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ORIGINAL ARTICLE

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Spatio-temporal expression of a *DAZ*-like gene in the Japanese newt *Cynops pyrrhogaster* that has no germ plasm

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Abstract To investigate the germ cell specification in urodeles, we cloned a *DAZ-like* sequence from the Japanese newt *Cynops pyrrhogaster*, *Cydazl*, and raised antibodies specific to Cydazl. *Cydazl* is a homologue of the human *DAZ* (deleted in azoospermia), *DAZL*, and *Xenopus dazl* genes, which are involved in gametogenesis or germ cell specification. During gametogenesis, expression of *Cydazl* mRNA and Cydazl protein was detected at first in the small previtellogenic oocytes in females but was not localized as seen in *Xenopus* and was restricted to secondary spermatogonia prior to meiosis in males. During early embryogenesis, maternal stores of the *Cydazl* transcript and protein were present in the entire embryos, not localized in any specific region. The zygotic expression was detected in hatching larvae (stage 50) by RT-PCR analysis whereas specific cells expressing *Cydazl* could not be determined by in situ hybridization at this stage. Strong expression of *Cydazl* and Cydazl were detected in primordial germ cells (PGCs) that had entered the gonadal rudiment at late stage 59. These results suggest that *Cydazl* does not function early in development, for the specification of germ cells, but functions later for differentiation of germ cells in the developing gonads during embryogenesis and for meiotic regulation, supporting the previous idea of an intermediate germ cell formation mode in urodeles.

Keywords *DAZ*-like · Primordial germ cells · Gametogenesis · Urodele

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Introduction

Historically, three modes of germ cell formation in the animal kingdom have been proposed (Nieuwkoop and Sutasurya 1981): epigenetic, intermediate and preformistic. In the epigenetic mode, sexual and asexual forms of reproduction alternate under the influence of environmental factors. During sexual reproduction, germ cells are formed from undifferentiated or dedifferentiated embryonic cells (i.e., totipotent embryonic cells; in Cnidaria and Platyhelminthes; see Nieuwkoop and Sutasurya 1981). In the intermediate mode, germ cells are formed at a rather late stage of development from pluripotent embryonic cells, which must have earlier passed through a phase of somatic development. Once formed, however, they are no longer replaceable by other cells (in mammals, urodeles, etc.; see Eddy and Hahnal 1983; Lawson and Hage 1994). In the preformistic mode, germ cells segregate from somatic cells at a very early stage of embryonic development. They are often predetermined by the presence of a germ-cell-specific germ plasm, so that either presumptive or true germ cells can be distinguished during most, if not all, of the life cycle (in insects, anurans, etc.; see Lehmann and Ephrussi 1994; Strome et al. 1994; Mahowald and Boswell 1983; Smith et al. 1983). As embryos develop, a hierarchy of developmental events is expressed in the progressive differentiation of cell types, regionalization of embryos, and establishment of morphological differences between regions. Perhaps the most fundamental of such events are the segregation of somatic cells from germ cells and the laying down of the body plan (Hall 1999). Therefore, the need to form primordial germ cells (PGCs) can act as a developmental constraint by inhibiting the evolution of embryonic patterning mechanisms that compromise their development (Dixon 1994). Conversely, events that stabilize the PGCs may liberate these constraints (Johnson et al. 2003). As described above, it is generally accepted that among the major extant amphibian lineage, one mechanism is found in urodeles (intermediate) and another in anurans (preformistic).

A special cytoplasmic structure, the so-called germ plasm, which was originally detected in the cytoplasm near the nucleus in early oocytes (Heasman et al. 1984), exists in anurans. The germ plasm located in the subcortical layer of the vegetal region of fully grown ovarian oocytes and fertilized eggs (Czolovska 1969) is used as a reliable marker of presumptive and true PGCs (Akita and Wakahara 1985; Kamimura et al. 1976; Whitington and Dixon 1975). At the electron microscopical level, germ plasm in anuran eggs and embryos contains numerous mitochondria and small electron-dense bodies called germinal granules (Williams and Smith 1971; Ikenishi and Kotani 1975), which are very similar to the polar granules in *Drosophila* (Mahowald 1968). However, no germ-cell-specific germ plasm has been found in urodele oocytes and eggs. Specific structures corresponding to anuran germinal granules or their derivatives were first recognized in the PGCs of *Ambystoma mexicanum* at stage 40 (late tailbud; Ikenishi and Nieuwkoop 1978). Such structures ("nuage" materials) were not found in PGCs prior to stage 40. Between stage 40 and stage 46 (prehatching), the amount of nuage materials markedly increased. Similar structures, not seen in somatic cells, were found in PGCs of feeding larvae of the newt, *Triturus* (presently termed *Cynops*) *pyrrhogaster* (Hamashima and Kotani 1977). From these observations, it thus seems that nuage materials appear in urodelan PGCs during later embryogenesis and that in urodeles PGCs do not develop from predetermined elements but, rather, arise epigenetically from common somatic cells during later embryogenesis by embryonic induction (see Nieuwkoop and Sutasurya 1979; Wakahara 1996).

The idea that somatic cells can be induced to form PGCs in urodeles was initially proposed by Kotani (1957, 1958). He removed presumptive lateral plate mesoderm from early gastrulae of the newt *Triturus* (presently *Cynops*) *pyrrhogaster* and replaced it with presumptive ectoderm from other early gastrulae. In a majority of the operated embryos (examined at larval stages) PGCs, albeit reduced in number, were observed in the genital ridges, suggesting that these PGCs were regulatively formed from the grafted ectoderm. This speculation has been supported by several lines of works such as xenoplastic recombinations of ectodermal caps with the ventral endoderm of urodele blastulae (Sutasurya and Nieuwkoop 1974), recombination experiments on the urodele ectoderm with anuran endoderm (Michael 1984), and in vitro induction of PGCs in explants of *Triturus* ectoderm (Kocher-Becker and Tiedemann 1971) by adding vegetalizing factors from chick embryos. Unfortunately, however, such studies were performed at histological or cytological levels without using germ-cell-specific molecular markers. In other words, it seems premature to conclude that PGCs or true germ cells in urodeles can be induced in vitro under the specific influence of mesoderm-inducing factors.

To investigate the mechanisms of germ cell specification in urodeles, it seems necessary to obtain proper reliable molecular markers of PGCs or true germ cells. Germ-cell-specific molecular markers have been reported in many organisms, including homologues of *vasa*, *nanos*, *dazl*, etc. (Lehmann and Ephrussi 1994; Extavour and Akam 2003). In urodeles, however, only a few markers for PGCs and/or true germ cells have been identified. In this study, we chose the *DAZL* gene as a molecular tool.

The *DAZ* (*Deleted in Azoospermia*) gene encodes an RNA-binding protein located on the Y chromosome of human males, and deletion of this locus correlates with azoospermia (Reijo et al. 1995). The *DAZ-like* (*DAZL*) gene, human autosomal *DAZ* gene, has also been cloned (Saxena et al. 1996), and homologues of this gene have been identified in some model organisms (*Caenorhabditis*: Karashima et al. 2000; *Drosophila*: Eberhart et al. 1996; zebrafish: Maegawa et al. 1999; *Xenopus laevis*: Houston et al. 1998; *Xenopus tropicalis*: Sekizaki et al. 2004; axolotl: Johnson et al. 2001; mouse: Cooke et al. 1996). In each of these species, *DAZL* RNA shows germ-cell-specific expression, and the DAZL protein is believed to play a key role in meiosis and the normal progression of gametogenesis in some systems, although the actual roles remain to be elucidated. In *Xenopus*, *Xdazl* RNA is localized to the germ plasm in oocytes and embryos (Houston et al. 1998). From depletion studies, it has been suggested that in *Xenopus* its product is required for early germ cell development during migration toward the gonad (Houston and King 2000), consistent with the known role of germ plasm. A *DAZL* homologue in the axolotl *Ambystoma mexicanum* has recently been cloned (*Axdazl*; Johnson et al. 2001). In axolotl oocytes and embryos, however, maternal *Axdazl* RNA is not localized, and the earliest cell-specific expression is found in PGCs after they become located in the vicinity of the genital ridges. These findings in the axolotl suggest that urodeles have no germ plasm and that the urodelan *DAZL* gene is induced by patterning signals in the embryo (see Johnson et al. 2003). In the axolotl study, however, protein expression of the *Axdazl* gene was not investigated.

In the present study, we cloned a *DAZ-Like* homologue in *Cynops pyrrhogaster* (*Cydazl*) and raised antibodies specific to Cydazl. The expression patterns of both *Cydazl* mRNA and Cydazl protein throughout the life cycle of this newt were analyzed, and the possible function of this gene in germ cell formation in *Cynops pyrrhogaster* was discussed.

Materials and methods

Adults and embryos

Adult *Cynops pyrrhogaster* males and females having spermatophores were purchased from a dealer (Hamamatsu Seibutsu Kyozai) and were kept under hibernation at 4° C until use. Fertilized eggs were obtained by three subcutaneous injections of human chorionic gonadotropin (250 IU/animal; Teikoku Zoki) into adult females. Embryos were maintained in 10% Holtfreter's solution until they reached the desired stages. Staging was done according to Okada and Ichikawa (1947).

RNA extraction

Total RNA was isolated from adult tissues and embryos of each stage with ISOGEN RNA extraction reagent (Nippon Gene). Poly $(A)^+$ mRNA was purified from total RNA using an OligotexTM-dT30 (Super) mRNA purification kit (Takara) according to the manufacturer's instructions.

cDNA cloning

Fragments of a cDNA clone encoding the conserved region of Dazl were isolated from testis total RNA by reverse transcription-polymerase chain reaction (RT-PCR) performed by using a touchdown protocol in which 57°C and 46.5°C were set as the extreme high and low annealing temperatures, respectively, using two degenerate oligonucleotide primers, RNP2-F (5′-GTITTYGTIGGIG-GIATHGA-3′) and RNP-0.5R (5′-GCIGGICCIARYT-TIARYTTYTTICC-3′) [Y (C or T); R (A or G); H (not G); I (inosine)]. RNP2-F and RNP0.5-R correspond to the highly conserved amino acid sequences in Dazl, VFVGGID and GKKLKLGPA, respectively. The reaction produced a single band of the expected size (210 bp). After sequence verification, this fragment was used as a probe to isolate a full-length *Cynops Dazl* (*Cydazl*) cDNA clone from a cDNA library constructed from *Cynops* adult testis with a lambda ZAPII predigested *Eco*RI/CIAP-treated vector kit (STRATAGENE) according to the manufacturer's instructions.

Fragments of a cDNA clone encoding the conserved region of β-actin were isolated from testis total RNA by RT-PCR using two degenerate oligonucleotides primers, β-actin-F1 (5′-TGGGAYGAYATGGARAARAT-3′) and β-actin-R1 (5′-GCCATYTCYTGYTCRAA-3′). β-actin-F1 and β-actin-R1 correspond to the highly conserved amino acid sequences in β-actin, WDDMEKI and FEQEMA, respectively. The reaction produced a single band of the expected size (450 bp). After sequence verification, an oligonucleotide primer set was designed for RT-PCR analysis (GenBank accession number of *Cynops* β-*actin* is AB164066).

Preparation of RNA probes

The full-length *Cydazl* cDNA cloned in a plasmid, pBluescript SK (−), was linearized with either *Xho*I (for sense RNA) or *Sma*I (for antisense RNA). Sense or antisense RNA probes were synthesized with T3 or T7 RNA polymerase (Gibco), respectively, in the presence of digoxigenin (DIG)-labeled UTP (Roche Diagnostics).

Northern blot analysis

Five micrograms of total RNA isolated from adult tissues was electrophoresed in 1% agarose gel containing 6% formaldehyde and blotted onto Hybond N^+ (Amersham). *Cydazl* mRNA was detected with an antisense or sense DIG-labeled RNA probe prepared as described above. The hybridized probe was visualized with alkaline phosphatase-conjugated anti-DIG Fab-fragments and a color-substrate solution [0.2 mM each of nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP), 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$] according to the instructions of the manufacturer (Roche Diagnostics).

RT-PCR analysis

Single-stranded cDNA was generated from the total RNA of adult tissues or poly $(A)^+$ mRNA of embryos of each stage with a SuperScript first-strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer's instructions. To amplify a cDNA fragment of *Cydazl*, the following oligonucleotide primer set was designed: Cydazl-F: 5′-CTGTCCCAAACTGACTGTG-3′ and Cydazl-R: 5′-CTGGCAAATCTATATCATAGCA-3′ (see Fig. 1b for the positions of the sequences for primers). cDNA fragments of β-*actin* and *EF1*-α (Takabatake et al. 1996) were amplified as controls using the following primer sets: β-*actin* forward, 5′-TGGGATGACATGGA-GAAGAT-3′ and reverse, 5′-GCCATCTCTTGTTC-GAAGTC-3′; *EF1*-α forward, 5′-ATCGACAAGAGAAC-CATCGA-3′ and reverse, 5′-GTGATCATGTTCTTGAT-GAA-3′. The PCR cycling conditions were as follows: 30 s at 94°C for melting, 1 min at 53°C for annealing, and 1 min at 72°C for extension. After 30 cycles (for adult tissues) or 40 cycles (for embryos) of amplification, PCR products were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining under UV illumination.

In situ hybridization

Testes and ovaries isolated from adults and embryos were fixed immediately in Bouin's solution overnight at 4°C. The samples were dehydrated in ethanol, embedded in paraffin, sectioned at 5–7 μm, placed onto gelatinized slides, and subjected to hematoxylin-eosin staining or in situ hybridization analysis. *Cynops* oocytes were staged according to the stages for axolotl oogenesis described by Beetschen and Gautier (1989), and *Xenopus* oocytes were staged according to Dumont (1972).

For in situ hybridization, the sections were deparaffinized, permeabilized, HCl-treated, proteinase K-digested, and incubated with prehybridization buffer. They were then hybridized with a sense or antisense RNA probe overnight at 50°C. After sequential washes, the samples were treated with RNase A (Sigma) and washed again. After blocking, the signals were detected with alkaline phosphatase-conjugated anti-DIG Fab fragments and visualized with a color-substrate solution (NBT-BCIP). These protocols for in situ hybridization were performed according to the instructions of the manufacturer (Roche

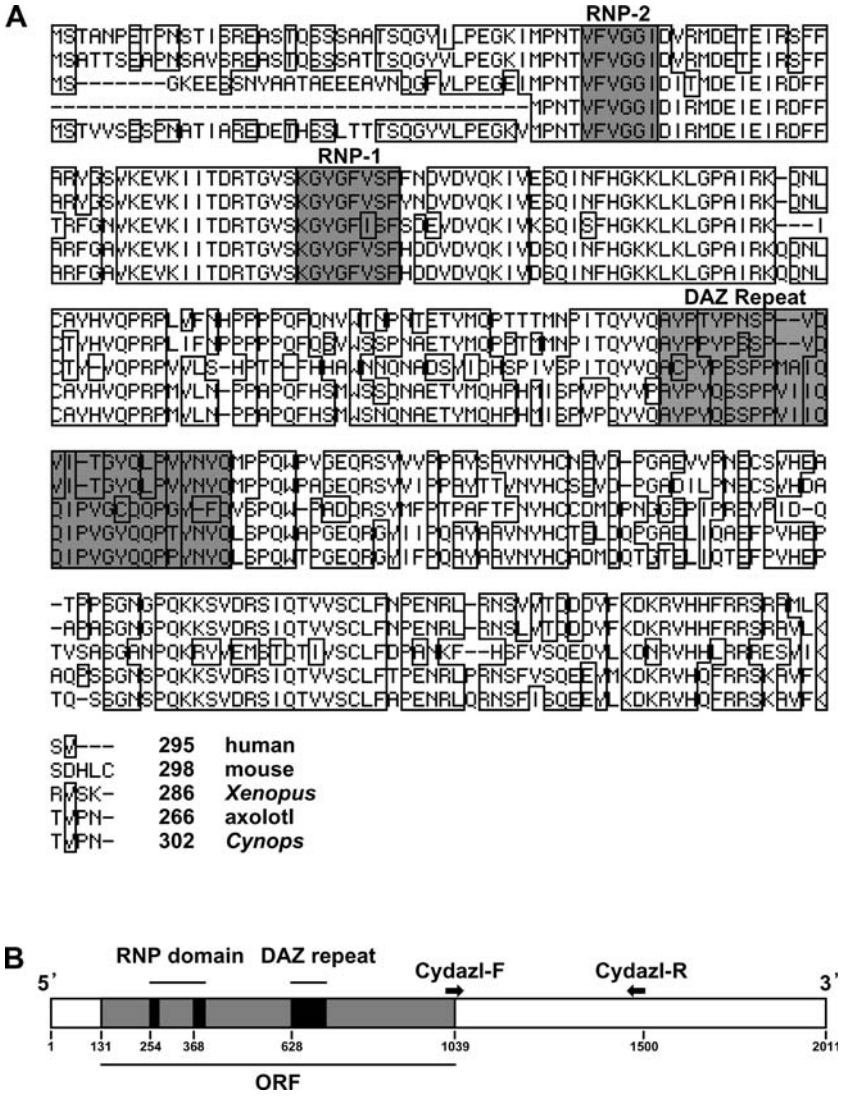

Fig. 1 Analysis of the predicted *Cynops* Dazl (Cydazl) amino acid sequence. **a** Comparison of the deduced amino acid sequence of *Cydazl* derived from the full-length cDNA with other vertebrate *Dazl* homologue proteins. The sequences of the human (*Dazl*), mouse (*dazla*), *Xenopus* (*Xdazl*), axolotl (*Axdazl*) and *Cynops* (*Cydazl*) *DAZ*-like gene products are aligned from top to bottom. Identical residues are *enclosed by boxes*. The RNP-1 hexamer and RNP-2 octamer motifs of the RNP domain and a region homologous to the DAZ repeat are *shaded* and indicated above the alignment. **b** Diagram showing domains and features of *Cydazl* mRNA. The *gray box* shows the open reading frame, while the *black boxes* show the positions encoding the RNP motifs (RNP-2 and RNP-1) with the putative *DAZ* repeat indicated *above the boxes*. The positions of the specific primers designed for RT-PCR analysis (Cydazl-F and Cydazl-R) are indicated by a *pair of arrows*. GenBank accession numbers: *Cynops* (*Cydazl*, this work) AB164065, human (*Dazl*) AAH27595, mouse (*dazla*) Q64368, *Xenopus* (*Xdazl*) AAC41242 and axolotl (*Axdazl*) AAK58846

Diagnostics) with the exception that 66% formamide was used.

Protein extraction

Adult tissues or embryos were homogenized with a pestle in an equivalent volume of the ice-cold extraction buffer (100 mM β-glycerophosphate, 15 mM $MgCl_2$, 5 mM EGTA, 100 μM *p*-amidinophenyl-methanesulfonyl fluoride, 3 μg/ml leupeptin, 1 mM dithiothreitol, 20 mM HEPES, pH 7.5). The homogenate was centrifuged at 15,000× *g* for 10 min at 4°C, and the supernatant was frozen in liquid nitrogen and kept at −80°C until use.

Production of recombinant proteins and antibodies

To construct a glutathione S-transferase (GST) fusion protein, the cDNA encoding the full open reading frame of *Cydazl* was amplified by PCR with a 5′-primer (5′-ATTCTAGAAATGTCTACTGTAGTCTCTGAA-3′) and a 3′-primer (5′-GTGCTCGAGGTTTGGGACAGTTTTGAATAC-3′). The PCR product was subcloned into the *Xba*I-*Xho*I site of a GST-expression vector, pGEX-KG (Guan and Dixon 1991).

To construct a histidine-tagged fusion protein, the cDNA encoding the full open reading frame of *Cydazl* was amplified by PCR with a 5′-primer (5′-TCGGATCCGAATGTCTACTGTAGTCTCTGAA-3′) and a 3′-primer (5′-GTGCTCGAGGTTTGGGACAGTTTTGAATAC-3′). The PCR product was subcloned into the *Bam*HI-*Xho*I site of pET21c (Novagen).

GST fusion protein (GST-Cydazl) and histidine-tagged fusion protein (Cydazl-His) were expressed in *Escherichia coli* (XL1 and BL21, respectively) and purified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electroelution in Tris-glycine buffer without SDS according to the method described previously (Hirai et al. 1992). The purified GST-Cydazl and Cydazl-His proteins were dialyzed and injected into mice to produce antibodies according to the method described previously (Yamashita et al. 1991). Polyclonal antibodies against the recombinants were affinity-purified with antigenic pro-

teins electroblotted onto an Immobilon membrane (Millipore).

Immunological detection of Cydazl

For immunoblotting analysis, proteins were separated by SDS-PAGE and transferred to an Immobilon membrane (Millipore). The blots were rinsed with TTBS (150 mM NaCl, 20 mM Tris-HCl, 0.1% Tween 20, pH 7.5), blocked with 5% dry milk in TTBS, and incubated with the affinity-purified anti-Cydazl antibodies (1:200 dilution in blocking buffer) for 2 h at 37°C. After washing three times for 5 min each, the antigen-antibody complex was visualized by an alkaline phosphatase-conjugated secondary antibody goat anti-mouse IgG (1:2,000; American Qualex Antibodies) and a color-substrate solution (0.2 mM each of NBT and BCIP, 100 mM diethanolamine, 5 mM $MgCl_2$, pH 9.5).

For immunohistochemistry, adjacent sections used for in situ hybridization and hematoxylin-eosin staining were deparaffinized, rehydrated, and washed in TTBS two times for 5 min each. Sections were blocked with 5% dry milk in TTBS for 1 h at RT and then incubated with the anti-Cydazl antibodies (1:100 dilution in blocking buffer) for 2 h at 37°C. After washing three times for 5 min each, the antigen-antibody complex was visualized by an alkaline phosphatase-conjugated secondary antibody (1:1000) and a color-substrate solution (0.2 mM each of NBT and BCIP, 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$).

Results

Cydazl encodes a protein that is highly related to the products of vertebrate *DAZ*-like genes

To obtain a cDNA fragment of the *Cynops pyrrhogaster Dazl* (*Cydazl*) sequence, we performed RT-PCR using a touchdown protocol. The reaction produced a single band of the expected size (210 bp) which showed significant homology to RNP domains of other *Dazl* sequences. Using the cloned fragment as a probe, the full-length cDNA sequence of *Cydazl* was obtained from a cDNA library constructed from the testis of a sexually mature male. The sequence (2,011 bp) encoded 302 amino acid residues as an ORF. Figure 1 shows the predicted amino acid sequences of *Cydazl* (*Cynops pyrrhogaster*, this study), *Axdazl* (*Ambystoma mexicanum*, Johnson et al. 2001), *Xdazl* (*Xenopus laevis*, Houston et al. 1998), mouse *dazla* (Cooke et al. 1996), and human DAZL (Reijo et al. 1995; Fig. 1a) and the domain structure of the gene product (Fig. 1b). Similarities of *Cydazl* to the homologues of human, mouse, *Xenopus* and axolotl were approximately 77%, 77%, 71% and 90%, respectively. Surprisingly, *Cydazl* was more similar to the mammalian homologues than that of *Xenopus* as pointed out by Johnson et al. (2001) in *Axdazl*. All members of the *DAZ* gene family encode RNA-binding proteins with two distinctive RNA recognition motifs (RNP-1 and RNP-2) that are highly conservative throughout vertebrates. Identities of the RNP domain of *Cydazl* to the homologues of human, mouse, *Xenopus* and axolotl were 88.8%, 89.9%, 87.6% and 100%, respectively. *Cydazl* also contained specific sequences corresponding to the DAZ repeat, which is encoded seven times in the human Y-linked *DAZ* gene (Reijo et al. 1995).

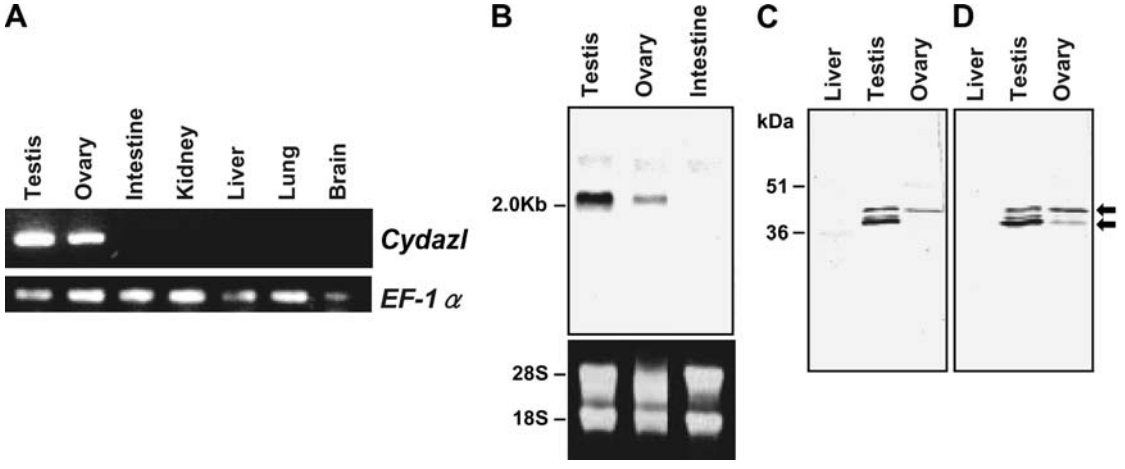

Fig. 2 Gonadal tissue-specific expression of *Cydazl* mRNA and Cydazl protein in the adult newt, *Cynops pyrrhogaster*. **a** Gonad-specific expression of *Cydazl* mRNA was confirmed by RT-PCR. The amplified fragment of *Cydazl* was detected by ethidium bromide staining as a single band. A fragment of *Cynops EF-1* α was amplified as an internal control. **b** Northern blot analysis of total RNA (5 μg) from the indicated adult tissues. Positions of 28S and 18S rRNA bands stained with ethidium bromide are shown *lower* *down* as a loading control. Gonad-specific expression of *Cydazl* mRNA was detected with an antisense DIG-labeled RNA probe as a single band. **c, d** Proteins extracted from the indicated adult tissues were western blotted and immunostained with anti-GST-Cydazl affinity-purified with Cydazl-His (**c**) and with anti-Cydazl-His affinity-purified with GST-Cydazl (**d**). Both antibodies recognize three forms of Cydazl protein specifically in gonadal tissues

Gonad-specific expression of *Cydazl* in adult tissues

Expression patterns of *Cydazl* mRNA and Cydazl protein were first analyzed in adult tissues. Gonad-specific expression of *Cydazl* mRNA in adult tissues was confirmed by RT-PCR (Fig. 2a) using a set of specific primers (Fig. 1b). *Cynops EF-1* α mRNA was employed as an internal control for an amplification standard. *Cydazl* expression was detected in the ovary and testis but not in somatic tissues (intestine, kidney, liver, lung and brain). Similar gonad-specific expression of *Cydazl* in adult tissues was also confirmed by northern blot analysis (Fig. 2b). A positive signal of the expected length (approximately 2.0 kb) was detected in the testis and ovary but not in the intestine.

To examine the Cydazl protein expression, we raised two kinds of mouse polyclonal antibodies against the full-length Cydazl protein using recombinant GST-fusion Cydazl protein (GST-Cydazl) and recombinant histidine-tagged Cydazl protein (Cydazl-His) as antigens. Both antibodies recognized the antigenic recombinant proteins with high specificity (data not shown), and the anti-GST-Cydazl affinity-purified with Cydazl-His and the anti-Cydazl-His affinity-purified with GST-Cydazl showed the same results (Fig. 2c, d), showing that three forms of Cydazl protein (39, 40 and 45 kDa) were specifically detected in the testis and ovary but not in somatic tissues. Based on the results of a previous study on Xdazl protein, Mita and Yamashita (2000) proposed that the modification of two forms of Xdazl (37 and 40 kDa) was caused by phosphorylation by MAP kinase, because the shift from 40 to 37 kDa was enhanced by phosphatase treatment. Therefore, the three molecular masses of Cydazl that were recognized in the testis and ovary by our antibodies may be caused by phosphorylation. The anti-Cydazl-His affinity-purified with GST-Cydazl was designated as anti-Cydazl and was used for the following analysis because it demonstrated the highest specific reactivity in the immunohistochemical analysis.

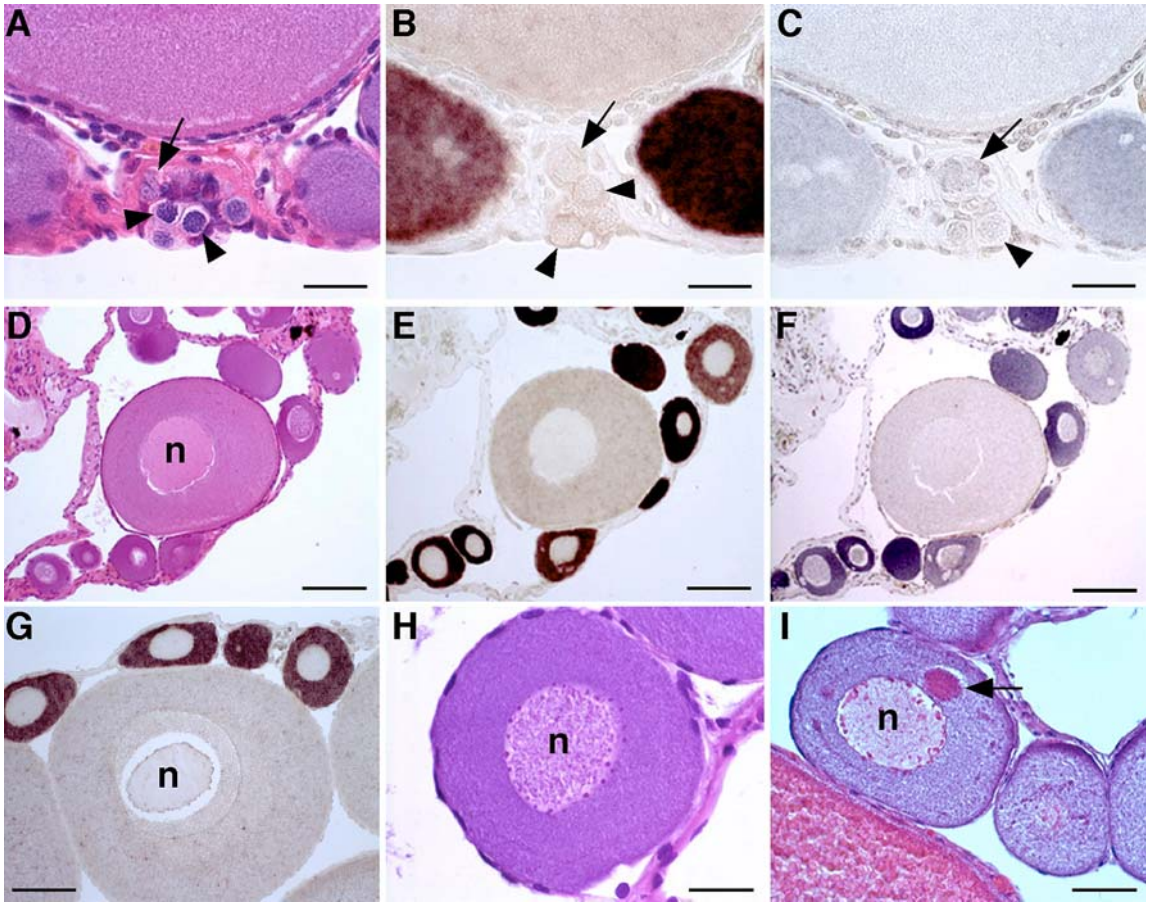

Fig. 3 *Cydazl* mRNA and Cydazl protein are expressed in the entire cytoplasm of oocytes in the adult ovary. Adjacent sections of the region containing the primary oogonium (*arrows*) and oocytes in early meiotic prophase I (*arrowheads*; **a, b, c**), and growing oocytes (stages I–II; **d, e, f**). Each section was stained with hematoxylin-eosin (**a, d**), hybridized to an antisense *Cydazl* digoxigenin-labeled probe (**b, e**) or immunostained with anti-Cydazl using enzymatic colorization by an AP-conjugated secondary antibody (**c, f**). No signal was detected in the primary oogonium and oocytes in early meiotic prophase I (**b, c**). Strong signals were detected in the entire cytoplasm of small previtellogenic oocytes (**e, f**). **g** A stage-IV oocyte hybridized to an antisense *Cydazl* probe shows no localization of *Cydazl* mRNA. **h, i** High magnification views of the sectioned *Cynops* stage-I oocyte (**h**) and a *Xenopus* oocyte of the same stage stained with hematoxylin-eosin (**i**). A mitochondrial cloud in the perinuclear region was clearly stained with eosin in the *Xenopus* oocyte (*arrow*) but not in the *Cynops* oocyte. *Scale bar*: 50 μm (**a–c**, **h**, **i**); 200 μm (**d–g**). *n* Nucleus

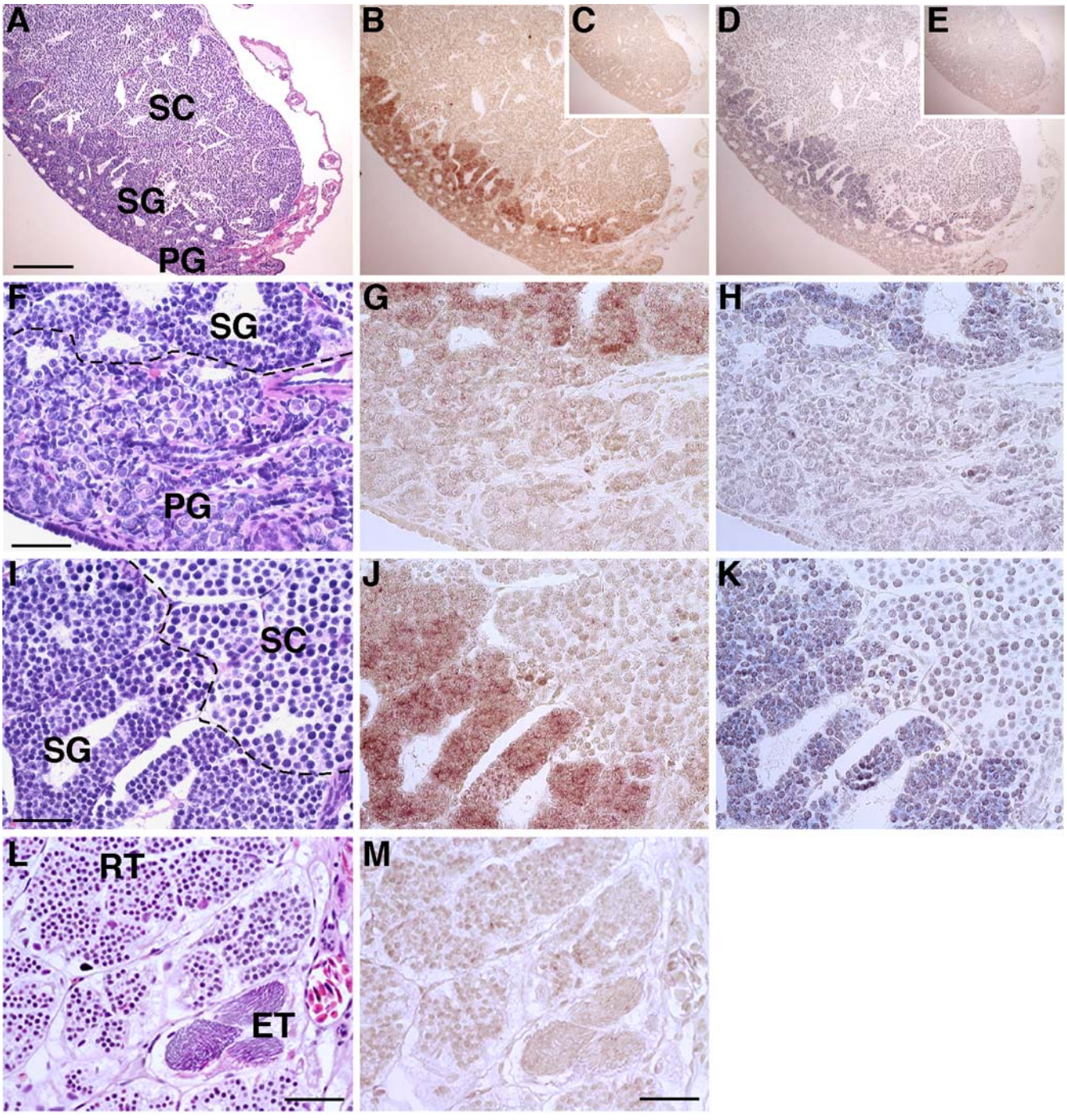

Fig. 4 *Cydazl* mRNA and Cydazl protein are expressed in secondary spermatogonia in the adult testis. Each adjacent section of the adult testis was stained with hematoxylin-eosin (**a, f, i, l**), hybridized to an antisense *Cydazl* DIG-labeled probe (**b, g, j, m**), hybridized to a sense probe (**c**), immunostained with anti-Cydazl using enzymatic colorization by an AP-conjugated secondary antibody (**d, h, k**) and without a primary antibody (**e**). **a** The developmental phases of spermatogenesis from primary spermatogonia (*PG*) to secondary spermatogonia (*SG*) and to spermatocytes (*SC*) are assigned into specific regions from bottom to top in the section. **b, d** Strong signals are localized in the region of secondary spermatogonia. **c, e** No visible signals were detected in any region in the negative controls. Higher magnification views of each adjacent section show specific localization of both *Cydazl* mRNA and Cydazl protein in the cysts of secondary spermatogonia (**j, k**) but no visible signals in the PGC-like primary spermatogonia (**g, h**), in spermatocytes (**j, k**), or in round spermatid (*RT*) and elongating spermatid (*ET*; **l, m**). *Scale bar*: 500 μm (*a–e*), 100 μm (*f–m*)

Stage-specific expression of *Cydazl* in developing gonads

To examine the expression patterns of *Cydazl* mRNA and Cydazl protein in oogenesis and spermatogenesis more closely, we performed in situ hybridization, immunohistochemistry, and hematoxylin-eosin staining on each adjacent section from the ovary and testis of sexually matured adults (Figs. 3, 4). In the ovary, *Cydazl* mRNA and Cydazl protein were not detected in PGC-like primary oogonia, secondary oogonia, oocytes in early meiotic prophase I or any somatic cells (Fig. 3a–c). Strong signals of both mRNA and protein were observed in the entire cytoplasm of small previtellogenic oocytes (stages I–II; Fig. 3d–f). However, no specific localization was observed in developing *Cynops* oocytes, unlike in the oocytes of *Xenopus laevis* (Houston et al. 1998), where *Xdazl* mRNA was detected as a mitochondrial cloud in developing oocytes and as small islands of germ plasm in the vegetal cortex of mature stage VI oocytes. Figure 3h and i shows hematoxylin-eosin staining on late stage I oocytes of *Cynops* and *Xenopus*, respectively. In *Xenopus* oocytes, a mitochondrial cloud was clearly observed by eosin staining (Fig. 3i). Contrary to this, in the *Cynops* oocytes, such a mitochondrial cloud could not be observed in any region throughout the oocyte maturation stages (Fig. 3h). Positive signals of *Cydazl* mRNA and Cydazl protein gradually decreased as vitellogenesis proceeded and the oocytes grew larger. Neither mRNA nor protein could be detected in growing oocytes (stages III-VI) by in situ hybridization using a DIG-labeled probe and immunohistochemistry (Fig. 3g).

Figure 4 shows the expression patterns of *Cydazl* mRNA and Cydazl protein in adult *Cynops* testis. Because the developmental phases of the male germ cells from spermatogonia to mature spermatozoa were assigned into specific regions in the newt testis, it was relatively easy to identify the stage of germ cells during spermatogenesis. Neither *Cydazl* mRNA nor Cydazl protein were detected in the PGC-like stem cells or in any somatic cells (Fig. 4f–h), as in the ovary. Positive signals of *Cydazl* mRNA and Cydazl protein expression were restricted to secondary spermatogonia prior to meiosis (Fig. 4a–k). Both *Cydazl* mRNA and Cydazl protein were absent in spermatocytes, spermatids, and mature spermatozoa (Fig. 4i–m). For all these experiments, sections hybridized with a *Cydazl* sense probe and without a primary antibody showed no visible signals (Fig. 4c, e).

Cydazl expression during embryogenesis

To examine the expression patterns of *Cydazl* mRNA and Cydazl protein in embryogenesis, we performed northern blot, RT-PCR and western blot analyses (Fig. 5). By northern blot analysis, *Cydazl* mRNA could hardly be detected during embryogenesis (data not shown). To detect the very low level of *Cydazl* mRNA with higher analytical sensitivity, we performed RT-PCR analysis. By RT-PCR analysis, we were able to detect *Cydazl* mRNA during the period of early embryogenesis (Fig. 5a). *Cydazl* mRNA inherited as a maternal transcript was detected in the fertilized eggs, and a similar expression level was retained to the neurula stage (stage 14). In the tailbud stage (stage 34), the level of *Cydazl* declined but then increased from the hatching larvae stage (stage 50). At this stage, however, the germ-cell-specific expression was not detected in our in situ hybridization (described later).

To examine the expression pattern of Cydazl protein, we performed western blot analysis (Fig. 5b). Cydazl proteins were detected in the fertilized eggs, and the expression level remained until the neurula stage (stage 14). After this stage, however, the expression level began to decline gradually, and expression was not detectable in the following stages.

Cydazl localization and cell-specific expression in PGCs during embryogenesis

To determine whether the expression of *Cydazl* mRNA and Cydazl protein show cell-specific localization during embryogenesis, we performed in situ hybridization, immunohistochemistry, and hematoxylin-eosin staining on each adjacent section of the embryos (stages 1, 2, 6,

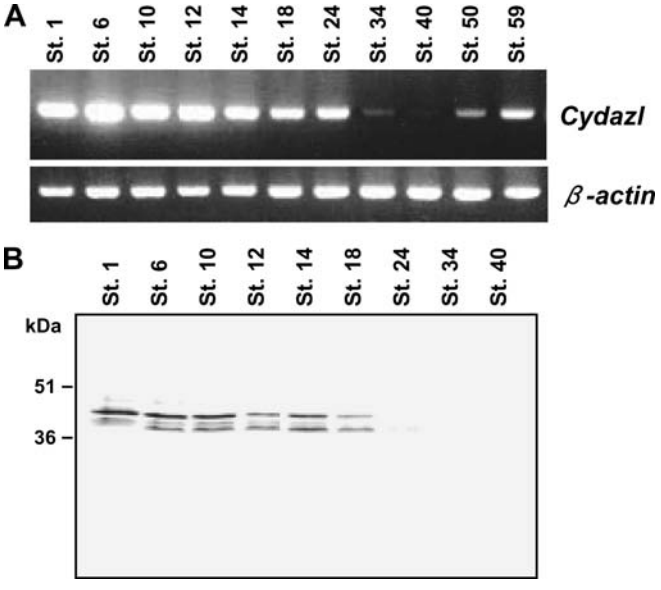

Fig. 5 Expression patterns of *Cydazl* mRNA and Cydazl protein during embryogenesis. **a** RT-PCR analysis to detect *Cydazl* mRNA expression during embryogenesis. Equivalent amounts of poly $(A)^+$ mRNA from whole embryos of each indicated stage were used. A fragment of β-*actin* was amplified as an internal control (*lower band*). *Cydazl* mRNA is present throughout the period of early embryogenesis declining gradually, and is upregulated at stage 50 onwards. **b** Immunoblot detection of Cydazl protein with anti-Cydazl using enzymatic colorization by an AP-conjugated secondary antibody. Proteins extracted from a whole embryo of each indicated stage were loaded per lane. Cydazl proteins were detected in the fertilized eggs (*St. 1*), and the level of expression remained until the neurula stage (*St. 14*), with some modification of molecular weight presumably due to phosphorylation. After stage 34, Cydazl proteins were not detected

8, 10, 12, 14, 18, 24, 34, 40, 50, 56, early 59, late 59 and 60). From fertilized egg to early-stage-59 swimming larvae, no cell-specific expression or localization of *Cydazl* mRNA and Cydazl protein could be detected in any region or cells (Fig. 6a–g). From stage 50, we could recognize PGCs migrating on the dorsal mesentery according to their typical histological character, that is, cells with a large, faintly stained nucleus with indentation and with little cytoplasm but with abundant melanin pigmentation. Melanin granules in the PGCs were of

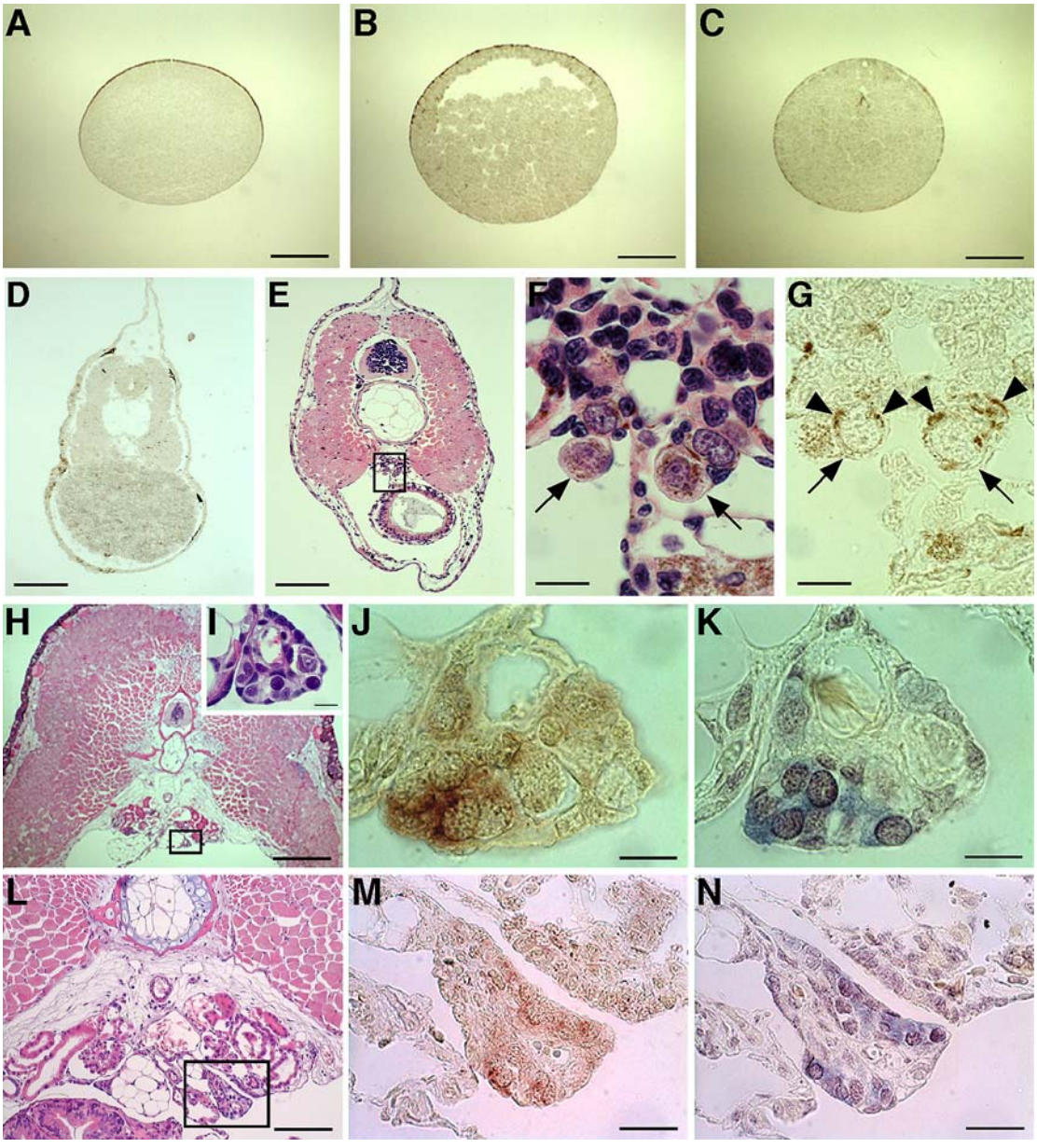

Fig. 6 *Cydazl* mRNA and Cydazl protein are specifically expressed in PGCs from late stage 59. **a–d** A section of the fertilized egg (St. 1; **a**), a sagittal section of the blastula (St. 10; **b**), a cross-section of the early tailbud (St. 24; **c**) and a cross-section of the hatching stage (St. 40; **d**) were hybridized to an antisense *Cydazl* DIG-labeled probe. No localization of signal was detected in any region or cells. **e–g** Cross-sections of the stage-56 embryo. **f, g** Higher magnification views of the indicated region in **e**. Adjacent sections were stained with hematoxylin-eosin (**e, f**) or hybridized to an antisense *Cydazl* probe (**g**). PGCs that have arrived at the genital ridges but are not surrounded by gonadal somatic cells (*arrows*) are shown in **f**. No signal was detected in PGCs at stage 56 (**g**). *Arrowheads* indicate melanin granules associated with the PGCs. **h–k** Cross-section of the late-stage-59 swimming larva. **i–k** Higher magnification views of the indicated region in **h**. Each adjacent section was stained with hematoxylin-eosin (**h, i**), hybridized to an antisense *Cydazl* probe (**j**) or immunostained with anti-Cydazl using enzymatic colorization by an AP-conjugated secondary antibody (**k**). Positive signals for Cydazl mRNA and Cydazl protein were first detected in PGCs at late stage 59. **l–n** Cross-section of stage-60 metamorphosed larva. **m, n** Higher magnification views of the indicated region in **l**. Each adjacent section was stained with hematoxylin-eosin (**l**), hybridized to an antisense *Cydazl* probe (**m**) or immunostained with anti-Cydazl (**n**). Both *Cydazl* mRNA and Cydazl protein are expressed in the germ cells surrounded by the developing gonadal tissue from late stage 59 (**h–k**). *Scale bar*: 500 μm (**a–c, h**); 200 μm (**d, e, l**); 20 μm (**f, g, i–k, m, n**)

sufficient abundance (Lesimple et al. 1990) to be misinterpreted as positive signals of in situ hybridization (Fig. 6g). Because the adjacent sections hybridized with a sense probe showed the same figures as those hybridized with an antisense probe, we could distinguish the positive signals from melanin granules. During embryogenesis, the first cell-specific expression of both *Cydazl* mRNA and Cydazl protein could be detected in PGCs of late-stage-59 swimming larvae (Fig. 6h–n). In this stage, PGCs that had reached the genital ridges were surrounded by gonadal somatic cells. In earlier stages (stage 56–early 59), however, the PGCs that had reached the genital ridges were still not surrounded by gonadal somatic cells and expressed neither *Cydazl* mRNA nor Cydazl protein (Fig. 6e–g). In stage-60 metamorphosed larvae, proliferating PGCs expressed both *Cydazl* mRNA and protein (Fig. 6l–n).

Discussion

DAZL homologues are highly conserved throughout vertebrates

The deduced amino acid sequences of *Cydazl* reported in this paper were nearly identical to those of the *Dazl* homologue in the axolotl (*Ambystoma mexicanum*, *Axdazl*; Johnson et al. 2001). Surprisingly, the sequences were more similar to those of mammals than of *Xenopus* (Fig. 1). This result was unexpected in view of the phylogenetic relationships of these animals, because it is generally accepted that gene sequences can be used as a direct measure of phylogenetic distance. Since entire sequences of the genome in urodele species have not been solved yet, it seems premature to conclude that every gene in urodeles is more similar to that of mammals than of anurans. This curious finding, however, may be explained by the view that common mechanisms of germ cell development are shared by urodele and mammalian embryos (Johnson et al. 2001; Wakahara 1996). Indeed, the mode of germ-cell specification in urodeles seems nearly identical to that in mammals but not to that in anurans (i. e., intermediate mode in mammals and urodeles but preformistic in anurans; Nieuwkoop and Sutasurya 1979). Whatever the modes of germ-cell formation are, however, the highly conservative sequences of *DAZ*-like gene homologues throughout the vertebrates (Fig. 1) suggest similar or basically identical functions of these genes in germ-cell specification during development (Ikenishi 1998; Wakahara 1996; King 2003).

Possible function of Cydazl in gametogenesis

In many organisms, *DAZL* RNA shows germ-cell-specific expression, and the DAZL protein is believed to play key roles in meiosis and normal progression of gametogenesis in some systems (Xu et al. 2001), although its actual roles remain to be elucidated. Loss-of-function studies have revealed roles of *DAZ* genes in germ-cell development in worms, flies, mice and humans. The specific phenotypes, however, vary among organisms. In *C. elegans*, the gene *daz-1* is required for oogenesis in hermaphrodites but is not required for spermatogenesis (Karashima et al. 2000). On the other hand, the *Drosophila boule* gene is required for entry into meiosis during spermatogenesis and male fertility but is not expressed in females (Eberhart et al. 1996). Male and female mice deficient in *Dazl* are sterile and exhibit defects in germ-cell development and survival (Ruggiu et al. 1997).

In this study, expression analyses of the *Cydazl* mRNA and Cydazl protein during gametogenesis showed different patterns between oogenesis and spermatogenesis (Fig. 7). That is, the expression was suppressed in oogonia and then up-regulated in the small previtellogenic oocytes (stages I–II) in the female. On the other hand, the positive expression was restricted to secondary spermatogonia prior to meiosis in the male (Figs. 3, 4). These results

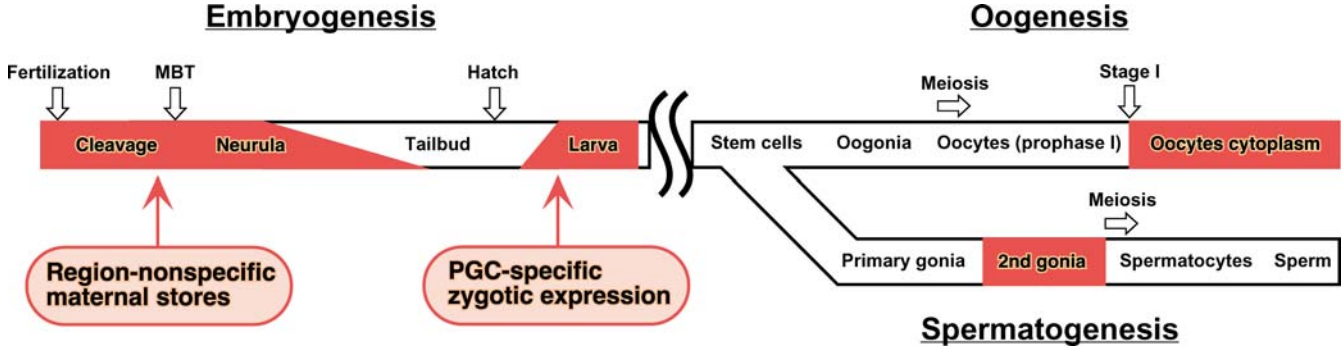

Fig. 7 Diagram showing expression patterns of the *Cydazl* gene throughout the *Cynops* life cycle. The periods when the *Cydazl* mRNA and Cydazl protein are expressed are shown in *red*. During early embryogenesis, the maternal stores of *Cydazl* RNA and Cydazl protein are present in the entire embryos but not localized in any specific region or cells. The zygotic expression of *Cydazl* mRNA begins at stage 50 (hatching larvae). Strong expressions of the mRNA and proteins in the PGCs are detected at late stage 59. The PGC-specific expression disappears in juveniles. During gametogenesis, the expression pattern of *Cydazl* mRNA and Cydazl protein in the female (oogenesis) is slightly different from that in the male (spermatogenesis). The expression is suppressed in oogonia and is then up-regulated in the small previtellogenic oocytes (stages I–II) in the female. Contrary to this, positive expression is restricted to secondary spermatogonia prior to meiosis in the male

suggest that the Cydazl protein has different functions for oogenesis and spermatogenesis. According to results of studies using model organisms described above, Cydazl is required for secondary spermatogonia to enter meiosis during spermatogenesis. In oogenesis, however, Cydazl probably does not have the same function as in spermatogenesis. So, what is the function of Cydazl expressed in the previtellogenic oocytes? The *Cydazl* transcript and protein expressed in the small oocytes are distributed evenly in the cytoplasm (Fig. 3d–g) but are not localized in any specific region as seen in *Xenopus*. In *Xenopus* oocytes, *Xdazl* RNA is concentrated primarily in the mitochondrial cloud, and it is then found in a wedge-like pattern extending to the cortex in stage II–III oocytes and becomes localized in the vegetal cortex by stage VI (Houston et al. 1998). Comparative observations in *Cynops* and *Xenopus* stage I oocytes stained with hematoxylin-eosin (Fig. 3h, i) clearly show that the mitochondrial cloud found in anuran eggs is not present in *Cynops* eggs. Therefore, it is concluded that *Cynops* eggs have no germ plasm as a specific structure in the cytoplasm but have abundant maternal stores of germ-cell-specific gene products, suggesting that all blastomeres in urodele early embryos have a potential to differentiate into PGCs. This potential, however, may not be sufficient to specify and/or to differentiate the germ cells but may be involved in the competence for some inductive signals that specify PGCs. Further studies, on for example depletion of maternal *Cydazl* mRNA, are required to elucidate this issue.

Possible function of Cydazl in PGCs

Houston and King (2000) clearly demonstrated that *Xdazl* was critically involved in PGC development in *Xenopus*. Xdazl protein was expressed in the germ plasm from blastula to early tailbud stages. Specific depletion of maternal *Xdazl* RNA resulted in tadpoles lacking or severely deficient in PGCs. In the absence of *Xdazl*, PGCs did not successfully migrate and reach the dorsal mesentery and germinal ridges. Thus, it was concluded that Xdazl was required for early PGC differentiation and was necessary for the migration of PGCs in *Xenopus* (Houston and King 2000). What is the function of *DAZL* homologues in urodeles that have no germ plasm or germ cell determinants in fertilized eggs? Results of expression analyses during embryogenesis (Fig. 5) showed that the maternal stores of *Cydazl* mRNA and Cydazl protein were present, albeit at very low levels, in the entire embryos but not localized in any specific region or cells (Fig. 6a–g). The temporal and spatial characteristics of the expression pattern of *Cydazl* are quite different from those of anuran *DAZL* genes, suggesting that the maternal Cydazl protein is not responsible for specification of PGCs during early development.

These results are in accordance with those of a study on *Axdazl* in axolotl (Johnson et al. 2001), showing that no cytoplasmic localization of the transcript was detected during early embryogenesis and that the zygotic expression in germ cells was observed later. In axolotl, positive signals of *Axdazl* RNA were detected in PGCs migrating to and those that had just arrived at the genital ridges of long tailbud embryos (Johnson et al. 2001). In *Cynops*, however, *Cydazl* transcript and protein could not be detected in PGCs migrating to and those that had just arrived at the genital ridges by either in situ hybridization or immunohistochemistry (Fig. 6d–g). They were first detected in germ cells that had arrived at the genital ridges and had been surrounded by gonadal somatic cells at late stage 59 (Fig. 6h–k) and were also detected in proliferating germ cells in developing gonads at stage 60 (Fig. 6l–n). These different expression patterns between axolotl and *Cynops* might be due to the different detection procedures employed and/or to possible differences in the organisms. In any case, our results indicate that Cydazl protein seems to have a role in the differentiation of germ cells during late embryogenesis.

Epigenetic, intermediate versus preformistic modes of germ cell formation

Because the PGCs in anurans are segregated earlier during embryogenesis and specified by a germ-cell-specific germ plasm, in vivo formation of PGCs could be possible by injecting germ plasm or the cytoplasmic fraction of it into blastomeres in anuran embryos (Wakahara 1977, 1978, 1990; Ikenishi et al. 1986). In vitro induction of PGCs in anurans, however, seems impossible without introducing a germ-cell determinant(s) or germ plasm into embryonic cells. Contrary to this, the PGCs of urodeles might be induced in undifferentiated ectoderm cells under the influence of mesoderm-inducing factors (Kocher-Becker and Tiedemann 1971; Sutasurya and Nieuwkoop 1974; Michael 1984). Unfortunately, however, almost all studies on mesoderm induction with chemical messengers have been done using *Xenopus* embryos. Activin, a peptide growth factor, can induce presumptive ectoderm from *Xenopus* blastula to differentiate into almost all mesodermal tissues in a dose-dependent fashion (Ariizumi et al. 1991a, 1999b). Furthermore, a complete set of mesodermal tissues was induced in presumptive ectodermal explants that were treated with combinations of activin and retinoic acid of various concentrations (Moriya et al. 1993). Nevertheless, there has been no report on the success of PGC induction in the animal cap assay using *Xenopus* undifferentiated ectoderm. Assuming that mesoderm induction in urodeles is regulated by a mechanism similar to that in *Xenopus* and that PGCs in urodeles are induced from the animal moiety of blastula, it would be possible to induce urodele PGCs in prospective ectodermal cells in vitro with combinations of chemical messengers such as activin, BMP-4 and retinoic acid. The next step in experiments will be artificial induction of PGCs or true germ cells in urodeles, and such experiments will be able to explain the inductive mechanisms of PGCs specification in urodeles, and are now proceeding using the molecular probe that has been cloned in this study.

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