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*Original Article*

# Lamina-Associated Polypeptide 2 (LAP2) expression in fish and amphibians

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**ABSTRACT Somatic and germinal cells of 15 fish and 33 amphibian species were examined by SDS-PAGE followed by immunoblotting to determine the expression of LAP2 (lamina-associated polypeptide 2). LAP2 expression in frogs, salamanders and fish does not vary with the mode of reproduction. In fish and frog cells, a rim-like LAP2 positive region was detected around the nucleus by indirect immunofluorescence microscopy. The cell distribution and expression patterns of LAP2 in fish, frogs and salamanders are comparable with those found in *Xenopus* and zebrafish. The mammalian somatic cell pattern, which may also occur in gymnophione amphibians, includes LAP2α, β and γ as major isoforms, whereas LAP2α does not occur in cells of fish, frogs and salamanders. In fish, LAP2γ is the major isoform of somatic cells, suggesting that LAP2γ may be ancestral. However, in the rainbow trout, as in frogs and salamanders, LAP2β was the major somatic isoform. Fish and frog sperm only express low molecular weight polypeptides. In contrast, fish and frog oocytes express an oocyte-specific LAP2 isoform of high molecular weight. In the toad *Bufo marinus* this isoform becomes upregulated in pre-vitellogenic oocytes of 150-200 μm in diameter. The absence of LAP2α and the differential expression of LAP2 isoforms in somatic and germ cells, as found in fish and frogs, may be ancestral vertebrate characters. In spite of differences in developmental time, the LAP2 isoforms of somatic cells are upregulated during gastrulation, suggesting that LAP2 may be implicated in the early development of fish and frog.**

KEY WORDS: *LAP2, somatic cell, germ cell, zebrafish, Xenopus*

## Introduction

From the time of their discovery by Foisner and Gerace (1993), the functions of LAP2 (lamina-associated polypeptide 2) in nuclear dynamics and architecture, disease, and development have been gradually elucidated (reviewed in Dechat *et al.,* 2000; Gruenbaum *et al.,* 2000; Wilson, 2000). Six isoforms of LAP2 (LAP2α, β, ε, δ, γ and ξ) occur in mammals. These isoforms are generated by alternative splicing of the same transcript, and all of them, with the exception of LAP2α and LAP2ξ, are type II integral membrane proteins (IMPs) of the inner nuclear membrane (reviewed in Dechat *et al.,* 2000). LAP2 isoforms bind to the nuclear lamina, are involved in postmitotic nuclear reassembly, may stabilize chromatin structure, and may target membranes to the chromosomes (reviewed in Dechat *et al.,* 2000). In addition, LAP2 isoforms are implicated in autoimmune diseases (Konstantinov *et al.,* 1995; Paulin-Levasseur *et al.,* 1996) and LAP2-related proteins are involved in the Emery-Dreifuss muscular dystrophy (Wilson, 2000). The differential expression and the regulation of LAP2 isoforms during *Xenopus* development suggest that these polypeptides may play a role in development (Lang *et al.,* 1999).

LAP2β has an N-terminal nucleoplasmic domain, a transmembrane domain, and a C-terminal domain located between inner and outer nuclear membranes (reviewed in Dechat *et al.,* 2000). The nucleoplasmic domain includes a lamina binding region (to lamin B1/B2 residues) and a 187 amino acid long N-terminal region that interacts with the chromatin. LAP2 isoforms share the LEM domain in the N-terminal region with the related proteins, emerin and MAN1 (Lin *et al.,* 2000). The LEM domain interacts with the chromosomal barrier of autointegration factor (BAF),

*Abbreviations used in this paper:* A6, *Xenopus* kidney cells; BAF, barrier of autointegration factor; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; IMP, integral membrane protein; kDa, kilodaltons; LAP2, lamina-associated polypeptide 2; LEM, LAP2-Emerin-MAN1 domain; NBT, nitro blue tetrazolium; $P_{200}$, *Xenopus* egg membranes; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBST, Tris buffered saline-tween; XLAP2β, *Xenopus* homologue of LAP2β.

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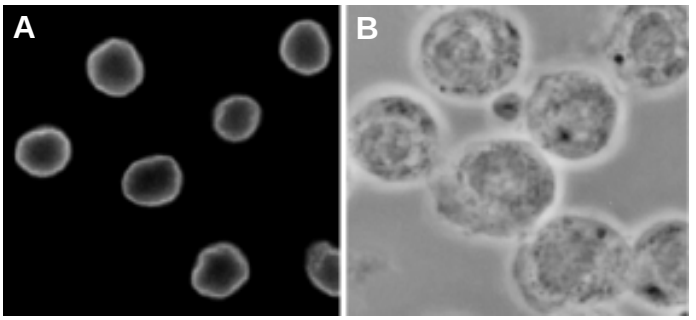


**Fig. 1. LAP2 distribution in fish cells. (A)** *Indirect immunofluorescence microscopy of rainbow trout hepatocytes with the MAN serum.* **(B)** *Phase contrast microscopy of the cells shown in (A). The average hepatocyte cell diameter is 14 μm.*

which seems to participate in the binding of LAP2 to the chromatin (Furukawa, 1999). LAP2 ε, δ, and γ are structurally similar to LAP2β, and lack only short regions of the LAP2β nucleoplasmic domain. In contrast, LAP2α only shares the N-terminus 187 amino acids with other LAP2 isoforms (Dechat *et al.,* 2000). It is postulated that the divergent LAP2α may contribute to stabilize higher order chromosome structure, whereas LAP2β may influence chromatin structure in ways that could modulate replication and possibly the competence for transcription (Gant *et al.*, 1999; Dechat *et al.,* 2000).

Two patterns of LAP2 expression have been detected, one in mammals (Harris *et al.,* 1994*;* Alsheimer *et al.,* 1998), and the other in *Xenopus* (Lang *et al.,* 1999). In mammals, the major LAP2 isoforms of somatic cells are LAP2α, β, and γ (Harris *et al.,* 1994; Alsheimer *et al.,* 1998; Goldberg *et al.,* 1999). In contrast, LAP2α has not been detected in *Xenopus* (Lang *et al.,* 1999). *Xenopus* somatic cells express one major LAP2 polypeptide (XLAP2: renamed XLAP2β), which is the homologue of the mammalian LAP2β (Gant *et al.,* 1999; Lang *et al.,* 1999), but it is unknown whether *Xenopus* expresses LAP2γ. In *Xenopus* oocytes and early embryos, XLAP2β is absent, and instead, an oocyte-specific LAP2 isoform has been found, whose cDNA has not been cloned. This isoform is down regulated after the gastrula stage, and becomes gradually replaced during development by XLAP2β, the isoform typical of somatic cells. The oocyte-specific LAP2 isoform has a higher molecular weight than XLAP2β, has a transmembrane domain, and therefore differs from the mammalian LAP2α (Lang *et al.,* 1999). In the zebrafish, somatic and oocyte-specific LAP2 isoforms have been found, with differential expression during development (Schoft *et al.*, unpublished). In contrast with *Xenopus*, LAP2γ is the major LAP2 isoform in zebrafish somatic cells and LAP2β is less abundant. The zebrafish LAP2β, γ, and oocyte-specific isoforms have been cloned (Schoft *et al.*, unpublished). Sequence information excludes the possibility that the zebrafish oocyte-specific LAP2 isoform is a homologue of the mammalian LAP2α (Schoft *et al.*, unpublished). As in *Xenopus*, LAP2α has not been detected in the zebrafish.

LAP2 expression patterns in fish and amphibians are of interest because these vertebrates display many distinct reproductive and developmental adaptations (Ballard, 1981; Wourms and Whitt, 1981; Duellman and Trueb, 1986; Harvey *et al.,* 1999). Taking advantage of the biodiversity of Ecuador, we have investigated the LAP2 expression in native fish and amphibians with modified modes of reproduction. The non-native fish studied are the swordfish (*Xiphophorus helleri*, and X*. maculatus*), the carp (*Cyprinus carpio*), the zebrafish (*Danio rerio*), and the rainbow trout (*Onchorhynchus mykiss*). The LAP2 expression in the small viviparous teleost *Priapichthys panamensis* was compared to 14 additional oviparous and viviparous teleost fish. Females of *P. panamensis* carry embryos of several developmental stages in the ovary (Sáenz *et al.*, unpublished), a condition known as superfetation (Haynes, 1995). Among the frogs, 17 species deposit their eggs in the water, such as the toad, *Bufo marinus*, and 13 species have terrestrial reproduction (amphibian reproductive modes are according to Duellman and Trueb, 1986). Frogs with terrestrial reproduction include frogs with direct development of the genus *Eleutherodactylus*, and the marsupial frogs of the genus *Gastrotheca* that brood the embryos inside a maternal pouch. In addition, the LAP2 expression of dendrobatid frogs was studied. Eggs of dendrobatid frogs are deposited on land, and when the tadpoles hatch, one of the adults transports them on its back to the water. Besides anurans, the comparison was extended to the lungless salamander *Bolitoglossa equatoriana* (Plethodontidae). *Bolitoglossa* eggs are terrestrial and undergo direct development. Two species of the limbless and tailless gymnophione amphibians genus *Caecilia* were also studied. Our comparative analysis of diverse fish and amphibian species suggests that LAP2 isoforms are important, not only within the cell, but also in development and evolution.

## Results

### *LAP2 Expression in Somatic Cells*

The human serum MAN (Paulin-Levasseur *et al.,* 1996) and the ZLAP2-serum1 (Schoft *et al.*, unpublished) reacted, albeit weakly in some cases, with fish LAP2 polypeptides. The MAN serum gave a stronger signal in amphibian cells. These tools allowed us to analyze LAP2 expression in fish and amphibians. The electrophoretically estimated molecular weights of the LAP2 isoforms varied among species of fish, frogs and salamanders (Tables 1, 2). The LAP2 signal, detected by immunofluorescence with the MAN antiserum, was located around the nuclear membrane in hepatocytes (Fig. 1 A,B), erythrocytes, brain, and white muscle cells (not shown) of the oviparous fish *Onchorhynchus mykiss* (rainbow trout). A similar LAP2 distribution was observed in the liver and testis of the viviparous fish *P. panamensis*, the toad *B. marinus* and the frog without tadpoles *Eleutherodactylus achatinus* (not shown). These patterns are equivalent to the LAP2 distribution seen in cells of *Xenopus* (Lang *et al.,* 1999).

The somatic LAP2 expression of oviparous and viviparous fish included LAP2β and LAP2γ polypeptides as major isoforms (Table 1). In fish, the expression levels of LAP2β and LAP2γ varied in different somatic tissues and species. LAP2γ was significantly more abundant than LAP2β in somatic cells of the zebrafish (Schoft *et al.,* unpublished) and most other fish, as illustrated by the LAP2 expression pattern in the heart of the viviparous fish, *P. panamensis* (Fig. 2A). LAP2γ was the only detected LAP2 isoform of certain tissues, such as the liver, heart, and spleen of the oviparous fish, *Moenkhausia* (Fig. 2B).

An exception to the fish somatic pattern was found in the rainbow trout. LAP2β was the major isoform of somatic cells. However the levels of LAP2β and LAP2γ expression varied in different cells (Fig. 2 C,D). For example, LAP2β was the major isoform found in the gills, brain, and heart cells (Fig. 2C), as well as

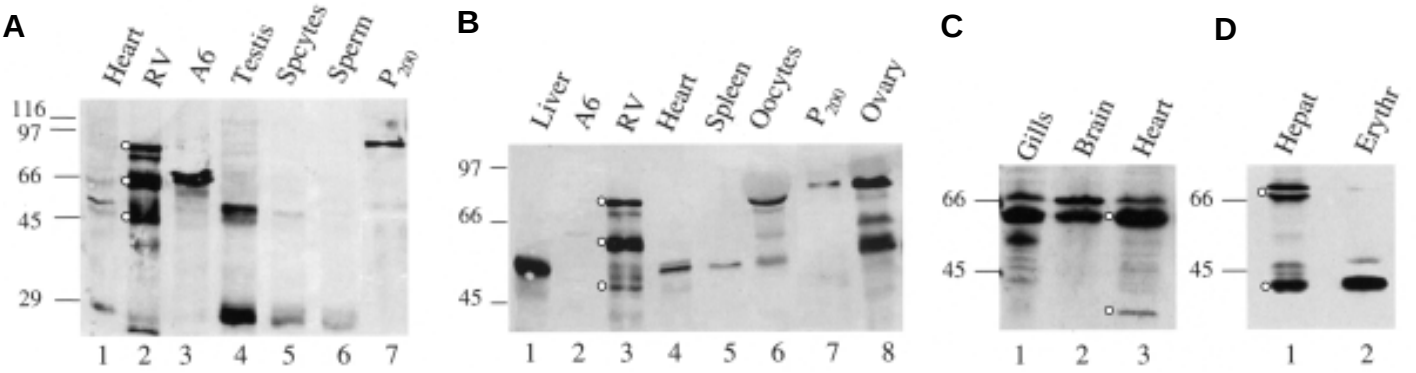


**Fig. 2. LAP2 expression in fish.** *Western blot analysis of fish polypeptides. Proteins were separated by SDS-PAGE with 11% acrylamide in (A), 10% acrylamide in (B), and 12% acrylamide in (C,D), and immunoblotted with the MAN serum.* **(A)** *LAP2 expression in an adult male of the viviparous fish* P. panamensis. *LAP2 expression in this fish (lanes 1 and 4-6) is compared to that in rat (lane 2) and Xenopus (lanes 3 and 7).* **(B)** *LAP2 expression in the oviparous fish* Moenkhausia. *LAP2β was not detected in liver, heart and spleen of this fish (lanes 1, 4 and 5, respectively). However it is abundant in the somatic cells of the ovary (compare lanes 6 and 8). Lane 6 corresponds to an oocyte-enriched fraction, contaminated with somatic cells.* **(C)** *LAP2 expression in the oviparous rainbow trout* (Onchorhynchus mykiss). *LAP2β was detected in the gills, brain and heart of this fish. A low expression level of LAP2γ occurred in the heart (lane 3).* **(D)** *LAP2 expression in hepatocytes and erythrocytes of the rainbow trout. The white dots in lane 2 of (A) and 3 of (B) indicate the rat LAP2α (78 kDa), LAP2β (58 kDa), and LAP2γ (40 kDa) isoforms. The white dots in lane 3 of (C) and lane 1 of (D) indicate LAP2β (65 kDa) and LAP2γ (40 kDa). Molecular masses of reference proteins are given (in kDa). In this and the following Figures, A6 corresponds to* Xenopus *kidney cells and P200 are* Xenopus *egg membranes. Erythr, erythrocytes; Hepat, hepatocytes; RV, rat somatic proteins; Spcytes, spermatocytes.*

in the spleen, kidney, white and red muscle cells (not shown). LAP2γ was not detected in most tissues. However low expression levels of LAP2γ were found in the heart (Fig. 2C), and spleen (not shown). The levels of LAP2γ expression were higher in some cases, such as in hepatocytes and erythrocytes (Fig. 2D). In hepatocytes, LAP2β and LAP2γ had similar levels of expression, whereas LAP2γ was the only LAP2 isoform of rainbow trout erythrocytes (Fig. 2D).

As in *Xenopus,* LAP2β was the major isoform found in somatic cells of frogs and salamanders (Table 2). This pattern is illustrated for the frog without tadpoles, *Eleutherodactylus unistrigatus* (Fig. 3A), the toad *B. marinus* (Fig. 3A), and the tree frog *Hyla lanciformis* (Fig. 3B). A small polypeptide was frequently found in frog spleen (Fig. 3A). All of the examined somatic tissues of *Osteocephalus yasuni* expressed a small polypeptide of about 40 kDa (not shown). Poeciliid fish somatic cells express a smaller polypeptide (Fig. 2A). However, the small polypeptides found in fish and frog cells cannot be regarded as LAP2 isoforms, because the MAN serum detects a small LAP2-unrelated polypeptide in *Xenopus* (Lang *et al.,* 1999).

LAP2α was not found in cells of the fish and amphibians analyzed. However in somatic cells of the gymnophione amphibian *Caecilia*, an immunoreactive polypeptide was detected with mobility on SDS-PAGE similar to the mammalian LAP2α (Fig. 3C; Table 2). Presently we do not know whether this large immunoreactive polypeptide has biochemical similarities with LAP2α. Moreover, *Caecilia* somatic cells express in addition two LAP2 isoforms, with electrophoretic mobilities that resemble the mammalian LAP2β and γ (Fig. 3C; Table 2). This pattern differs from the LAP2 expression pattern of fish and frog somatic cells and resembles the pattern of rat somatic cells.

### *LAP2 Expression in Sperm and Testis*

The sperm of fish and frogs lacks the LAP2 isoforms that are typical of somatic cells (LAP2β and γ in fish and LAP2β in frogs). Instead, one or two small polypeptides were characteristically found in fish and frog sperm cells (Table 1, 2), as shown for the viviparous fish *P. panamensis* (Fig. 2A), and the tree frog *H. lanciformis* (Fig. 3B). Two sperm-specific small polypeptides were usually found in the testis of hylid frogs, whereas in other frog and fish species, usually only one small sperm-specific polypeptide was detected (Table 2). Fish and frog spermatocytes expressed the sperm-specific polypep-

TABLE 1

**APPARENT MOLECULAR SIZE (kDa) OF LAP2 ISOFORMS IN FISH WITH DIFFERENT REPRODUCTIVE MODES**

| Fish species | LAP2 isoforms | | | | Small polypeptide (<40 kDa) | |
|---|---|---|---|---|---|---|
| | α | β | γ | Oocyte | Sperm | Oocyte |
| **Viviparous fish** | | | | | | |
| CYPRINODONTIFORMES: POECILIIDAE | | | | | | |
| *Priapichthys panamensis* | No | 64 | 46 | 87 | Yes | Yes |
| *Xiphophorus helleri* | No | 58 | 48 | 88 | Yes | Yes |
| *X. maculatus* | No | 60 | 48 | 81 | Yes | Yes |
| **Oviparous fish** | | | | | | |
| CHARACIFORMES: CHARACIDAE | | | | | | |
| *Aphyocharax* sp. | No | Nd | 45 | 84 | Nd | Nd |
| *Bryconamericus caucanus* | No | 62 | 44 | 80 | Yes | Yes |
| *Moenkhausia oligolepis* | No | Nd | 46 | 84 | Yes | Yes |
| *Moenkhausia* sp. | No | 59 | 48 | 84 | Nd | Yes |
| *Rhoadsia altipinna* | No | Nd | 46 | 76 | Yes | Yes |
| CHARACIFORMES: CURIMATIDAE | | | | | | |
| CURIMATIDAE sp. | No | Nd | 46 | Nd | Nd | Nd |
| CYPRINIFORMES: CYPRINIDAE | | | | | | |
| *Cyprinus carpio* | No | 68 | 47 | Nd | Yes | Yes |
| *Danio rerio* (zebrafish)[1] | No | 63 | 45 | 84 | Yes[2] | Nd |
| PERCIFORMES: CICHLIDAE | | | | | | |
| *Aequidens rivulatus* | No | Nd | 52 | 78 | Nd | Yes |
| PERCIFORMES: ELEOTRIDAE | | | | | | |
| *Dormitator latifrons* | No | Nd | 50 | Nd | Yes | Yes |
| SALMONIFORMES: SALMONIDAE | | | | | | |
| *Onchorhynchus mykiss* | No | 65 | 40 | Nd | Nd | Nd |
| SILURIFORMES: LORICARIIDAE | | | | | | |
| *Ancistrus* sp. | No | Nd | 45 | Nd | Nd | Yes |

[1]Schoft *et al.*, unpublished. [2]This work, not shown. Nd, not determined; No, not present; Yes, present

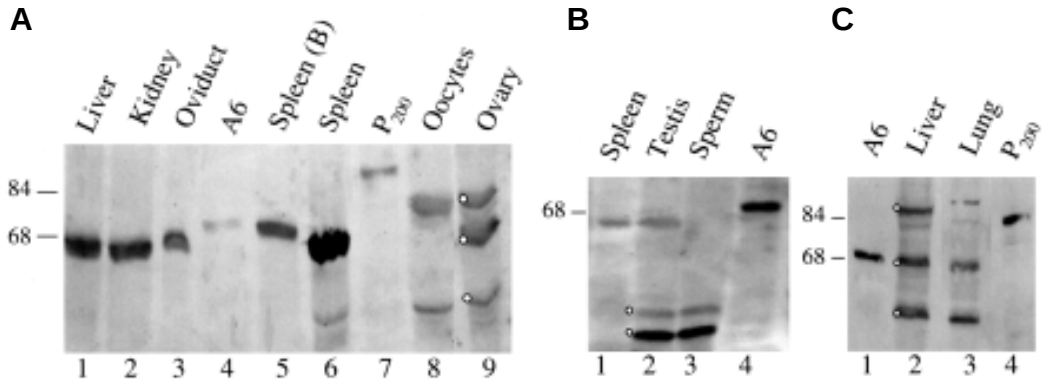


**Fig. 3. LAP2 expression in Amphibia.** *Proteins were separated by SDS-PAGE with 10% acrylamide and immunoblotted with the MAN serum.* **(A)** *LAP2 expression in* Eleutherodactylus unistrigatus, *a frog with direct development, in comparison to* Bufo marinus *and* Xenopus, *frogs with aquatic reproduction. LAP2 expression in* E. unistrigatus *(lanes 1-3, 6, 8, and 9), and in the spleen of* B. marinus *(spleen B, lane 5) is compared to that of* Xenopus *(lanes 4 and 7). Somatic tissues of* E. unistrigatus *and* B. marinus *(lanes 1-3, and 5-6) express LAP2β.* E. unistrigatus *oocytes (lane 8) express the oocyte-specific LAP2 isoform and a small polypeptide which is also found in the spleen of this frog. In the ovary (lane 9), follicle cell LAP2β was found in addition to the oocyte specific LAP2 polypeptides (seen in lane 8). The white dots indicate the oocyte-specific isoform (73 kDa), follicle cell LAP2β (58 kDa) and a small oocyte-specific polypeptide (<40 kDa).* **(B)** *LAP2 expression in a male* Hyla lanciformis, *a frog with aquatic reproduction. LAP2 expression in this frog (lanes 1-3) is compared to that of* Xenopus *(lane 4). LAP2β was detected in the spleen and testis (lanes 1-2). However sperm only express two small polypeptides of about 33 and 26 kDa, which are also seen in the testis (indicated by white dots in lane 2).* **(C)** *LAP2 expression in the gymnophione amphibian* Caecilia orientalis *(lanes 2-3) is compared to that observed in* Xenopus *(lanes 1 and 4). The samples were derived from an aquatic* C. orientalis *larvae at hatching. The white dots in lane 2 indicate the three polypeptides of* C. orientalis *(about 90, 68 and 43 kDa, respectively) which crossreacted with LAP2 antibodies of the MAN serum. Molecular masses of reference proteins are given (in kDa).*

tides and the LAP2 isoforms typical of somatic cells (Fig. 2A). The testes in fish, frogs, and salamanders contain somatic, spermatogenic and sperm cells and expressed the LAP2 isoforms of somatic cells and the sperm-specific polypeptides. This pattern is seen in the fish *P. panamensis* and the frog *H. lanciformis* (Figs. 2A, 3B). In the carp (*Cyprinus carpio*) similar amounts of LAP2β and LAP2γ were found in testes and spermatocytes. Carp somatic cells, in contrast, expressed higher amounts of LAP2γ than of LAP2β, as seen in somatic cells of most other fish. No sperm-specific polypeptide was detected in the carp testis (not shown).

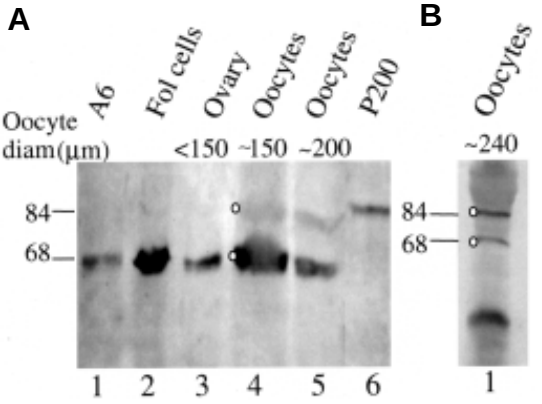


**Fig. 4. LAP2 expression during oogenesis in *Bufo marinus*.** *Proteins were separated by SDS-PAGE with 10% acrylamide and immunoblotted with the MAN serum. The size of the oocytes analyzed is comparable to that of* Xenopus *stage I oocytes (Dumont, 1972).* **(A)** *LAP2 expression in follicle cells, a juvenile ovary and previtellogenic oocytes of up to approximately 200 μm in diameter. LAP2 expression in Bufo (lanes 2-5) is compared to that in* Xenopus *(lanes 1 and 6).* **(B)** *LAP2 expression in oocytes of up to approximately 240 μm in diameter. The oocyte diameters given in (A) and (B) are the maximum diameter of oocytes found in each ovarian sample. The 68 kDa band in oocyte samples comes from small oocytes and possible contamination with follicle cells. The white dots in lane 4 of (A) and lane 1 of (B) signal the oocyte-specific LAP2 (84 kDa) and LAP2β (68 kDa). Molecular masses of reference proteins are given (in kDa). Fol cells, follicle cells.*

### *LAP2 Expression in Ovary and Oocytes*

Fish, frog, and salamander oocytes expressed an oocyte-specific LAP2 isoform, whose apparent molecular weight is higher than that of LAP2β (Table 1, 2). The LAP2 isoforms, characteristic of somatic cells (LAP2β and LAP2γ in fish, and LAP2β in frogs and salamanders), were not found in oocytes, as shown for the oviparous fish *Moenkhausia* (Fig. 2B) and the frog with direct development *E. unistrigatus* (Fig. 3A). The LAP2β band seen in *Moenkhausia* oocytes (Fig. 2B) comes from contamination with follicle cells. A small polypeptide was sometimes found in fish and frog oocytes (Table 1, 2), as seen for *E. unistrigatus* (Fig. 3A), and *Xenopus* (Lang *et al.,* 1999).

In contrast with oocytes, fish ovary expressed three LAP2 isoforms, which are the LAP2β and γ isoforms of follicle cells, and the oocyte-specific LAP2 polypeptide (Fig. 2B). This pattern is different from that of mammalian cells, because LAP2α did not occur in fish. In frog ovaries, the two polypeptides detected are LAP2β of follicle cells and the high molecular weight oocyte-specific LAP2 polypeptide. Additionally, in ovaries of some frogs, a small polypeptide was also present (Fig. 3A).

The onset of expression for the oocyte-specific LAP2 isoform was determined in juvenile ovaries of *B. marinus*. In ovaries containing oocytes of up to 150 μm in diameter, only LAP2β was detected, whereas the oocyte-specific LAP2 isoform became detectable in oocytes of 150-240 μm in diameter (Fig. 4 A,B). Thereafter, the oocyte-specific LAP2 was the major isoform found in oocytes throughout *Bufo* oogenesis (not shown). *Bufo* oocytes of 200 μm in diameter were previtellogenic, translucent, and with a large germinal vesicle that contains numerous nucleoli. In sections, the oocyte chromosomes resembled lampbrush chromosomes (not shown). This observation suggests that the upregulation of the oocyte-specific LAP2 isoform occurs in oocytes that may have reached the diplotene stage.

### *LAP2 Expression During Development*

The gradual replacement of the oocyte-specific LAP2 by the isoforms typical of somatic cells starts during gastrulation in *Xenopus* and in the zebrafish (Lang *et al.,* 1999; Schoft *et al.*,

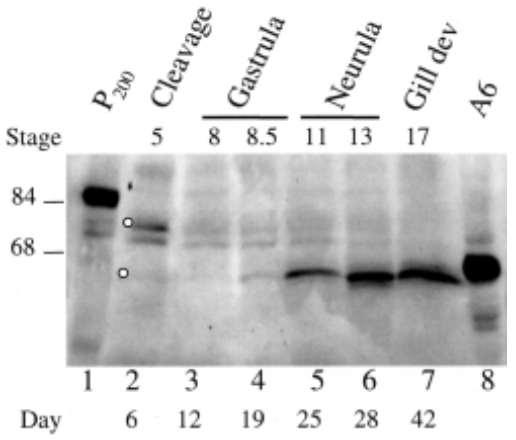


**Fig. 5. LAP2 expression during development of the marsupial frog *Gastrotheca riobambae*.** *Proteins were separated by SDS-PAGE with 10% acrylamide and immunoblotted with the MAN serum. Each lane contains proteins from one embryo. LAP2 expression in Gastrotheca embryos (lanes 2-7) is compared to that in* Xenopus *(lanes 1 and 8). The oocyte-specific LAP2 (74 kDa) and LAP2β (62 kDa) are signaled by dots in lane 2. The intermediate size polypeptide may correspond to a degradation product of the oocyte-specific LAP2, as in* Xenopus *(Lang et al., 1999). Molecular masses of reference proteins are given (in kDa). Gill dev, gill development.*

unpublished). Similarly, the switch of LAP2 expression from oocyte-specific to the somatic pattern occurred during gastrulation in the frogs *Gastrotheca* (Fig. 5), and *Colostethus machalilla*, and in the viviparous fish *P. panamensis* (not shown). In embryos of the marsupial frog *Gastrotheca*, the oocyte-specific LAP2 polypeptide was the major LAP2 isoform of cleavage stage embryos. LAP2β became upregulated in the advanced gastrula, and gradually replaced the maternal oocyte-specific LAP2 during development (Fig. 5). The main difference found among species was in the timing of developmental events. Whereas *Xenopus* embryos require 13.25 hours to reach the late gastrula (stage 12 of Nieuwkoop and Faber, 1994), *C. machalilla* takes 3 days (Ávila, *et al.,* unpublished), and *Gastrotheca* (Fig. 5) requires 19 days (del Pino and Escobar, 1981). In *Gastrotheca,* low levels of LAP2β may be expressed during cleavage, as suggested by the faint LAP2β band seen in cleavage and early gastrula embryos (Fig. 5). The time to reach the gastrula stage is unknown for the viviparous fish *P. panamensis*.

## Discussion

Three patterns of LAP2 expression (Table 3) are found in the vertebrates, exemplified by the zebrafish, *Xenopus*, and the rat (Alsheimer *et al*., 1998; Lang *et al*., 1999; Schoft *et al.,* unpublished). The major LAP2 isoforms of fish somatic cells are LAP2β and γ, with varying levels of expression in different tissues and species (Table 3). The prevalent pattern is the abundance of LAP2γ in fish somatic cells, such as it occurs in the zebrafish (Schoft *et al*., unpublished). However, in the rainbow trout LAP2β is the major isoform of somatic cells. Similarly, LAP2β is the major isoform of somatic cells in frogs and salamanders. In contrast, somatic cells of gymnophione amphibians express three LAP2 isoforms with mobilities similar to the mammalian α, β and γ polypeptides (Fig. 3C; Table 3). Presently we do not know whether the large immunoreactive polypeptide of gymnophione amphibians represents a LAP2 isoform that possesses biochemical properties comparable with that of the mammalian α-polypeptide or a membrane bound polypeptide like LAP2β. So far, the presence of three major LAP2 isoforms (LAP2α, β, and γ) in somatic cells appears as characteristic of the mammals (Table 3). LAP2γ may represent an ancestral isoform, as it is predominant among fish (Table 1). LAP2β and LAP2α may be derived isoforms, given their presence in fish and frog cells and in mammalian cells, respectively (Tables 1, 2).

The germ cell LAP2 expression differs from the somatic patterns. Rat sperm expresses LAP2α (Alsheimer *et al.,* 1998). However fish and frog sperm cells express only small LAP2-related polypeptides (Table 3). An oocyte-specific LAP2 isoform is characteristic of fish and frog oocytes and early embryos, whereas, in the mammals this LAP2 isoform has not been detected (Table 3). We found the presence of LAP2α, β, and γ in the mouse ovary, as in somatic cells (not shown). However the large proportion of somatic cells may hinder the detection of an oocyte-specific LAP2 isoform in the mouse ovary. LAP2 expression may be linked to the characteristics of differentiated cells, such as the different lamin types or to the chromatin structure. In fact, cells with different functions, such as sperm, oocytes and early embryos, express different LAP2 isoforms (Table 3) and different lamins (Benavente and Krohne, 1985; Stick, 1988; Alsheimer *et al.,* 1998; Lang *et al.,* 1999). Moreover, the peculiarities of chromatin structure in the highly modified sperm, oocytes and embryonic cells may require the expression of different isoforms of LAP2.

This comparative analysis suggests that the different reproductive modes found in fish and amphibians are not related to the patterns of LAP2 expression (Tables 1, 2). We expected to find a wide variety of LAP2 expression patterns in the fish, given that they represent the most diverse vertebrate group. The analyzed fish (Table 1) are classified into eight families, representing five teleost orders according to Froese and Pauly (2000). However, an unvarying pattern was observed in the zebrafish, and in oviparous and viviparous teleost fish (Table 1). The major deviation from the fish common pattern occurs in the rainbow trout (Table 1), whose somatic cell LAP2 expression resembles the frogs rather than the fish. However, the reproductive mode of the rainbow trout provides no indication of its deviant mode of LAP2 expression. The rainbow trout LAP2 pattern may be shared by other Salmonidae, and therefore, it is important to analyze the LAP2 pattern in other members of this group. Similarly, representatives of other fish taxa should be analyzed to determine whether further LAP2 deviation exists among fish.

The analyzed frogs and salamanders (Table 2) have several modes of reproduction and represent six different anuran families, and one urodele family (Duellman and Trueb, 1986). However, the LAP2 expression pattern does not deviate from *Xenopus* (Table 2). Surprisingly, the somatic cell pattern of gymnophione amphibians differs from the other amphibians and appears comparable to that of the mammals (Table 2). The deviant LAP2 expression of gymnophione amphibians correlates with other features of this group. In fact, the gymnophione are the most divergent amphibians, lacking limbs and tail (Duellman and Trueb, 1986). Moreover, the caecilian vertebral development has unique characters, differs from frogs, and in general resembles the mammals (Wake and Wake, 2000). The rainbow trout and gymnophiones deserve further analysis to determine the molecular affinities of their divergent LAP2 polypeptides.

LAP2α does not occur in cells of the zebrafish (Schoft *et al.*, unpublished), 14 additional teleost fish (Table 1), *Xenopus* (Lang *et al.,* 1999), 30 frog species, and a salamander (Table 2). These findings contrast with the abundance of LAP2α in mammalian cells (Lang *et al.,* 1999; Dechat, *et al.,* 2000). It is unknown whether LAP2α is expressed in somatic cells of gymnophione amphibians (Table 2). Fish, frogs and salamanders have differential expression of LAP2 in somatic and germ cells, whereas only sperm has differential LAP2 expression in the mammals (Table 3). The absence of LAP2α and the differential expression of LAP2 in somatic, germ cells, and during development are common among the lower vertebrates, and may correspond to ancestral vertebrate characters. Moreover, the transition of LAP2 expression from oocyte-specific to the somatic pattern occurs at gastrulation in the species analyzed, in spite of differences in the timing of developmental events. These observations signal the likely involvement of LAP2 in the early development of fish and frogs.

TABLE 2

**APPARENT MOLECULAR SIZE (kDa) OF LAP2 ISOFORMS IN AMPHIBIANS**

| Species | LAP2 isoforms | | | | Small polypeptide (<40 kDa) | |
|---|---|---|---|---|---|---|
| | α | β | γ | Oocyte | Sperm | Oocyte |
| **ANURA** | | | | | | |
| **A. Eggs deposited in water[1]** | | | | | | |
| BUFONIDAE | | | | | | |
| *Atelopus spumarius* | No | 68 | Nd | Nd | Yes | Nd |
| *Bufo haematiticus* | No | 68 | Nd | 84 | Yes | Yes |
| *B. margaritifer* | No | 68 | Nd | 84 | Yes | Yes |
| *B. marinus* | No | 68 | Nd | 84 | Yes | Yes |
| *Dendrophryniscus minutus* | No | 68 | Nd | 84 | Nd | Yes |
| HYLIDAE | | | | | | |
| *Hyla bifurca* | No | 59 | Nd | Nd | Yes | Nd |
| *H. carcarata* | No | 64 | Nd | 79 | Yes | Yes |
| *H. granosa* | No | 62 | Nd | Nd | Yes | Nd |
| *H. lanciformis* | No | 64 | Nd | 79 | Yes (2 bands) | Yes |
| *H. pelucens* | No | 58 | Nd | 70 | Yes (2 bands) | Yes |
| *H. rosembergi* | No | 59 | Nd | Nd | Yes (2 bands) | Nd |
| *Osteocephalus yasuni* | No | 60 | Nd | 74 | Yes (2 bands) | Yes |
| *Scinax ruber* | No | 65 | Nd | Nd | Nd | Nd |
| *Scinax quinquefasciata* | No | 61 | Nd | 75 | Nd | Yes |
| LEPTODACTYLIDAE | | | | | | |
| *Baricholos pulcher* | No | 58 | Nd | 73 | Nd | Yes |
| *Physalaemus* sp. | No | 58 | Nd | Nd | Yes | Nd |
| PIPIDAE | | | | | | |
| *Xenopus laevis*[2] | No | 68 | Nd | 84 | Nd | Nd |
| **B. Eggs arboreal, tadpoles drop into water[1]** | | | | | | |
| CENTROLENIDAE | | | | | | |
| *Centrolene grandisone* | No | 64 | Nd | Nd | Nd | Nd |
| **C. Eggs terrestrial, tadpoles carried to water by an adult[1]** | | | | | | |
| DENDROBATIDAE | | | | | | |
| *Colostethus machalilla* | No | 62 | Nd | 75 | Nd | Yes |
| *C. infraguttatus* | No | 63 | Nd | 77 | Nd | Yes |
| *Epipedobates boulengeri* | No | 68 | Nd | 84 | Nd | No |
| *E. tricolor* | No | 68 | Nd | 84 | Nd | Yes |
| **D. Eggs terrestrial, direct development[1]** | | | | | | |
| LEPTODACTYLIDAE | | | | | | |
| *Eleutherodactylus achatinus* | No | 58 | Nd | 73 | Yes | Nd |
| *E. appendiculatus* | No | 58 | Nd | 73 | Nd | Nd |
| *E. eugeniae* | No | 58 | Nd | 73 | Nd | Yes |
| *E. longirostris* | No | 58 | Nd | 73 | Nd | Yes |
| *E. simonbolivari* | No | 58 | Nd | 73 | Nd | No |
| *E. unistrigatus* | No | 58 | Nd | 73 | Yes | Yes |
| *E. w-nigrum* | No | 58 | Nd | Nd | Yes | Yes |
| **E. Eggs carried in dorsal pouch of female, tadpoles aquatic[1]** | | | | | | |
| HYLIDAE | | | | | | |
| *Gastrotheca riobambae* | No | 62 | Nd | 74 | No | Yes |
| *G. pseustes* | No | 64 | Nd | Nd | No | Nd |
| **URODELA** | | | | | | |
| **Eggs terrestrial, direct development[1]** | | | | | | |
| PLETHODONTIDAE | | | | | | |
| *Bolitoglossa equatoriana* | No | 59 | Nd | Nd | Nd | No |
| **GYMNOPHIONA** | | | | | | |
| **Reproduction unknown[1]** | | | | | | |
| CAECILIIDAE | | | | | | |
| *Caecilia guntheri* | 87[3] | 65[3] | 40[3] | Nd | Nd | Nd |
| *C. orientalis* | 90[3] | 68[3] | 43[3] | Nd | Nd | Nd |
| **MAMMALIA** | | | | | | |
| *Rattus rattus*[2] | 78 | 58 | 40 | No[4] | Nd | Nd |

[1]Reproductive modes according to Duellman and Trueb (1986). [2] According to Lang *et al.,* (1999). [3] It is unknown whether these polypeptides have biochemical similarities with the mammalian LAP2α, β and γ. [4] In the mouse (not shown). Nd, not determined; No, not present; Yes, present

## Materials and Methods

### *LAP2 Standards of Zebrafish, Xenopus and Rat*

The following *Xenopus* protein standards were used for comparison: A6 (total protein of kidney epithelial cells), $P_{200}$ (total protein of egg membranes prepared according to Lang *et al.,* 1999). The molecular weight of XLAP2β, found in A6 cells is 68 kDa, and the oocyte-specific-specific XLAP2 found in $P_{200}$ is 84 kDa (Lang *et al.*, 1999). In addition, the zebrafish ovary and rat somatic cells were used for comparison. The zebrafish LAP2β, γ, and oocyte-specific LAP2 have molecular weights of 63, 45, and 84 kDa, respectively (Schoft *et al.*, unpublished). Rat somatic cells (RV) contain three main LAP2 polypeptides: LAP2α of 78 kDa, LAP2β of 58 kDa and LAP2γ of 40 kDa (Lang *et al.,* 1999). Molecular weight protein standards of 205, 116, 97.4, 66, 45 and 29 kDa were used in SDS-PAGE.

### *Cells, Tissues and Embryos*

From each animal, small pieces (of about 10 $mm^3$) of organs, such as the heart, spleen, liver, kidney, and the gonads were homogenized in sample buffer (Laemmli 1970). Sperm was isolated from spermatogenic and somatic cells by gently squeezing the testis in phosphate buffered saline (PBS: 137 mM NaCl, 3 mM KCl, 1.5 mM $KH_2PO_4$, 7 mM $Na_2HPO_4$, pH 7.4). The resulting liquid was transferred to a new tube and allowed to rest on ice for 10 min. The upper layer after this period was enriched in swimming mature sperm, whereas the sediment contained mostly round spermatocytes (microscopically detected). Each fraction was mixed with sample buffer. Oocyte enriched fractions were obtained by gently squeezing a piece of ovary in PBS, followed by centrifugation for 5 seconds at 15,000 g. The supernatant, enriched in oocytes, was mixed with sample buffer. The *Bufo* oocyte preparations included oocytes of several sizes, and the maximum oocyte diameter of each ovarian sample was recorded. To obtain follicle cells enriched preparations, an ovarian fragment was squeezed and washed several times in PBS to remove oocytes, the remaining tissue was homogenized and mixed with sample buffer. Individual embryos of the marsupial frog *Gastrotheca* were homogenized in PBS on ice and centrifuged at 15,000 g for 5 seconds. The supernatant was mixed with sample buffer. *Gastrotheca e*mbryos were staged according to del Pino and Escobar (1981). Rainbow trout hepatocytes were isolated as previously described (Mommsen *et al.*, 1994). Red blood cells were obtained from the caudal vein and mixed with heparin to prevent the formation of cell aggregates. They were separated from leucocytes in a washing solution (10 mM Tris-HCl, 147 mM NaCl, 15 mM $Na_3$Citrate, pH 7.5) by low speed centrifugation. Both cell types were solubilized in sample buffer. The rainbow trout analyzed, weighed around 250 g, and were kindly supplied by Dr. Thomas Moon (Department of Biology, University of Ottawa).

### *Antibodies*

The following primary antibodies were used: The human antiserum designated MAN (Paulin-Levasseur *et al.,* 1996), and the guinea pig polyclonal antibody against the zebrafish amino terminal region present in all zebrafish LAP2 isoforms (ZLAP2-serum1; Schoft *et al.*, unpublished). Antibodies of both sera react with the common aminoterminal domain of LAP2 isoforms. The MAN antiserum was diluted 1:10,000 to 1:20,000 for immunoblotting in Tris buffered saline-Tween buffer (TBST: 140 mM NaCl, 0.3% Tween-20, 10

TABLE 3

**LAP2 EXPRESSION IN FISH, AMPHIBIANS AND MAMMALS[1]**

| Pattern | *Major LAP2 isoforms* | | | | *SP* |
|---|---|---|---|---|---|
| | α | β | γ | O | |
| 1. FISH | | | | | |
| SOMATIC[2] | | ▬ | ▬ | | |
| OOCYTE | | | | ▬ | |
| SPERM | | | | | ▬ |
| 2. FROGS AND SALAMANDERS | | | | | |
| SOMATIC | | ▬ | | | |
| OOCYTE | | | | ▬ | |
| SPERM | | | | | ▬ |
| 3. MAMMALS | | | | | |
| SOMATIC | ▬ | ▬ | ▬ | | |
| OVARY[3] | ▬ | ▬ | ▬ | | |
| SPERM | ▬ | | | | |

[1]Each block indicates the occurrence of a LAP2 isoform. [2]The LAP2β and LAP2γ levels of expression vary among tissues and species. [3]According to our observations in the mouse. O, oocyte; SP, small polypeptide

mM Tris-HCl, pH 8.0), the ZLAP2-serum1 was diluted 1:2,000 to 1:3,000 in 5% non-fat dry milk in TBST. For microscopic immunolabelling the dilution of the MAN antiserum was of 1:5,000. The anti-human and anti-guinea pig antibodies conjugated to alkaline phosphatase (from Dianova) were used as secondary antibodies for immunoblotting in all species, except for the rainbow trout. The biotinylated secondary antibody used for immunoblotting of trout proteins was sheep anti-human IgG (whole antibody from Amersham Canada, Oakville, Ontario). The secondary antibody used for immunofluorescence was goat anti-human IgG conjugated to CY3 (Jackson Immunoresearch, West Grove, Pa.).

### *SDS-PAGE and Immunoblotting*

Proteins were separated in 5% stacking and 10% to 12% resolving SDS-polyacrylamide gel electrophoresis (PAGE) as previously described (Laemmli 1970). The separated polypeptides were electrophoretically transferred from gels to nitrocellulose membranes, and processed for immunoblotting following the procedure given in Lang *et al.,* (1999). Blots of rainbow trout polypeptides were reacted with the appropriate biotinylated secondary antibody, followed by incubation with horseradish peroxidase-conjugated streptavidine (from Amersham Canada) at a dilution of 1:4,000 in PBS-Tween for 45 min. at 20°C. The blots were then developed using the chemiluminescence kit (Amersham Canada). The reactivity was visualized on hyperfilm ECL (Amersham Canada). For other species, the presence of the bound secondary antibodies was detected with NBT/BCIP (nitro blue tetrazolium, and 5-Bromo-4-chloro-3-indolyl Phosphate) color reaction. The nitrocellulose membrane was incubated in the dark with 66 µl NBT solution (50 mg/ml in 70% dimethylformamide) and 33 µl BCIP solution (50 mg/ml in 100% dimethylformamide) in 10 ml alkaline buffer (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5). Incubation was done inside a plastic bag to avoid bubbles of air, from hours to several days until the bands developed. The color reaction was stopped with 2% formalin in distilled water. The membranes were stored wet inside plastic bags, and scanned with a Hewlett Packard Scan Jet 4C using the Adobe Photoshop (Adobe Systems Inc.). Molecular weights of the LAP2 isoforms were estimated with Total Lab version 1.1 (Phoretix).

### *Tissue Sections*

For immunolabelling, rainbow trout brain and liver were fixed in methacarn (60% methanol, 30% chloroform and 10% acetic acid) for 24 hours at 4ºC as described previously by Dardick *et al.,* (1988). Rainbow trout white muscle remained unfixed. Methacarn-fixed tissues were cryoprotected by successive immersions, each for 2 h, in sterile 5, 10 and 15% sucrose/PBS. Fixed tissues were then stored in 15% sucrose/PBS at 4ºC until used. Immediately after excision from the animal, small pieces of white muscle were placed in plastic molds, embedded in Shandon cryomatrix medium (Fisher Scientific, Ottawa, Canada) and frozen in dry ice. White muscle blocks were stored at -80°C until used. For sectioning, methacarn-fixed tissues were embedded in Shandon cryomatrix medium and frozen in 1,1,1,2-tetrafluoroethane (Histofreeze from Fisher Scientific, Ottawa, Canada). Freezing was allowed to conclude on dry ice. Frozen tissues, including white muscle, were placed in an Ames-cryostat and allowed to equilibrate with the cryostat chamber temperature. Sections of 8 µm in thickness were cut at –20ºC and collected on 0.5% gelatin-coated slides. Slides were stored at -20ºC until histochemical staining was performed.

### *Indirect Immunofluorescence Staining*

Rainbow trout hepatocytes and erythrocytes were allowed to attach to coverslips coated with 0.01% poly-L-lysine. Samples were fixed, permeabilized and stained as previously described (Chaly *et al.*, 1984). Frozen sections of unfixed and fixed tissues were warmed to room temperature prior to staining. Sections were then incubated with the primary antibody for 1 hour, rinsed in PBS, treated with the secondary antibody for one hour, and extensively washed in PBS. Following reaction with antibodies, all cells and tissue preparations were stained for one minute in DNA-specific 4'-6-diamino-2-phenylindole (0.1 mg/ml diluted 1:3,000, Molecular Probes, Eugene, Oregon, US). All samples were rinsed extensively in PBS and mounted in PBS (pH 7.0) containing 0.1% *p*-phenylene diamine and 50% glycerol. For controls, all cells and tissues studied were incubated with PBS instead of the primary antibody. Conventional epifluorescence microscopy was carried out on a Zeiss Axiophot and observations were recorded on Ilford XP2-400 film.

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