Zebrafish *Danio rerio* Proliferating Cell Nuclear Antigen (PCNA): Cloning and Characterization

Jae-Seong Lee*1,2,† and Myung Chan Gye*3

*1Institute for Molecular Biology and Genetics, Seoul National University, Seoul 151-742, South Korea
*2School of Biochemistry, The University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom
*3Department of Biology, Kyonggi University, Suwon 442-760, South Korea

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The zebrafish *Danio rerio* proliferating cell nuclear antigen (PCNA) gene was cloned after screening of embryonic cDNA library of 48 to 72 h after fertilization using random primer labelled human PCNA probe. Zebrafish PCNA gene cloned is 1247 bp including 5'-, 3'-untranslated regions and open reading frame (261 amino acids) and showed a high homology of amino acids to human (94.6%), mouse (92.7%), rat (93.5%), *Xenopus* (91.6%) and *Drosophila* (71.6%). It also expressed a transcript of approximately 1.3 kb in various tissues (head, liver, intestine, ovary) and cultured fibroblast cell line. This report help in the understanding of the PCNA's role in fish at the replication initiation folk and elongation.

**Key words:** zebrafish, *Danio rerio*, PCNA, cDNA, cloning, expression

Proliferating cell nuclear antigen (PCNA) is important for DNA replication, DNA elongation and DNA repair.1-3 It interacts directly with cyclin/cdk complexes, allowing progression through the G1/S boundary of the cell cycle,4 and functions by forming a homotrimeric ring-shaped structure producing a clamp for a processive DNA polymerase complex, consisting of DNA pol δ, DNA template, and replication factor-C (RF-C).5 However, in fish the cell cycle regulation and auxiliary machinery of DNA replication are not fully understood. Only Ortego et al.6 reported that expression of PCNA was identified in 3 small fish such as medaka *Oryzias latipes*, guppy *Poecilia reticulata*, and western mosquito fish *Gambusia affinis* for a parameter of experimental carcinogenesis or environmental monitoring. In addition, DNA replication in fish is not understood until now. Therefore, for a better understanding on replication mechanism in fish system, zebrafish was chosen and PCNA gene was isolated along with attempts of purification of replication protein A (RPA; known as single-strand DNA binding protein) involved in DNA replication. Currently, cloned PCNA gene is available in diverse species such as human, rat, *Xenopus*, *Drosophila* and plants (carrot, oilseed, maize),7-13 but in fish systems, it is not reported yet. Therefore, this report will be valuable to approach phylogenetically with acceleration of understandings of mechanisms of fish DNA replication. In this report, it is suggested that zebrafish PCNA gene has a high homology of amino acid sequence to mammals, implying that it might have a similar function in DNA replication as shown in mammals.

**Materials and Methods**

**Screening of Zebrafish Embryonic cDNA Library**

For cloning of zebrafish PCNA gene, approximately $4 \times 10^5$ cDNA clones from zebrafish embryonic cDNA library of 48 to 72 h after fertilization were screened by plaque hybridization using radiolabelled human PCNA probe as described earlier.14 Autoradiography was with Agfa X-ray film and intensifying screen at $-80^\circ$C for 24 h. Positive PCNA clones were further purified.

**Cloning and Sequencing**

Seven zebrafish λZAPI/PCNA cDNA clones were isolated after the primary, secondary and tertiary screening of zebrafish embryonic cDNA library. The longest clone was chosen for further analysis. Zebrafish λPCNA insert was digested with EcoRI, and subcloned to pBS/EcoRI for restriction enzyme mapping. After construction of a restriction enzyme map of zebrafish PCNA clone, each fragment was sequenced by the dideoxy chain termination method using Sequenase version 2.0 (United States Biochemicals) and by ABI automated DNA sequencer through primer walking using synthetic primers based on the PCNA sequences analyzed. For searching the similarity and characteristics of zebrafish PCNA sequence, the BLAST program was run.

**Northern Blot Analysis**

To investigate expression patterns of zebrafish PCNA gene, Northern blot analysis was carried out with various tissues and cultured fibroblast cell line. Total RNAs were
extracted by homogenization of zebrafish tissues (head, liver, intestine, ovary) and cultured fibroblast cell line with TRizol® reagent according to the manufacturer’s suggestion. Approximately 20 μg of total RNA was transferred on Hybond® nitrocellulose membrane (Amerham). The blot was prehybridized at 65°C for 2 h in standard hybridization buffer as reported earlier.13) The blot was hybridized with [α-32P) dCTP and [α-32P] dATP-labelled human PCNA probe using random primer labelling. Hybridization was performed overnight in the same buffer at 65°C. The blot was washed thoroughly with washing buffer (0.2× SSC, 0.1% SDS). Finally, the blot was wrapped in Saran® wrap and exposed with X-ray film (Agfa) at −80°C for 24 h.

Expression of Zebrafish His-tagged PCNA Protein in E. coli BL21 (DE3) pLysS

To see the expression and immunological cross-species reactivity of zebrafish PCNA protein, zebrafish PCNA gene was subcloned to pET-16b vector for producing zebrafish PCNA-His tagged recombinant protein. For confirming the cross-reactivity against human PCNA antibody (Pharminigen), zebrafish PCNA-His protein was induced in E. coli BL21 (DE3) pLysS with 1 mm IPTG at 25°C overnight at 0.6 of OD600. After fractionation of proteins extracted in 12% SDS-polyacrylamide gel, the proteins were transferred to nitrocellulose membrane, and subjected to blocking using 3% bovine serum albumin (fraction ‡W) supplementing 0.02% Triton X-100. The human PCNA antibody (anti-mouse) was added in hybridization solution (TBS/0.02% Triton X-100) at 1:1,000. After brief washing of the membrane, secondary antibody anti-mouse Ig horseradish peroxidase (HRP; Amersham Life Science, England) was added at 1:2,000 for ECL (Amer- sham Life Science, England) development.

Results and Discussion

Screening of Zebrafish Embryonic cDNA Library

After screening of approximately 4×10⁸ cDNA clones from zebrafish embryonic cDNA library of 48 to 72 h after fertilization using random primer labelled human PCNA cDNA probe, 7 clones were initially isolated. After second and third round of hybridization of those candidates, all the clones were confirmed to have the PCNA gene.

Cloning and Sequencing

The longest clone containing zebrafish PCNA gene was chosen for further analysis. For subcloning, the insert containing zebrafish PCNA gene cloned to ZAPII vector/ EcoRI was digested with EcoRI, and subcloned to pBS/EcoRI. The insert was sequenced by the dideoxy chain termination method using Sequenase version 2.0 (United States Biochemicals) and by ABI automatic DNA sequencer. Zebrafish PCNA gene cloned is 1247 bp including 5’- and 3’-untranslated regions and open reading frame. The zebrafish PCNA gene encodes 261 amino acids (aa) (Fig. 1). For searching the similarity of zebrafish PCNA, the BLAST program was run. The zebrafish PCNA gene has a high homology to other species such as human (94.6%), mouse (92.7%), rat (93.5%), Xenopus (91.6%), Drosophila (71.6%) as shown in Fig. 2. This result implies that zebrafish PCNA protein would have a similar function to those of mammalian counterparts.1-5) Northern Blot Analysis

To investigate the expression pattern of the zebrafish PCNA gene, Northern blot analysis was carried out with various tissues and cultured fibroblast cell line. They expressed a transcript of approximately 1.3 kb (Fig. 3A). For transcript, it is reported that other species also have a single transcript of approximately 1.0 to 1.3 kb.7,8,10) Thus, zebrafish has a single gene which is functionally and ubiquitously expressed in diverse tissues and cultured fibroblast cell line.

Expression of Zebrafish His-tagged PCNA Protein in E. coli BL21 (DE3) pLysS

Zebrafish PCNA-His protein was purified using Ni-NTA agarose column (Qiagen) (Fig. 3B). After Western blot to zebrafish PCNA protein produced in E. coli BL21 (DE3) pLysS, a single band appeared at the position of approximately 30 kDa (Fig. 3C). The findings suggest that zebrafish PCNA protein has a similar role with human counterparts at replication initiation and elongation. Furthermore, zebrafish PCNA-His protein expressed in E. coli BL21 (DE3) pLysS can be used to test in vitro DNA replication and elongation with RPA protein which is purified from zebrafish crude extracts using conventional chromatographies using affi-gel blue chromatography, single-strand DNA chromatography and Resource Q...
Zebrafish Danio rerio PCNA Gene

Fig. 2. Comparison of the predicted zebrafish PCNA with human and other species (Drosophila [A34752], Xenopus [AAA49926], mouse [I48707], rat [CAA68261], human [NP002583]).

Dashes represent identities between zebrafish and other species PCNA.

Fig. 3. A. Expression patterns of zebrafish PCNA in head (H), liver (L), intestine (I), ovary (O) and cultured fibroblast cell line (C).

B. Expression and purification of zebrafish PCNA-His protein in E. coli BL21 (DE3) pLysS after induction with 1 mM IPTG at 25°C overnight in a 1 l culture in LB medium with 50 μg/ml carbenicillin; M, marker; FT, flow-through; C, Western blot of zebrafish PCNA-His against human PCNA antibody after induction with 1 mM IPTG in E. coli BL21 (DE3) pET-16b/zebrafish PCNA gene.

sepharose ion-exchange chromatography. In summary, this report shows a high homology of amino acid sequence of zebrafish PCNA gene compared to other species and its encoding protein can react against human PCNA antibody. This result suggests that zebrafish PCNA protein can function like human PCNA in DNA replication and elongation.

References


