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ISOLATION OF cDNAS ENCODING THE CATALYTIC DOMAIN OF POLY(ADP-RIBOSE) POLYMERASE FROM *XENOPUS LAEVIS* AND CHERRY SALMON USING HETEROLOGOUS OLIGONUCLEOTIDE CONSENSUS SEQUENCES*

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SUMMARY: We have isolated and sequenced cDNAs encoding the catalytic domain of poly(ADP-ribose) polymerase (PARP) from *Xenopus laevis* and *Oncorhyncus masou* (cherry salmon). The cDNAs were amplified by polymerase chain reaction using heterologous oligonucleotides corresponding to the conserved sequences of mammalian cDNAs as primers. The deduced amino acid sequences of *Xenopus laevis* and cherry salmon cDNA showed 84.4% and 75.6% similarities to that of human PARP, respectively. In both species, mRNA for PARP was identified as a single band of 4 kb, and PARP mRNA was abundant in ovary and brain. Thus, mixed oligonucleotide-primed amplification is a useful method in the cloning of cDNAs from different species, and the catalytic domain of PARP is conserved structurally among phylogenetically different species, suggesting an importance of poly(ADP-ribosyl)ation.

Poly(ADP-ribose) polymerase (PARP) [EC 2.4.2.30] is a zinc-finger protein and binds to single strand breaks (nicks) of DNA (1-3). The enzyme activity of PARP is stimulated by single and double strand breaks of DNA (4). Poly(ADP-ribosyl)ation is supposed to have functions in DNA repair, cell growth, and differentiation (1-3). The cloning of cDNAs for PARP from mammals (5-10) opened the way for approaches at molecular level to determine the biological significance of poly(ADP-ribosyl)ation. PARP activity has been found in different phylogenetic classes, including non-vertebrates (1). Using antiserum against mammalian enzyme, immunoreactive polypeptides have also been found in vertebrates. Identification and characterization of PARP in several eukaryotes using above strategies seems to be difficult because of proteolysis and purity of the enzyme.

The functional analysis would be straightforward if cDNAs become available from lower vertebrates, like *Xenopus laevis* (*X. laevis*) and cherry salmon, in which the processes of oocyte

*The nucleotide sequences of cherry salmon PARP (Accession No. D13809) and Xenopus PARP (Accession No. D13810) are deposited to Genbank .

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Abbreviations used are: aa, amino acid(s); bp, base pair(s); kb, kilobase(s); PARP, poly(ADP-ribose) polymerase [EC 2.4.2.30]; PCR, polymerase chain reaction.

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maturation, spermatogenesis, fertilization, and development have been characterized. Our approaches on cloning of PARP cDNAs from frog, fishes, insects and yeast using mammalian cDNAs or antibody against human PARP had not been successful, probably due to the divergence in the nucleotide and aa sequences of PARP between mammals and non-mammals (unpublished data). In fact, mammalian and chicken PARP cDNAs have failed to give any specific cross-hybridization in the insect gene (11).

We report here the cloning procedures of partial cDNAs encoding PARP from different species by a modified PCR using mixed oligonucleotide primers, and using so obtained *Xenopus* and salmon cDNAs, we have analyzed their structural conservation and gene expression.

MATERIALS AND METHODS

Mixed oligonucleotide primers and PCR. Fig. 1 shows the location of oligonucleotide primers and probes used to amplify the cDNAs. These were designed according to the conserved aa sequences of mammalian PARP (3) as follows: primer 5, 5'-CA(AG)GCNAA(AG)GTNGA(AG)ATG-3'; primer 8, 5'-(GC)(AT)NGT(AG)TG(AG)CA(AG)TA(AG)TTNGC-3'; primer/probe 9, 5'-CC(TC)TTNCC(AG)AACAT(AG)TANCC-3'; probe 7, 5'-GA(TC)GTNAA(TC)TA(TC)GA(AG)AA(AG)(CT)T-3'. One μg quantities of cDNA were amplified for 35 cycles with 2.5 units of *Thermus aquaticus* (Taq) DNA polymerase (Perkin-Elmer Cetus) and 200 pmols of each oligonucleotide primer in total volume of 50 μl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 2.5 mM $MgCl_2$, 0.05% Tween 20, 200 μg/ml gelatin plus each dNTP at 500 μM and 50 μl of mineral oil (Sigma) (12). The samples were placed in an automated heating/cooling block (ATTO, Tokyo) programmed for a temperature-step cycle of 92°C (2 min), 55°C (7 min), and 72°C (2 min) with 7 min-extension at 72°C after the final cycle.

Southern blot hybridization of PCR products. PCR products were separated by electrophoresis in 2% agarose gel. Then the products were transferred to a Hybond-N^+ (Amersham) membrane with 0.4 N NaOH. The blots were hybridized with oligonucleotide probe end-labeled with [γ-^{32}P]ATP (>6,000 Ci/mmol, DuPont) at 25°C, 37°C and 42°C in a hybridization mixture containing 30% (v/v) formamide, 6x SSC, 0.1% sodium dodecyl sulfate, 5x Denhardts' solution (1x Denhardts'=0.02% (w/v) each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone) (13).

DNA sequencing of PCR products. A DNA fragment produced by PCR was recovered with DE81 paper after agarose gel electrophoresis. This PCR product was "blunt-ended" by DNA blunting kit (Takara, Kyoto), and the resulting "blunt-ended" DNA was subcloned into the *Sma*I site of pUC 118/119 (Takara, Kyoto). DNA sequencing was performed in phage M13 by the dideoxynucleotide chain termination method (14) using deoxycytidine 5'-[α-[^{35}S]thio] triphosphate (1,000-1,500 Ci/mmol, DuPont) and Sequenase (United States Biochemicals), as described by the manufacturer. All sequences were determined once or more for both strands of at least 5 plasmids.

Southern blot of PARP genes from vertebrates and non-vertebrates. High molecular weight DNAs were extracted from tissues of human, newt (*cynopus*), frog (*X. laevis*), cherry salmon, white salmon, Iwana fish (*Clupeiformes salvelinus*), Ayu fish (*Clupeiformes plecoglossus*), and fruit fly (*Drosophila melanogaster*). Five μg of DNA was digested with *Bam*HI, separated on a 0.8% agarose gel. The blots were hybridized with the [α-^{32}P]dCTP (3,000 Ci/mmol, DuPont) labeled PCR product from cherry salmon. Hybridization was carried out at 42°C in 50% (v/v) formamide, 6x SSC, 0.1% sodium dodecyl sulfate, 5x Denhardts' solution. Wash conditions were 2x SSC, 0.1% sodium dodecyl sulfate at 55°C.

Northern blot of PARP mRNA from *X. laevis* and cherry salmon. RNA was extracted from tissues by lithium chloride/urea method (15). Forty μg of RNA samples were denatured in a 50% (v/v) formamide and 6% (v/v) formaldehyde buffer at 65°C, resolved on a 1.2% agarose gel containing 6% (v/v) formaldehyde, and transferred to a Biodyne A nylon membrane (Paul BioSupport). Before blotting, quality of RNA was determined by ethidium bromide staining of

rRNA in agarose gel. Northern blots were prehybridized and hybridized to PCR products from *X. laevis* and cherry salmon labeled with ^{32}P as described (13).

RESULTS AND DISCUSSION

Since we failed to clone cDNA encoding PARP from lower eukaryotes other than mammals (frog, fishes, fruit fly, and yeast) by probing with human PARP cDNA or by anti-human PARP antibody screening of a expression library, we decided to isolate parts of cDNAs by heterologous oligonucleotide-primed amplification by PCR. Such redundant primers have been used successfully to isolate cDNA or members of multigene families (16). We employed this strategy to clone PARP cDNAs from diverse species. Among mammalian PARP cDNAs, the deduced aa sequences were conserved in the catalytic region, NAD-binding domain, in the carboxyl termini of PARP. So we chose several sequences that were well conserved among mammals, and designed the heterologous oligonucleotide primers and probes. From the published sequences of PARP (5-10), it would be expected that mixed oligonucleotide-primed amplification using primers 5 and 8 produces a 457 bp DNA fragment (Fig. 1).

A DNA fragment of about 460 bp was detected in *X. laevis*, cherry salmon and human cDNAs upon the amplification using primers 5 and 8 by ethidium bromide staining of gel (data not shown). The human cDNA was amplified in parallel as a control, since at least one of the primer sequences in mixed primers should match completely. Then we proved the specificity by Southern blot hybridization using mixed oligonucleotide probes. As shown in Fig. 2, the PCR products amplified with primers 5 and 8 hybridized with probe 9 which had the same sequence as primer 9. Under hybridization conditions of low stringency (25°C), the DNA fragments from each species hybridized with probe 9, but as the hybridization temperature was raised, hybridization signals from *Xenopus* and salmon cDNA were eliminated. At 42°C only human cDNA showed a positive signal. When primers 5 and 9 were used, about 400 bp fragment that would be expected was amplified in all three cDNAs. This PCR product was analyzed on Southern blot using probe 7 (Fig. 1), and the same results were obtained (data not shown). These results showed that the amplified fragment should represent a PARP cDNA from each species, and not be from human gene or cDNA. So, these PCR products were sequenced. It may be noted that when total genomic DNA was used as templates for PCR with our oligonucleotides as primers, we were unable to obtain sequences that proved specific for PARP after sequencing. Thus, the mixed oligonucleotide-primed PCR using cDNAs as templates may be a useful tool for cloning cDNA from evolutionarily different species with diverse sequences.

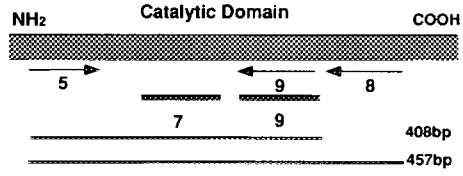

Fig. 1. Schematic representation of the location of oligonucleotide primers and probes used for PCR cloning. The solid box is corresponding to the catalytic domain of PARP. Primers and probes are indicated as arrows and bold lines, respectively.

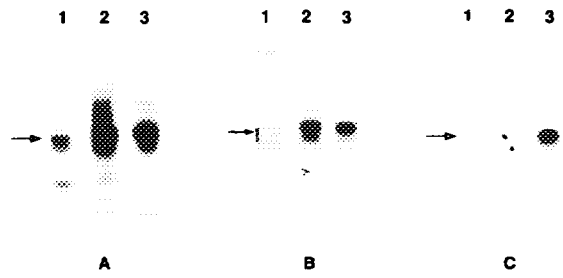

Fig. 2. Southern blot analysis of PCR products amplified with primers 5 and 8. The PCR products were hybridized to oligonucleotide probe 9 at 25°C (panel A), 37°C (panel B), and 42°C (panel C). Lane 1; products from cherry salmon cDNA library. Lane 2; products from *Xenopus* cDNA library. Lane 3; products from human cDNA library.

In this method, the mixed oligonucleotide primers do not necessarily contain the completely matched sequence in the target cDNAs. So we discuss the homology of sequences excluding the primer region. The sequences of the DNA fragments from *Xenopus* and salmon amplified with primers 5 and 9 are shown in Fig. 3. Each was a 405 bp fragment including primer sequences and had one open reading frame. The nucleotide sequences of partial cDNAs encoding *Xenopus* and salmon PARP have 73.5% and 72.4% identity, respectively, with that of human PARP cDNA (5) in the corresponding region. The nucleotide sequence homology between *X. laevis* and cherry salmon is 66.8%. These diversities might have affected hybridization efficiency in our first

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A GlnAlaLysValGluMetLeuAspAsnLeuLeuAspIleGluValAlaTyrSerLeuLeu  20
  CAAGCGAAGGTCGAGATGTTGGACAACCTACTGGACATTGAAGTGGCCTACAGTCTGCTG  60
  LysGlyGlyAlaGluAspAsnLysLysAspProIleAspIleAsnTyrGluLysLeuLys  40
  AAAGGAGGGGCCGAGGATAACAAGAAGGACCCTATCGACATCAACTATGAGAAACTCAAA 120
  ThrLysIleGluValValAspLysThrThrLysGluAlaGluIleIleLeuGlnTyrVal  60
  ACCAAGATTGAGGTTGTTGATAAGACCACAAAGGAGGCAGAGATCATTCTGCAGTATGTC 180
  LysAsnThrHisAlaAlaThrHisAsnThrTyrThrLeuValValGluGluIlePheLys  80
  AAGAACACACATGCTGCTACACACAACACCTACACACTGGTTGTTGAGGAGATCTTCAAG 240
  IleValArgGluGlyGluTyrGlnLysTyrArgProPheGlnAspLeuProAsnArgGln 100
  ATCGTTAGGGAGGGAGAGTACCAGAAGTACCGGCCCTTCCAGGATCTGCCCAATCGACAG 300
  LeuLeuTrpHisGlySerArgAlaThrAsnTyrAlaGlyIleLeuSerGlnGlyLeuArg 120
  CTTCTGTGGCACGGATCTCGTGCCACCAACTACGCTGGTATCCTTTCTCAGGGTCTGCGT 360
  IleAlaProProGluAlaProValThrGlyTyrMetPheGlyLys     135
  ATCGCCCCTCCTGAAGCCCCCGTGACGGGCTACATGTTCGGCAAG     405

B GlnAlaLysValGluMetLeuAspAsnLeuLeuAspIleGluValAlaTyrSerLeuLeu  20
  CAGGCAAAGGTGGAGATGCTGGATAATCTGCTCGACATTGAAGTTGCTTACAGCCTGTTG  60
  ArgGlyGlyAlaAspAspGlyGluLysAspProIleAspValLysTyrGluLysIleLys  40
  AGAGGTGGCGCCGATGATGGTGAAAAGGATCCCATTGATGTGAAATATGAAAAGATTAAG 120
  ThrAspIleLysValValAlaLysAspSerGluGluSerArgIleIleCysAspTyrVal  60
  ACTGACATTAAGGTTGTTGCTAAAGATTCAGAAGAATCCAGAATTATATGCGATTATGTC 180
  LysAsnThrHisAlaAspThrHisAsnAlaTyrAspLeuGluValLeuGluIlePheLys  80
  AAGAACACGCACGCTGATACGCACAATGCATATGATCTTGAGGTCCTCGAGATATTCAAA 240
  IleAspArgGluGlyGluTyrGlnArgTyrLysProPheLysGlnLeuHisAsnArgGln 100
  ATCGACCGTGAAGGTGAATATCAGCGGTATAAACCATTTAAACAGCTACACAACCGCCAG 300
  LeuLeuTrpHisGlySerArgThrThrAsnPheAlaGlyIleLeuSerGlnGlyLeuArg 120
  CTGCTTTGGCACGGCTCCCGCACCACGAATTTTGCAGGAATATTGTCTCAGGGTCTCCGA 360
  IleAlaProProGluAlaProValThrGlyTyrMetPheGlyLys     135
  ATTGCTCCGCCAGAAGCTCCTGTTACCGGCTATATGTTCGGCAAG     405
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Fig. 3. Nucleotide and deduced aa sequences of cDNAs for cherry salmon (A) and *X. laevis* (B) PARP corresponding to the catalytic domain. The underline indicates the position of primer. The numbers represent the arbitrary ones of nucleotide and aa sequences starting from the 1st codon in primer 5.

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Human     *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  778
Salmon    GlnAlaLysValGluMetLeuAspAsnLeuLeuAspIleGluValAlaTyrSerLeuLeu
Xenopus   GlnAlaLysValGluMetLeuAspAsnLeuLeuAspIleGluValAlaTyrSerLeuLeu
Bovine    *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
Rat       *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
Mouse     *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
Chicken   *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

Human     Arg *  * SerAsp * SerSer *  *  *  *  * ValAsn *  *  * Leu *  798
Salmon    LysGlyGlyAlaGluAspAsnLysLysAspProIleAspIleAsnTyrGluLysLeuLys
Xenopus   ArgGlyGlyAlaAspAspGlyGluLysAspProIleAspValLysTyrGluLysIleLys
Bovine    Arg *  * SerAsp * SerSer *  *  *  *  * ValAsn *  *  * Leu *
Rat       Arg *  * SerAsp * SerSer *  *  *  *  * ValAsn *  *  * Leu *
Mouse     Arg *  * SerAsp * SerSer *  *  *  *  * ValAsn *  *  * Leu *
Chicken   Arg *  * AsnAsp * GlyAsp *  *  *  *  * IleAsn *  *  * LeuArg

Human      * AspThrLys *  * AspArgAspSerGlu * AlaGlu *  * ArgLys *  *  818
Salmon    ThrLysIleGluValValAspLysThrThrLysGluAlaGluIleIleLeuGlnTyrVal
Xenopus   ThrAspIleLysValValAlaLysAspSerGluGluSerArgIleIleCysAspTyrVal
Bovine     * AspThrLys *  * AspLysAspSerGlu * AlaGlu *  * ArgLys *  *
Rat        * AspThrLys *  * AspArgAspSerGlu * AlaGluVal * ArgLys *  *
Mouse      * AspThrLys *  * AspArgAspSerGlu * AlaGluVal * ArgLys *  *
Chicken    * AspThrLys *  * Asp * AspSerGlu * AlaLys *  * LysGln *  *

Human      *  *  *  * ThrThr *  *  * Ala * Asp * Glu * IleAsp * *  *   838
Salmon    LysAsnThrHisAlaAlaThrHisAsnThrTyrThrLeuValValGluGluIlePheLys
Xenopus   LysAsnThrHisAlaAspThrHisAsnAlaTyrAspLeuGluValLeuGluIlePheLys
Bovine     *  *  *  *  * Thr *  *  * Ala * Asp * Glu * ValAsp *  *  *
Rat        *  *  *  * ThrThr *  *  * Ala * Asp * Glu * IleAsp *  *  *
Mouse      *  *  *  * ThrThr *  *  * Ala * Asp * Glu * IleAsp *  *  *
Chicken    *  *  *  * AlaThr *  *  * Ala * Asp * Lys * Val *  *  *  *

Human      * Glu *  *  * GluCys * Arg * Lys *  * LysGln * His *  * Arg 858
Salmon    IleValArgGluGlyGluTyrGlnLysTyrArgProPheGlnAspLeuProAsnArgGln
Xenopus   IleAspArgGluGlyArgTyrGlnArgTyrLysProPheLysGlnLeuHisAsnArgGln
Bovine     * Glu *  *  * GluSer * Arg * Lys *  * LysGln * His *  * Arg
Rat        * Glu *  *  * GluSer * Arg * Lys *  * ArgGln * His *  * Arg
Mouse      * Glu *  *  * GluSer * Arg * Lys *  * ArgGln * His *  * Arg
Chicken    * Glu *  *  * GluSer * Arg * Lys *  * LysGln * His *  * Gln

Human      *  *  *  *  *  *  * Thr *  * Phe *  *  *  *  *  *  *  *   * 878
Salmon    LeuLeuTrpHisGlySerArgAlaThrAsnTyrAlaGlyIleLeuSerGlnGlyLeuArg
Xenopus   LeuLeuTrpHisGlySerArgThrThrAsnPheAlaGlyIleLeuSerGlnGlyLeuArg
Bovine     *  *  *  *  *  *  * Thr *  * Phe *  *  *  *  *  *  *  *  *
Rat        *  *  *  *  *  *  * Thr *  * Phe *  *  *  *  *  *  *  *  *
Mouse      *  *  *  *  *  *  * Thr *  * Phe *  *  *  *  *  *  *  *  *
Chicken    *  *  *  *  *  *  * Thr *  * Phe *  *  *  *  *  *  *  *  *

Human      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  893
Salmon    IleAlaProProGluAlaProValThrGlyTyrMetPheGlyLys
Xenopus   IleAlaProProGluAlaProValThrGlyTyrMetPheGlyLys
Bovine     *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
Rat        *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
Mouse      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
Chicken    *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
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Fig. 4. Comparison of deduced aa sequences of PARPs from cherry salmon and *X. laevis*, with those of chicken, mouse, rat, bovine, and human. Only the differences from the sequence of cherry salmon PARP are indicated in the chicken, mouse, rat, bovine, and human sequences. The aa sequences corresponding to the primers are underlined. The numbers represent the aa sequence of human PARP.

attempt to clone these cDNAs by cross-hybridization using human cDNA as a probe.

The comparison of deduced aa sequences of the catalytic domains among salmon, *Xenopus*, chicken, rat, mouse, bovine, and human PARP is summarized in Fig. 4. Sequence of *Xenopus* PARP was 84.4% identical to that of human PARP. The similarity of the deduced aa sequence between salmon and human PARP is 77.0%, that is lower than that between *X. laevis* and human. Salmon PARP has substitutions of 14 aa residues which are conserved among other species. The aa homology between *X. laevis* and salmon is 75.6%. It is of great interest that the conservation of aa sequences within the NAD-binding domain is found not only among mammals, but also apparently in fish and amphibia. Especially the carboxyl terminal region (aa 855-886) exhibits extensive similarity. This conserved region is presumed to be a part of the structure involved in dinucleotide interaction.

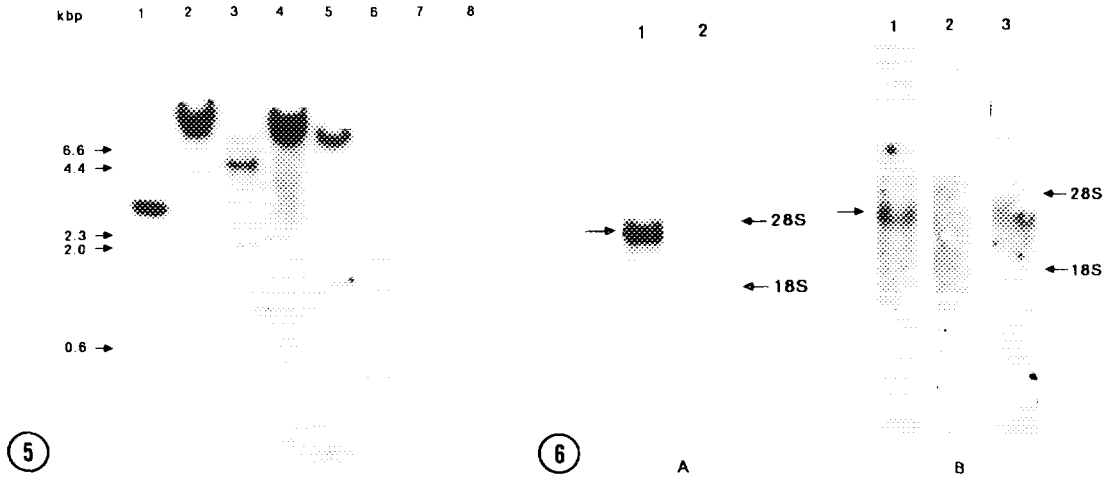

Fig. 5. Southern blot analysis of PARP gene from vertebrates and non-vertebrates probed by cherry salmon PCR product amplified with primers 5 and 9. Lane 1, *D. melanogaster*; Lane 2, *C. salvelinus*; Lane 3, *C. plecoglossus*; Lane 4, white salmon; lane 5, cherry salmon; Lane 6, *X. laevis*; Lane 7, *cynopus*; Lane 8, human.

Fig. 6. Northern blot analysis of PARP mRNA from *X. laevis* and cherry salmon. RNA was extracted from immature oocytes and liver of *X. laevis*, and ovary at mature stage, brain, and liver of cherry salmon. Northern blots were hybridized to PCR products from *X. laevis* (panel A) and cherry salmon (panel B) amplified with primers 5 and 9. Panel A: lane 1, oocytes; lane 2, liver. Panel B: lane 1, brain; lane 2, liver; lane 3, ovary.

On Southern blotting, cherry salmon PCR product hybridized to genomic DNA from fruit fly (*D. melanogaster*), and fishes (*C. salvelinus, C. plecoglossus*, and salmons) (Fig. 5). But no cross-hybridization was observed in human, newt, or frog DNA. When *Xenopus* PCR product was used as a probe, only *Xenopus* DNA gave hybridization signal (data not shown). Thus it appeared that salmon PARP sequence is evolutionarily closely related to insects.

Fig. 6 shows Northern blot analysis using total RNA from ovary, oocytes, brain and liver from *X. laevis* and cherry salmon. The amplified DNA fragments from *Xenopus* and salmon consistently hybridized to an mRNA species of about 4 kb. In ovary, oocytes, and brain, mRNA for PARP was abundant, whereas it was sparse in liver. It should be mentioned that the high level expression in *Xenopus* oocytes was observed. The mRNA species that would be required during oogenesis and embryonic development should accumulate in the oocytes. Accumulation of PARP mRNA during oocyte maturation might also be required for oogenesis and embryonic development.

Recently, we cloned the cDNA homologous to mammalian PARP from *D. melanogaster* (17), and genetical analysis is in progress. In *X. laevis* and fish, micro-injection of RNA or antibody into oocytes has been established. PARP cDNAs cloned from *X. laevis* and cherry salmon are useful as tools to study the functions of poly(ADP-ribosyl)ation in oocytes maturation, fertilization, and development.

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