by a tough elastic non-cellular laminated layer, which in turn is surrounded by

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a host-produced adventitial layer [Thompson and Lymbery, 1995]. From GL, protoscoleces (PScs), larval forms of the parasite infective to definitive hosts (canines), are asynchronously and clonally formed. The study of the early development of PScs from the GL and/or brood capsules (daughter internal cysts) of hydatid cysts in natural infections, has implications both in diagnosis and in therapeutic practice [Eckert et al., 1995]. As an example, it was reported that the antiparasitic drug praziquantel affects PSc differentiation only when applied at the beginning of that process [Urrea-Paris et al., 2002]. Consequently, systematic studies on the morphological, cellular, and molecular basis of protoscolex development are important to understand the strategy of parasite infectivity as well as to find putative targets for a rationale drug design. Given the phylogenetic position of this parasitic platyhelminth, studies related to the mechanisms controlling protoscolex development are also intended to enlighten fundamental principles about morphogenesis and morphological innovation during evolution.

During early PScs development, cell buds are generated in the GL of the hydatid cyst which grows toward the cyst cavity or lumen, thus, giving rise to differentiated mature protoscoleces or to brood capsules. These latter structures are daughter cysts, which are morphologically similar to the mother cyst, except for the absence of the adventitial layer; these structures are able to produce protoscoleces by the same cellular mechanism as described above. The process of budding leads to the formation of PScs [Thompson, 1976]. Eventually, infective PScs are released to the cyst lumen.

Recently, seven stages in the PScs development from the GL in natural infections have been reported [Galindo et al., 2002, 2003]. On the other hand, some molecular aspects of *E. granulosus* development have also been described [Oliver et al., 1992; Ehrlich et al., 1994; Martínez et al., 1997; Chalar et al., 1999].

During these studies, several questions have arisen in relation to the spatial organization of the cells and the appearance of differentiated structures during protoscolex development. In this report, we address some of these questions by studying the cellular organization of both nascent buds and developing protoscoleces attached to the GL. Our data provide new information on the cellular mechanisms of bud formation and growth that lead to protoscolex maturation. Moreover, our results may contribute to the morphological location of molecular markers involved in the *E. granulosus* early development which could eventually be helpful in finding putative targets for a rationale drug design.

MATERIALS AND METHODS

Samples

E. granulosus fresh fertile hydatid cysts were obtained from ovine and bovine livers or lungs obtained from abattoirs in Chile and Uruguay. Cysts were processed as described elsewhere [Galindo et al., 2002]. Viability of the protoscoleces, free or attached to the GL, as well as the presence of early and elongated buds was monitored as described [Galindo et al., 2003]. Other parameters of viability, such as a whitish color and the thickness of the GL [Bortoletti and Ferreti, 1973] were also considered.

Hematoxylin Staining

Pieces of GL from fertile hydatid cysts were fixed in 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS) pH 7.2 at 4°C for 24 h, and then embedded in paraffin. Sections (5 μm) were stained in hematoxylin–eosin and visualized under a Zeiss light microscope. Digital images were obtained using a Coolpix 995 Nikon camera.

Propidium Iodide (PI) Staining

Protoscoleces and associated brood capsules and GLs were isolated from open cysts and fixed in freshly prepared 4% (w/v) paraformaldehyde in PBS for 24 h at room temperature. The fixative was removed, replaced by methanol, and the material was stored at −20°C. Methanol was removed by four washes in PBS, 10 min each, and an RNAse solution (400 μl, 150 μg/μl final concentration) was added to remove RNA. PI (750 μl, 2 μg/ml final concentration) was added to 20 μl aliquots of parasites. After 1 h at room temperature in the dark, the stained parasites were washed twice with PBS, included in VectaShield™, mounted on slides and visualized in a Bio Rad MRC 600 confocal scanning laser microscope. Digital images corresponding to series of sections separated by 0.9 μm, were obtained.

Additionally, slices of parasites were stained by the same procedure after being deparaffinated in xilol and ethanol, and were visualized by differential interference contrast (DIC) under an epifluorescence microscope.

Number of Nuclei in Protoscoleces

The number of slices occupied by each nucleus was measured in a sample of seven parasites (five mature and two immature PScs). Routinely, 20 nuclei per stack were analyzed. Number of nuclei were estimated by the average of several independent counts in sections separated by 3.6 μm and then 2.7 μm (i.e., four and then three sections), using an appropriate software (Image J 1.25s, available at http://rsb.info.nih.gov/ij), and then adjusting the number by hand. As an example, and for a typical confocal stack of images comprising 100 sections, we start counting at section 10, then at section 14, then at 17 and so on.

Nuclei Isolation

The procedure of Blobel and Potter [1966] was applied, with some modifications. Briefly, GL of hydatid cysts was homogenized at 4°C in 0.05 M Tris HCl, pH 7.4, 0.025 M KCl, 0.003 M $MgCl_2$, 0.002 M $CaCl_2$, and 0.32 M sucrose. Protease inhibitors (5 mM PMSF, 2.5 mM TLCK, and 1 mM EDTA) were added to the homogenate. A crude nuclear fraction was obtained by centrifugation at 650*g* for 15 min at 4°C. The sediment was washed twice in the homogenizing buffer and suspended in 1.6 M sucrose in the same homogenizing buffer. Two milliliter of this suspension was layered onto 2 ml 2.2 M sucrose. The gradient was centrifuged at 70,000*g* for 60 min at 4°C in a Sorvall ultracentrifuge model OTD65-B using an AH-650 rotor. Nuclei were collected from the 1.6 to 2.2 M sucrose interphase, diluted in PBS and sedimented by centrifugation at 10,000*g*. Purity of the nuclei was evaluated by light microscopy, DNA quantification, and oxygen consumption.

Estimation of Nuclear DNA Content

Nuclei were suspended in PBS and counted in a Neubauer counting chamber. For DNA estimation, an aliquot of the suspension was mixed with an equal volume of 10% trichloroacetic acid (TCA) and the sediment was recovered after spinning at 10,000*g*. The sediment was washed twice in ice-cold 5% TCA, once in 80% ethanol and once in ethanol-ether (3:1, v/v). DNA was extracted from the sediment in 5%TCA at 90°C and its concentration determined according to Burton [1956]. The content of DNA per nucleus was estimated by dividing the amount of DNA in a volume of nuclei suspension by the number of nuclei in the same volume.

RESULTS

Distribution of Nuclei During Early Developmental Stages of Nascent Protoscoleces

As previously shown [Galindo et al., 2003], cells from an early bud are separated from cells of the GL (Fig. 1A), being concentrated at the lateral and apical regions of the bud. This heterogeneous distribution of cells originates a space free of cells or foramen which, in latter stages, moves on toward the apical zone of the bud (Fig. 1B), adopting a Y-shape in the slice (Fig. 1C), and a cup-shape as observed in three dimensions (Fig. 2A). These observations were confirmed by confocal and by conventional light microscopy. Figure 2A shows that nuclei are found at the border of the bud (right, upper and lower figures) while a foramen is present at the center of the bud (left, upper, lower and center figures).

Additionally, serial sections of a nascent bud stained with hematoxylin–eosin show that the nuclei are concentrated at the apical and lateral regions of the bud while a foramen is evident at the base and center of that structure (Fig. 2B, a–f). In a more developed stage of a nascent protoscolex, the cup-like foramen is well defined (Fig. 3A,B), leaving nuclei (3A) and differentiating structures (3B) at the apical and lateral zones of the bud.

Distribution of Nuclei in Developed Protoscoleces Attached to the GL

In Figure 4, the distribution of nuclei in two fully developed protoscoleces attached to the GL of a fertile hydatid cyst is shown. In Figure 4A, nuclei are concentrated at the suckers (Su) and at the rostelar pad (RP); this last structure is found below the hooks (H). The foramen (F) follows the shape of a cup (Fig. 4A,B), as it was observed in early and grown buds (Figs. 1, 2, and 3). Interestingly, calcareous corpuscles (Cc) are clearly evident at the base of the grown protoscolex (Fig. 4B, arrow), surrounded by a small cluster of cells. Cc are not seen in early buds, thus appearing as a structure proper of developed protoscoleces.

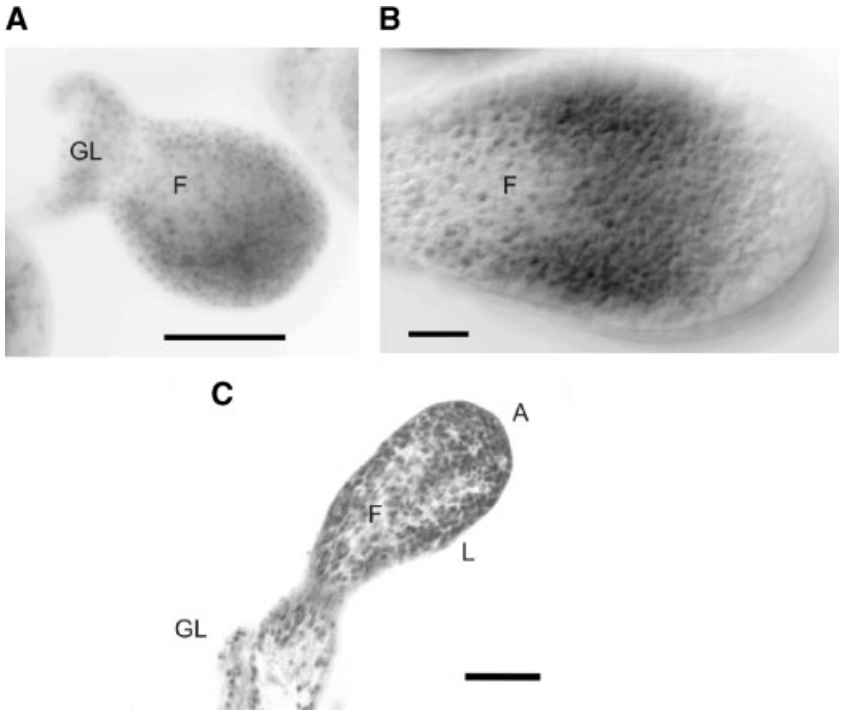

Fig. 1. Different development stages of buds attached to the germinal layer of fertile hydatid cysts. **A:** Early bud, bar 50 μm. **B** and **C**: Elongated buds, bars 50 and 100 μm. A and B, PI-stained buds, inverse image; C, Hematoxylin–eosin stained bud. A, apical region; L, lateral region; F, foramen; GL, germinal layer.

Number of Nuclei in Protoscoleces Attached to the GL and in Free Protoscoleces

To determine the average diameter of nuclei, i.e., the number of confocal sections each nucleus occupies, 20 nuclei were analyzed in each stack, as indicated under "Materials and Methods." By this procedure, a value of 6.9 ± 0.3 sections per nucleus was found. Estimations of the number of nuclei yielded 1733 ± 126 in mature protoscoleces, and 1663 ± 42 in juvenile protoscoleces attached to the GL (Table I). The maximal number of nuclei was found typically in middle sections and it was in a range between 200 and 300 nuclei (Fig. 5). Finally, we have estimated the number of nuclei present in elongated grown bud stages (as in Fig. 2B) and found to be roughly the same as in mature and juvenile PScs (not shown).

The distribution of nuclei in mature protoscoleces, as seen in whole-mount preparations observed by confocal microscopy, is not homogenous, but appears to be concentrated in two defined structures: the suckers and the rostellar pad (Fig. 5). Nuclei are also found adjacent to the protoscolex tegument, surrounding the foramen and shaping the body of the protoscolex; in this region, several nuclei associated to Cc are found. Protoscoleces stained with haematoxylin–eosin and observed under a light microcope show similar nuclei distribution (Fig. 4).

Isolation of Nuclei and DNA Nuclear Content in the GL of Hydatid Cysts

Interestingly, most of the nuclei were found in the interphase 1.6–2.2 M sucrose, contrarily to the observed sedimentation of vertebrate nuclei below the 2.2 M sucrose cushion [Blobel and Potter, 1966]. Purity of the nuclei was evaluated by light microscopy, DNA quantification, and oxygen consumption (not shown). A mass of 3.1 pg of DNA per nucleus was found as an average of six independent nuclear counts and DNA determinations (Table II).

DISCUSSION

Our results support and extends previous study from Galindo et al. [2002, 2003] in emphasizing some new aspects of protoscolex development. The non differentiated bud is a hollow structure with most of the cells concentrated at the lateral and lateral-apical regions of the

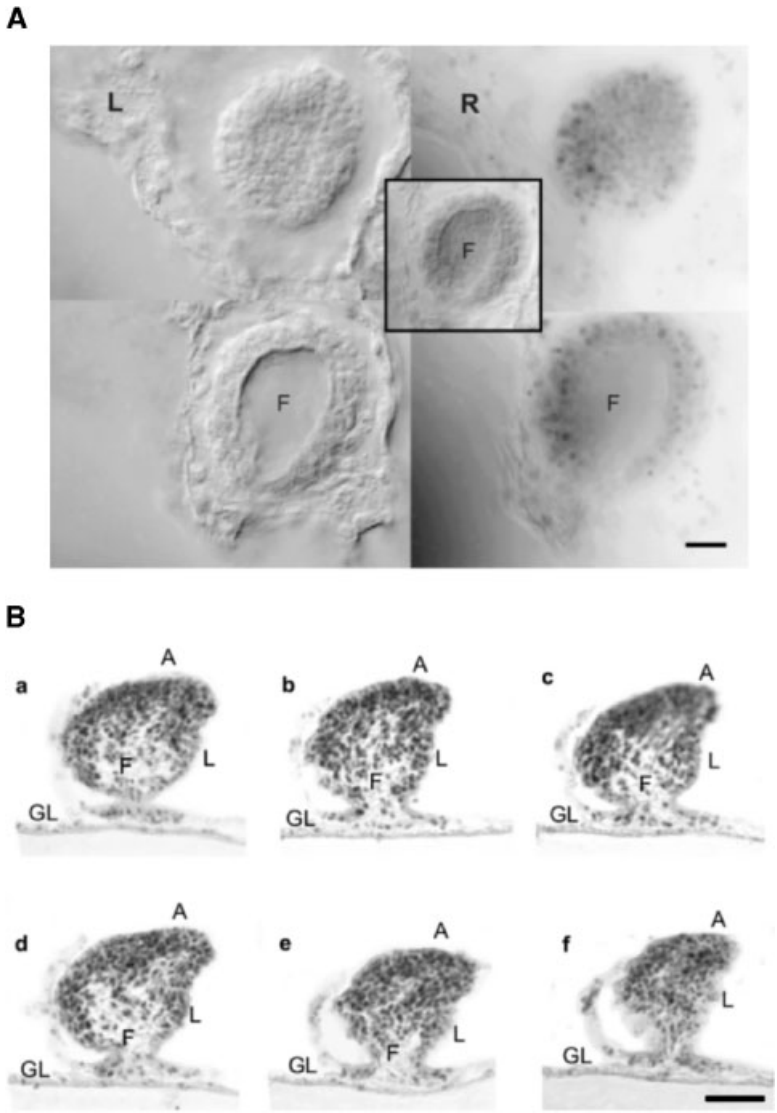

Fig. 2. **A**: Serial observations (**top** to **bottom**) of a whole-mount preparation of an early bud. L, DIC microscopy; R, PI-stained fluorescence microscopy, inverse image; Center, merged image. Bar: 50 μm. **B** (**a**–**f**): Serial sections of a bud attached to the germinal layer of a fertile hydatid cyst. The sample was fixed in 4% paraformaldehyde, embedded in paraffin and 5 μm serial sections were stained with hematoxylin–eosin. Bar: 100 μm. A, apical region; L, lateral region; F, foramen; GL, germinal layer.

nascent protoscolex. This is evident from the spherical early bud stage onwards. Cells are mainly clustered in lateral regions at the apex of the bud (where presumptive suckers will develop) and in an anterior and central small region (where the rostellar pad and hooks will emerge). As observed earlier by Rogan and Richards [1987] and Galindo et al. [2002], a furrow is visible delineating this specific central territory, before hooks (which constitute the first completely differentiated structure in the developing protoscolex) begin to form, and separating the apical zone of the nascent PSc from the forming body. Once hooks appear, suckers begin to develop as lateral depressions and projections at the apex of the developing bud [as in Galindo et al., 2002]. Thus, hooks and suckers cellular territories are previously defined by clustering of determined cells.

The central hollow space, the foramen, seems to collapse by the displacement of the central structure towards the stalk. The foramen shapes the place of the future invagination and retraction processes of the scolex, an important mechanism associated to the fixation of mature infective protoscolesces to the intestinal

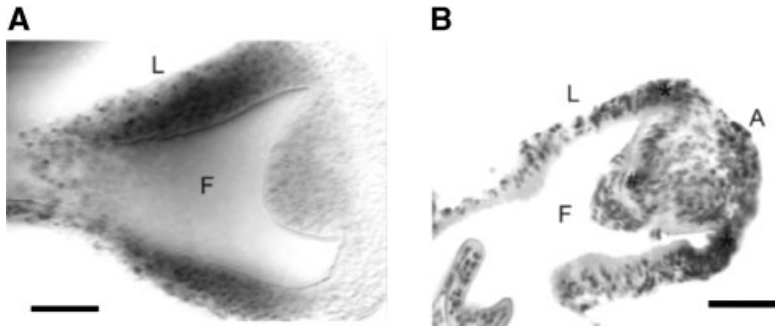

Fig. 3. **A**: Whole-mount preparation of a grown bud. Merged DIC and inverse PI-stained images. Bar: 100 μm. **B**: Light microscopy of a grown bud attached to the germinal layer of a fertile hydatid cyst. The sample was fixed in 4% paraformaldehyde and embedded in paraffin. Five micrometer sections were stained with hematoxylin–eosin. Bar: 100 μm. A, apical region; L, lateral region; F, foramen; #, primordial hooks; *, primordial suckers.

microvilli of canine. On the other hand, the bud stage is devoid of Cc, which are evident in fully developed protoscoleces attached to the GL as well as in mature free PScs in the hydatid fluid. In mature infective protoscoleces Cc may account for as much as 14.3% of the dried tissue weight [Thompson and Lymbery, 1995]. Interestingly, Cc disappear during protoscolex development to adult worm in the canine intestine. Thus, our observation suggests that developing protoscoleces may be infective to canids from the stage at which hooks, suckers and Cc are fully formed.

The number of nuclei remains unaltered from elongated grown bud stage onwards; this observation strongly suggests that no significant cell proliferation occurs during the formation of specific structures in protoscolex development. That would mean that from elongated grown buds on, cell differentiation is the main process leading to the formation of hooks and suckers, the attachment apparatus of the protoscolex to microvilli of the canine intestine.

Sedimentation of *E. granulosus* nuclei in the interphase of 1.6/2.2 M sucrose suggests a lower density of these cellular structures as compared to vertebrate nuclei. This is probably related to the lower amount of DNA per nucleus in *E. granulosus*, approximately half the amount of DNA per nucleus found in mammals. Furthermore, the nuclei of *E. granulosus* seems to be smaller than the one of mammals (not shown). The amount of DNA per nucleus in platyhelminthes varies widely from 0.06 pg per haploid nuclei in *Stenostomum brevipharyngium* to 20.52 pg in *Otomesostoma auditivum* [Gregory, 2001]. However, most of the platyhelminthes show an amount of DNA per nucleus in the range 1.0–5.0 pg which fits well with the amount of DNA per nucleus we have found for *E. granulosus*. Nematodes, round worms of medical interest, show a much lower amount of

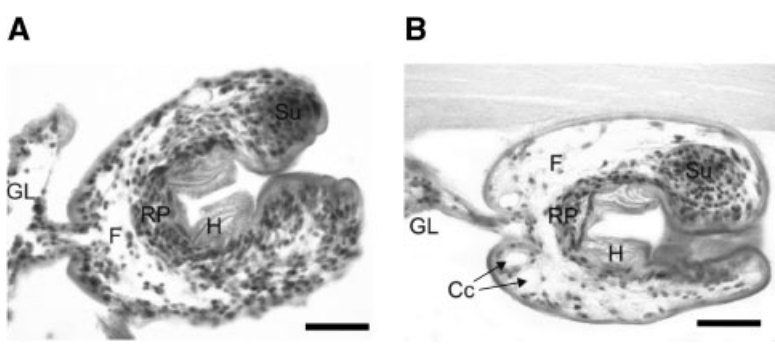

Fig. 4. Light microscopy of two protoscoleces (**A** and **B**) attached to the germinal layer of a fertile hydatid cyst. The samples were fixed in 4% paraformaldehyde, embedded in paraffin and 5 μm sections were stained with hematoxylin–eosin. Bar: 100 μm. F, foramen; RP, rostellar pad; H, hooks; Su, suckers; Cc, calcareous corpuscles; GL, germinal layer.

TABLE I. Number of Nuclei in Mature and Juvenile PScs

Samples	Nuclei number
Mature PSc1	1,940
Mature PSc2	1,703
Mature PSc3	1,656
Mature PSc4	1,617
Mature PSc5	1,750
Mean value ± SD	1,733 ± 126
Juvenile PSc1	1,633
Juvenile PSc2	1,693
Mean value ± SD	1,663 ± 42

A sample of seven whole-mounted, PI-stained parasites (five mature and two immature PScs) was analyzed. Numbers of nuclei were estimated by taking the average of several independent countings in confocal microscopy sections separated by turns of four and then three sections, as each nucleus occupies an average of 6.9 ±0.3 sections. The final number was then adjusted statiscally. See additional details in "Materials and Methods."

DNA per nucleus than plathyhelminthes, ranging from 0.027 pg per haploid nuclei in *Meloidogyne graminicola* to 2.1 in *Parascaris univalens* [Gregory, 2001].

We have already cloned and characterized some molecules that could play a relevant role during PSc development. In particular, several homeobox containing genes have been isolated, some of them corresponding to the Hox-type [Lalanne, 2003] and others to the *NK* homeobox family [Oliver et al., 1992]. Hox genes are involved in patterning during the embryonic development [De Robertis, 1994], particularly in the anterior-posterior axis, whereas *NK*-type homeobox genes have been involved in the control of differentiation processes in several organisms [Kim and Niremberg, 1989; Price et al., 1992; Jagla et al., 1993; reviewed in Duboule, 1994]. One of these *NK*-type homeobox containing genes, *Eghbx3 (Stalker)*, shows an expression pattern located in a specific PSc region (the stalk) and the associated GL [Martínez et al., 1997].

The observation of sequential developmental steps in the organization of cells, from the initial bud formation to the PSc attached to the GL, suggests the occurrence of possible inductive processes like those involving transforming growth factors (TGFs), bone morphogenetic factors (BMPs), and fibroblast growth factors (FGFs) signaling, as reported in other systems [Hogan, 1996]. In this regard, it is worth mentioning that BMP 2/4 has been found in a recent survey of full-length-enriched cDNA libraries from *E. granulosus* PScs (Cluster ID EGC00109, http://nema.cap.ed.ac.uk/Lopho/LophDB.php; Fernández et al., 2002). Similarly, TGF-beta receptor-interacting protein

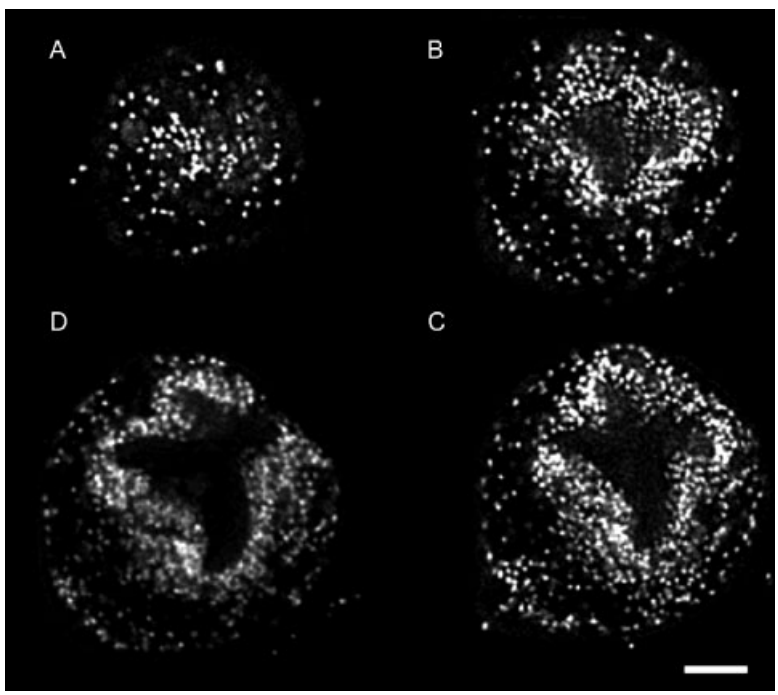

Fig. 5. Confocal microscopy observation of a whole-mount PI-stained mature protoscolex. Several representative slices from a stack, left to right, clockwise: **A**, 35/109; **B**, 45/109; **C**, 55/109; and **D**, 65/109. Bar: 50 μm.

TABLE II. DNA Content per *E. granulosus* Nucleus

Experiment	pg DNA/per nucleus
1	3.0
2	2.1
3	3.9
4	3.3
5	3.6
6	2.9
Mean number ± SD	3.1 ± 0.6

Nuclei were isolated from the germinal layer of hydatid cysts, suspended in PBS and counted. In an aliquot of the same suspension, the amount of DNA was measured. The content of DNA per nucleus was estimated by dividing the amount of DNA in a volume of nuclei suspension by the number of nuclei in the same volume.

(EMC00137) and TGF-beta resistance-associated protein TRAG (EMC00457) were found in *E. multilocularis*, a closely related species.

In conclusion, our results point to the protoscolex morphogenesis as a highly complex and dynamic process involving different steps that should inspire the search of key molecular players responsible for the development of the protoscolex stage of *E. granulosus*, infective to canine. This could eventually be helpful in the search for putative targets for a rationale drug design destined to interrupt the biological cycle of the parasite. Given the phylogenetic position of this cestode, studies on the developing stages of *E. granulosus* should enlighten fundamental principles about growth, morphogenesis and morphological innovation during evolution.

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