The genes *pme-1* and *pme-2* encode two poly(ADP-ribose) polymerases in *Caenorhabditis elegans*

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Poly(ADP-ribose) polymerases (PARPs) are an expanding, well-conserved family of enzymes found in many metazoan species, including plants. The enzyme catalyses poly(ADP-ribosyl)ation, a post-translational modification that is important in DNA repair and programmed cell death. In the present study, we report the finding of an endogenous source of poly(ADP-ribosyl)ation in total extracts of the nematode *Caenorhabditis elegans*. Two cDNAs encoding highly similar proteins to human PARP-1 (huPARP-1) and huPARP-2 are described, and we propose to name the corresponding enzymes poly(ADP-ribose) metabolism enzyme 1 (PME-1) and PME-2 respectively. PME-1 (108 kDa) shares 31 % identity with huPARP-1 and has an overall structure similar to other PARP-1 subfamily members. It contains sequences having considerable similarity to zinc-finger motifs I and II, as well as with the catalytic domain of huPARP-1. PME-2 (61 kDa) has structural similarities with the catalytic domain of PARPs in general and shares 24 % identity with huPARP-2. Recombinant PME-1 and PME-2 display PARP activity, which may partially account for the similar activity found in the worm. A partial duplication of the *pme-1* gene with pseudogene-like features was found in the nematode genome. Messenger RNA for *pme-1* are 5′-tagged with splice leader 1, whereas those for *pme-2* are tagged with splice leader 2, suggesting an operon-like expression for *pme-2*. The expression pattern of *pme-1* and *pme-2* is also developmentally regulated. Together, these results show that PARP-1 and -2 are conserved in evolution and must have important functions in multicellular organisms. We propose using *C. elegans* as a model to understand better the functions of these enzymes.

Key words: development, gene expression, post-translational modification, worm.

INTRODUCTION

Poly(ADP-ribosyl)ation is an important post-translational modification (for review see [1–3]), which is thought to regulate many nuclear functions, including DNA repair, chromatin structure, DNA synthesis and programmed cell death [2]. Poly(ADP-ribose) metabolism involves two major enzyme categories: poly(ADP-ribose) polymerases (PARPs) and poly(ADP-ribose) glycohydrolase (PARG) [4]. The PARP family of enzymes includes the canonical PARP-1, as well as some recently discovered, structurally divergent members, including PARP-2 [5], PARP-3 [6], VPARP [7], tankyrase-1 and -2 [8,9] and TiPARP [10]. An alternative form of PARP-1, short PARP-1, has also been identified [11]. The potential roles of these enzymes are currently under investigation.

PARP-1 (EC 2.4.2.30) is a 116 kDa nuclear enzyme composed of three functional domains: an N-terminal DNA-binding domain containing two zinc-finger motifs (I and II), a central automodification domain and a C-terminal catalytic domain containing an active site termed the PARP signature motif [12]. PARP-2, a 62 kDa enzyme, also modular and found in the nucleus, is composed of an N-terminal DNA-binding domain and a C-terminal PARP signature motif [5]. Both PARP-1 and PARP-2 synthesize poly(ADP-ribose) from the substrate NAD^+ and covalently attach the growing polymer to glutamic residues of acceptor proteins [5,13]. Their enzymic activities are stimulated by DNA strand breaks, suggesting a biological role in the cellular response to DNA damage. In fact, mice and cells lacking PARP-1 display hypersensitivity to ionizing radiation [14]. The precise role of PARP-2 in DNA repair has not yet been defined; however, the structure of its DNA-binding domain is different from PARP-1 and may indicate a distinct mechanism of activation.

Poly(ADP-ribose) polymers may contain as many as 100 residues, arranged linearly and containing branched portions [15]. Poly(ADP-ribose) is covalently linked by its first residue to various nuclear proteins, including PARPs, topoisomerases and histones (the major *in vivo* acceptors) [2], but it can also interact non-covalently with the latter and other proteins as well [16,17]. The modified acceptor proteins undergo distinct changes in function, including transient inhibition of enzymic activity and modifications of architectural function. The latter, in the case of histones, causes chromatin decondensation [2]. Poly(ADP-ribose) is transiently present in the cell and is readily degraded by PARG [18,19], which exhibits both endoglycosidic and exoglycosidic activities [20,21]. In mammals, PARG is represented by a unique cytoplasmic protein that can be rapidly shuttled to the nucleus where its substrate is localized [22].

The presence of PARP enzymes has been reported up the evolutionary scale in species ranging from simple slime moulds [23] to mammals, including humans [24]; to study further poly(ADP-ribose) metabolism, we chose to investigate the presence of PARP enzymes in the nematode *Caenorhabditis elegans*. One reason, in particular, for doing so is that this well-characterized worm species has had its entire genome sequenced [25] and thus provides an excellent source for the search of

Abbreviations used: IPTG, isopropyl β-D-thiogalactoside; ORF, open reading frame; PARG, poly(ADP-ribose) glycohydrolase; PARP, poly(ADP-ribose) polymerase; huPARP, human PARP; PME-1, poly(ADP-ribose) metabolism enzyme 1; RT, reverse transcriptase; SL, splice leader; UTR, untranslated region; ZF, zinc finger.

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The nucleotide sequence data for *pme-1* and *pme-2* have been submitted to the GenBank® Nucleotide Sequence Database under the accession numbers AF499444 and AF500111 respectively.

proteins containing the PARP signature motif. The present study describes the characterization of genes encoding two proteins that display PARP activity from the nematode *C. elegans*. Although the potential existence of PARP in *C. elegans* has been mentioned elsewhere [26,27], we report here a thorough analysis of genes, the mRNAs and the encoded proteins. This is the first time that an endogenous source of poly(ADP-ribosyl)ation has been found and characterized at the gene level in the nematode *C. elegans*.

EXPERIMENTAL

C. elegans culture and extracts

Worms were handled and cultured as described previously [28]. Animals were grown at 20 °C on agar plates seeded with *Escherichia coli* strain OP50. For liquid cultures, worms were first grown on ten 9 cm plates until bacteria were cleared from the surface (usually 3 days). The worms were then transferred to 1 litre S basal medium [0.1 M NaCl, 0.05 M KH_2PO_4/K_2HPO_4 (pH 6), 5 μg/ml cholesterol, 0.01 M potassium citrate (pH 6), 0.05 mM EDTA, 0.025 μM $FeSO_4 \cdot 7H_2O$, 0.01 μM $MnCl_2 \cdot 4H_2O$, 0.01 μM $ZnSO_4 \cdot 7H_2O$, 0.001 μM $CuSO_4 \cdot 5H_2O$, 3 mM $CaCl_2$, 3 mM $MgSO_4$] supplemented with 7 g of *E. coli* strain NA22 paste. Cultures were continuously shaken at 250 rev./min for 4–5 days. Worms were then harvested in 200 ml centrifuge bottles, put on ice for 30 min and centrifuged for 5 min at 300 ***g*** at 4 °C. The resulting pellet (usually 5–10 ml) was washed twice with M9 buffer (22 mM KH_2PO_4/42 mM Na_2HPO_4/86 mM NaCl/1 mM $MgSO_4$). The worms were finally washed with 0.1 M NaCl and then resuspended in 20 ml of 0.1 M NaCl, mixed with 20 ml of 60 % (w/v) sucrose and centrifuged for 3 min at 300 ***g*** at 4 °C. Floating worms were recovered and washed twice in 0.1 M NaCl.

For the worm extract, freshly washed worms (5 ml) were resuspended in 10 ml of extraction buffer [100 mM Tris/HCl (pH 8), 10 mM $MgCl_2$, 10 % (v/v) glycerol, 1.5 mM dithiothreitol] and passed twice at 8000 p.s.i. (1 p.s.i. = 6.9 kPa) in a French pressure cell (SLM-AMINCO®; Rochester, NY, U.S.A.) maintained at 4 °C. The extract was centrifuged for 10 min at 1000 ***g*** at 4 °C. The protein concentration of the supernatant was determined by the method of Bradford [29], and the supernatant was assayed for PARP activity.

Staged worm culture

Worms from a 1 litre culture were cleaned by sucrose flotation, and the resulting pellet was treated with 40 ml of hypochlorite alkaline solution [143 mM NaOH, 1.4 % (w/v) sodium hypochlorite] for approx. 5 min with vortex-mixing every 1 min. The mixture was then passed twice through an 18G needle. The eggs were quickly centrifuged and washed three times with 50 ml of M9 buffer, transferred to 1 litre S medium (without food) and allowed to hatch overnight with shaking at 250 rev./min at 20 °C. The L1 larvae were supplemented with strain NA22 bacteria and collected 2 h later by centrifugation at 300 ***g***. L2, L3 and L4 larvae were collected after 12, 24 and 36 h respectively, and young adults after 48 h. Each worm pellet was flash-frozen in liquid nitrogen and stored at −75 °C.

Cloning of cDNAs encoding *C. elegans* PARP-1 and -2

Reverse transcriptase (RT)–PCR was done with the pre-amplification system for first-strand cDNA synthesis (SuperScript II kit; Gibco BRL). Total RNA was prepared as described below. First-strand synthesis was performed using oligo(dT)12–18 oligonucleotides. To amplify full-length *pme-1* cDNA [potential human PARP-1 (huPARP-1) homologue] from first-strand synthesis, specific oligonucleotides based on the putative Y71F9AL.18 gene sequence were used. The forward primer was ATCGATCGGAGCTCATGATTCATTCCAACGAGCCA (predicted initiation codon underlined) containing a *Sac*I restriction site, and the reverse primer was CTTTAGCTGATATTCTATTTGAGTCGACCTAGCTGAT (predicted termination codon underlined) containing a *Sal*I restriction site. After amplification (94 °C, 30 s; 55 °C, 30 s; 72 °C, 2 min 30 s; 35 cycles with Pwo DNA polymerase), the PCR product (2856 bp) was gel-purified (Qiaquick gel extraction kit; Qiagen), digested by *Sac*I–*Sal*I restriction enzyme and cloned in a pQE-31 expression vector. The construction was named pQE-AME-2 and was sequenced using Dye Terminator Kit on ABI automated sequencer 373A. Alternatively, an expressed sequence tag (yk399b10) encoding the full-length *pme-1* cDNA was obtained from Dr Yuji Kohara Laboratory (Japan). The phagemid yk399b10 was excised and circularized using a standard method [30], and the resulting plasmid was named pYK399b10. The primary nucleotide sequence of the insert of pYK399b10 was confirmed by automated sequencing and was found to be identical with the sequence of *pme-1* cDNA found in pQE-AME-2. The *pme-1* cDNA from pYK399b10 was placed into pQE-30 expression vector using the same method as stated above. The resulting construct was named pQE-PME-1 [PME-1, poly(ADP-ribose) metabolism enzyme 1], and the recombinant protein produced with this construct was named His_6-PME-1. *E. coli* M15-pREP4 bacteria were transformed with pQE-PME-1. To amplify the full-length *pme-2* cDNA (potentially a huPARP-2 homologue) from first-strand synthesis, specific oligonucleotides based on the putative E02H1.4 gene sequence were used. The forward primer was GACAGAAGGTACCATGTCTATAATCAACGACGA (predicted initiation codon underlined) containing a *Kpn*I restriction site, and the reverse primer was TGAGAAATTAGCATCTGCAGTTAAGCCATCATTTCCTTAG (predicted termination codon underlined) containing a *Pst*I restriction site. After amplification (94 °C, 45 s; 52 °C, 45 s; 72 °C, 3 min; 25 cycles with *Taq* DNA polymerase), the PCR product (1644 bp) was gel-purified (Qiaquick gel extraction kit), digested with *Kpn*I–*Pst*I restriction enzymes and cloned in pQE-30 expression vector. The construction was named pSD1.2 and was sequenced by automated sequencing. The recombinant protein produced with this construct was named His_6-PME-2. *E. coli* M15-pREP4 bacteria were transformed with this expression plasmid. Alternatively, a random-primed ^{32}P-labelled probe using the PCR-amplified cDNA (1644 bp) was used to screen a *C. elegans* mixed-stage cDNA library (gift from R. Barstead, Oklahoma Medical Research Foundation, Oklahoma City, OK, U.S.A.) using standard methods [30]. Approximately 300000 plaques were screened and 39 positively hybridizing plaques were picked. pBluescript SK phagemid vectors containing the cDNA inserts were subsequently isolated (Stratagene). The inserts of three of these clones, which appeared to contain identical insertions of 1.8 kb after *Bam*HI–*Xho*I restriction digestion, were sequenced by automated sequencing.

huPARP-1 cDNA

huPARP-1 cDNA was kindly provided by Dr Alexandre Bürkle (Deutsches Krebsforschungszentrum, Germany) as an eukaryotic expression construct named pPARP31. pPARP31 was subjected to site-directed mutagenesis, replacing an aspartate residue with an alanine residue at position 214 (Quickchange kit; Stratagene), digested with *Sma*I–*Asc*I, gel-purified (Qiaquick; Qiagen) and

treated with DNA polymerase (Klenow fragment). The resulting DNA fragment contained a deletion of the first 17 codons of the original full-length huPARP-1 cDNA. It was introduced into the bacterial expression vector pQE-30 digested with *Sma*I restriction enzyme, generating a construction named pSD6.3. The bacteria *E. coli* XL1-Blue-MRF′ were transformed with pSD6.3.

PCR amplification of the 5′-terminal regions

To determine the trans-splicing nature of PME-1 and PME-2 messengers, as well as their true 5′-end, PCR was performed on an oligo(dT)-primed cDNA library made from polyadenylated [poly(A)$^+$] RNA from mixed-stage worm culture. A PCR amplification of the predicted 5′-end of *pme-1* was done using a gene-specific forward primer (ATGATTCATTCCAACGAGCCA), with predicted initiation codon underlined, and a reverse gene-specific primer (CCTCCACATACTCCCCAACCG) generating a PCR product of 568 bp. Two other separate amplifications were done using splice leader 1 (SL-1) primer (GTTTAATTACCCAAGTTTGAG) or SL-2 primer (GGTTTTAACCCAGTTACTCAAG) as forward primers and the same gene-specific reverse primer. The PCR sequencing was a 'two-step'-type amplification: first, a denaturing step at 94 °C for 90 s; secondly, a two-step sequence of 94 °C for 10 s, followed immediately by a step at 55 °C for 10 s repeated 25 times and a final step at 72 °C for 5 min. The same method was used for *pme-2*: a PCR amplification of the predicted 5′-end of *pme-2* was done using a gene-specific forward primer (GACAGAAGGTACCATGTCTATAATCAACGACGA), with predicted initiation codon underlined, and a gene-specific reverse primer (TGAGAAATTAGCATCTGCAGTTAAGCCATCATTTCCTTAG) generating a PCR product of 1647 bp. Two other separate amplifications were done using the SL-1 primer or SL-2 primer as forward primers and the same gene-specific reverse primer. The PCR sequence was as follows: 94 °C for 90 s, a sequence of three steps consisting of 94 °C for 30 s, 48 °C for 30 s and 72 °C for 120 s repeated 35 times and a final step at 72 °C for 5 min. PCR products generated by this method were excised from agarose gels and sequenced using automated sequencing.

RNA preparation and Northern-blot analysis

Total RNA was prepared from frozen worm pellets by first pulverizing them in a mortar with liquid nitrogen and then homogenizing the resulting powder using a Polytron in a sonicating buffer [500 mM NaCl/200 mM Tris/HCl (pH 7.5)/10 mM EDTA/1 % SDS/100 mM 2-mercaptoethanol] and extracted once with an equal volume of phenol (pH 4.3). The RNA in the aqueous phase was extracted 3–5 times with an equal volume of phenol/chloroform/3-methylbutan-1-ol (125:24:1, by vol.), pH 4.3. The aqueous phase was then combined with 0.1 vol. of 3 M sodium acetate (pH 5.2) and 2 vol. of 100 % ethanol, mixed and stored at −75 °C. Poly(A)$^+$ RNA was extracted from total RNA by a poly(dT) column using an mRNA purification kit (Pharmacia, Piscataway, NJ, U.S.A.). Poly(A)$^+$ RNA (approx. 2 μg) was loaded on to a 1 % agarose gel containing 2.2 M formaldehyde and transferred to a Nytran membrane (Schleicher and Schuell, Keene, NH, U.S.A.) following standard methods [30]. One membrane was probed with the ^{32}P-labelled *pme-1* cDNA *Hin*dIII fragment corresponding to nucleotides 260–1141, and the other blot was probed with *pme-2* cDNA fragment corresponding to nucleotides 656–1621. After 2 weeks, both membranes were probed with *cyt-1* cDNA *Eco*RI fragments as a loading control [31]. These fragments share no sequence similarity to *pme-1* and *pme-2*.

Bacterial cultures and extracts

Cultures of M15/pREP4 bacteria transformed with pQE-PME-1, M15/pREP4 transformed with pSD1.2 and XL1-Blue-MRF′ transformed with pSD6.3 were grown overnight at saturation at 37 °C. Fresh cultures (400 ml) were started with 5 ml overnight cultures and grown at 37 °C to A_{600} 0.600. Cultures were then induced with 1 mM isopropyl β-D-thiogalactoside (IPTG) at 37 °C for 4 h, except for M15/pREP4 + pQE-PME-1, which was induced at 20 °C, and XL1-Blue-MRF′ + pSD6.3 for 3.5 h. Untransformed bacteria were also treated with 1 mM IPTG at 37 °C for 4 h (M15/pREP4) and 3.5 h (XL1-Blue-MRF′). Cultures were pelleted at 3000 ***g*** for 10 min at 4 °C, resuspended in 10 ml extraction buffer and passed twice at 16000 p.s.i. in a French Pressure Cell (SLM-AMINCO®) maintained at 4 °C. Extracts were centrifuged for 10 min at 1000 ***g*** at 4 °C, and the protein concentration of supernatants was determined [29]. Each supernatant was analysed by SDS/PAGE and assayed for PARP activity.

Determination of poly(ADP-ribose) synthesis in worm and bacterial extracts

PARP activity was measured as described earlier [32,33] with modifications: samples assayed (70 μl) were mixed with 70 μl of 2× reaction buffer [200 mM Tris/HCl (pH 8.0), 20 mM $MgCl_2$, 20 % (v/v) glycerol, 3 mM dithiothreitol, 400 μM NAD], 10 μl of DNase I-activated DNA (1 mg/ml) and 1 μl of [^{32}P]NAD (1000 Ci/mmol; 10 mCi/ml; Amersham Pharmacia Biotech). Reactions were left for 30 min at 30 °C and then terminated with the addition of 649 μl of 100 mM Tris/HCl (pH 8.0), 100 μl of 3 M NaOAc (pH 5.2) and 700 μl of propan-2-ol. Samples were kept on ice for 30 min and centrifuged at 16000 ***g*** for 10 min at 4 °C. Pellets were washed twice with 100 % ethanol and dissolved at 60 °C in 1 ml of 1 M KOH, 50 mM EDTA for 1 h.

Dihydroxyboronyl Bio-Rex affinity chromatography and polyacrylamide gel analysis

Dihydroxyboronyl Bio-Rex resin was synthesized as described previously [33,34]. Chromatography was performed as described by Shah et al. [33] except for the elution, which was done with 5 ml of water at 37 °C. The eluate was desiccated for 16 h and the residual pellets (10–30 μl) were dissolved in 40 μl of loading buffer [50 % urea/25 mM NaCl/4 mM EDTA (pH 7.5)/0.02 % xylene cyanol/0.02 % Bromophenol Blue]. Electrophoresis was performed on a 20 cm × 20 cm × 0.15 cm 20 % (w/v) polyacrylamide gel (polyacrylamide/bisacrylamide, 1986:24) in Tris/borate/EDTA [0.09 M Tris/0.09 M boric acid/2 mM EDTA (pH 8.3)] at 60 V until Bromophenol Blue had reached 12 cm from the origin. The gel was dried and exposed.

Computer analysis

The deduced amino acid sequences of huPARP-1 (accession no. P09874) signature domain (amino acids 859–908; Figure 2B) and bovine PARG (accession no. U78975) were used to search the *C. elegans* sequence database at the Sanger Centre and the *C. elegans* expressed sequence tag database (Dr Yuji Kohara, Genome Biology Laboratory, National Institute of Genetics, Mishima, Japan). Database searches were done using the TBLASTN of the BLAST program [35]. Analyses of sequence data and sequence comparisons were performed using the CLUSTALW program of the Lasergene package (DNASTAR, Madison, WI, U.S.A.).

RESULTS

Cloning of the full-length *pme-1* and *pme-2* cDNAs in *C. elegans*

We searched the *C. elegans* protein data bank for the major enzymes involved in the metabolism of poly(ADP-ribose), i.e. PARPs and PARG. A search made with the huPARP-1 signature motif LLWHGSRTTNFAGILSQGLRIAPPEAPVTGYMFG-KGIYFADMVSKSANYY returned three loci: Y71F9AL.18, AC8.1 and E02H1.4 (Figure 1) with putative gene products sharing significant sequence similarity to PARP-1 and -2. A fourth locus ZK1005.1, encoding a putative tankyrase, was also found and is currently under investigation. Two loci (H23L24.5 and F20C5.1) were found to encode putative PARGs (characterization of these is currently in progress and will be reported later). Together, these genes form what we call PMEs. The present study focuses on the characterization of loci Y71F9AL.18 and E02H1.4.

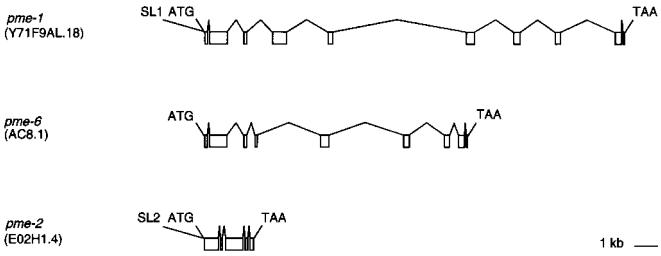

Figure 1 Schematic representation of genomic organization of *pme-1*, *pme-6* and *pme-2* genes

Start codon (ATG), stop codon (TAA) and SL are indicated. Open boxes indicate exons.

Locus Y71F9AL.18, on chromosome I, spans over 16.6 kb and contains 10 exons (Figure 1). The full-length cDNA sequence contains 3009 nucleotides, including a 5′-untranslated region (5′-UTR) consisting of 130 nucleotides. The four nucleotides, CCGG upstream of the initiation codon ATG, do not match the vertebrate consensus sequence or the *C. elegans* consensus sequence [36] for translational initiation. There is an in-frame stop codon in the 33-nucleotide region upstream of the transla-

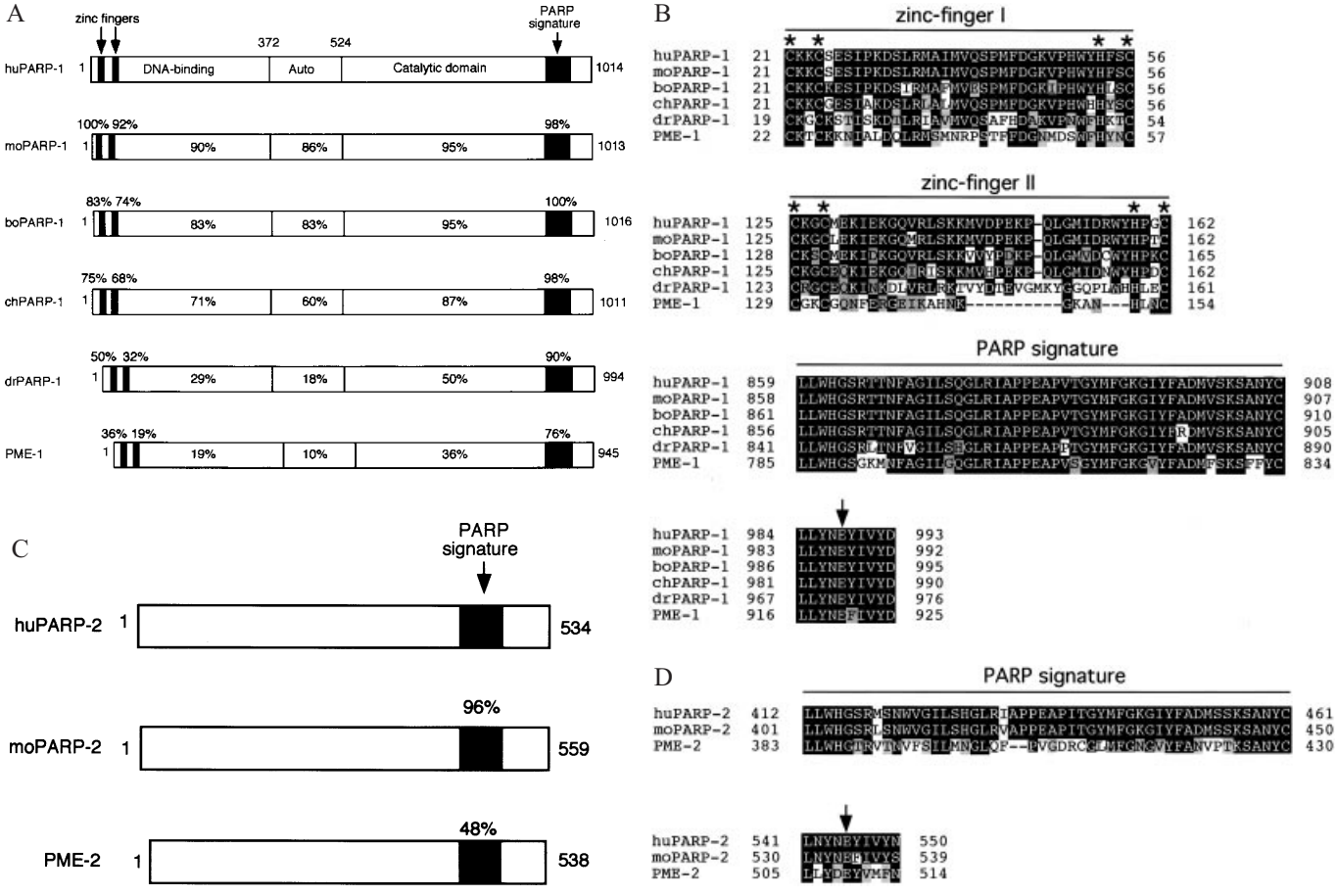

Figure 2 PME-1 and PME-2 are structurally related to higher-eukaryote PARP-1 and PARP-2 respectively

PARP-1 is a modular enzyme containing three functional domains: DNA-binding, automodification (Auto) and a catalytic domain. It also contains special features such as zinc-finger motifs (I and II) and a highly conserved motif in the catalytic domain called the PARP signature. (**A**) Schematic primary structures of PME-1 and various orthologues [huPARP-1, accession no. P09874; mouse PARP-1 (moPARP-1, accession no. P11103); bovine PARP-1 (boPARP-1, accession no. P18493); chicken PARP-1 (chPARP-1; accession no. P26446); *Drosophila* PARP-1 (drPARP-1; accession no. P35875); and PME-1 (accession no. AF499444)]. (**B**) Alignment of the deduced amino acid sequences of PME-1 and various orthologues. The ★ indicates amino acids involved in Zn^{2+} co-ordination, the ↓ indicates E988 as a conserved amino acid involved in the polymerization step of poly(ADP-ribosyl)ation [37]. (**C**) Schematic primary structures of PME-2 (accession no. AF500111) and orthologues huPARP-2 (accession no. Q9UMR4) and moPARP-2 (accession no. O88554). (**D**) Alignment of the deduced amino acid sequences of PME-2 and various orthologues. The ↓ indicates a glutamate residue that plays a similar role to E988 in PME-1. Numbers indicate positions of amino acid residues. The percentage indicates identity with huPARP-1 or huPARP-2 functional domains and with motifs such as zinc-finger motifs I and II and the PARP signature. Amino acids that are identical are boxed in black. Conserved substitutions are indicated in grey.

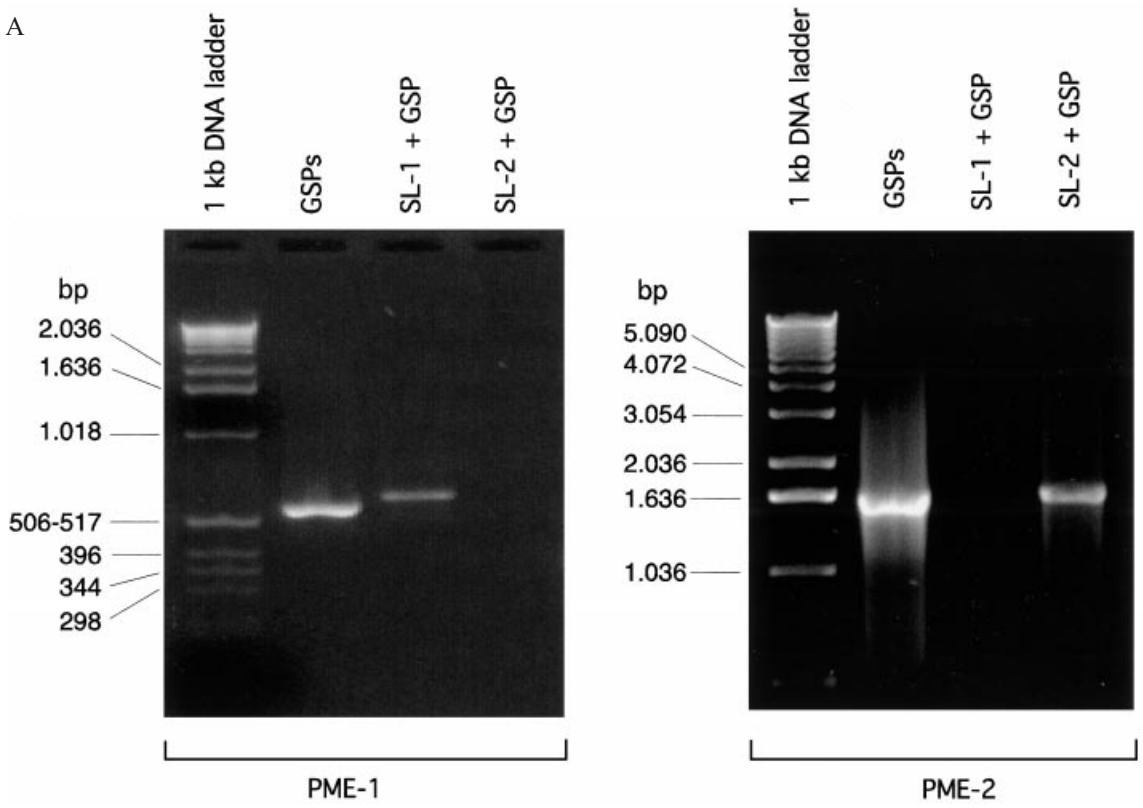

Figure 3 PME-1 and PME-2 transcripts are trans-spliced by SL-1 and SL-2 splicing factors

(**A**) RT–PCR amplification of 5′-end of PME-1 and PME-2 transcripts. A pair of gene-specific primers (GSPs) for PME-1 and PME-2 were selected to yield 568 and 1647 bp PCR products for PME-1 and PME-2 respectively. A second and a third PCR amplification using SL-1 and SL-2 primers as forward primers and the same reverse GSP for PME-1 and PME-2 yielded 609 and 1646 bp products for SL-1/PME-1 and SL-2/PME-2 respectively. (**B**) Sequence determination of PCR products SL-1/PME-1 and SL-2/PME-2. Large upper-case letters indicate SL sequence, small upper-case letters indicate genomic UTR and lower-case letters indicate translated sequence.

tional start codon. Two potential *C. elegans* poly(A)$^+$ addition signals [36], namely AAAAAA (nt 2968–2973) and UAUGAA (nt 2984–2989), are located 41 and 21 nucleotides upstream of the poly(A)$^+$ tail. The predicted protein of locus Y71F9AL.18 would have a calculated molecular mass of 107.972 kDa and an estimated pI of 7.9. Its structural similarities to huPARP-1 and other eukaryotic PARP-1 (Figure 2A) include a DNA-binding domain with two zinc-finger motifs, an automodification domain and a catalytic domain with a PARP-signature motif. Amino acid identity between the Y71F9AL.18 gene product and huPARP-1 is 31% over their entire sequences. The first zinc finger (ZF-I) is composed of 36 amino acids, of which C23C25 and H54C57 are involved in the co-ordination of a zinc ion (Figure 2B). The actual length of the ZF-I could be biologically important, as it is highly conserved throughout the species. The second zinc finger (ZF-II) is composed of 26 amino acids (10 less than its human counterpart), of which C129C132 and H151C154 co-ordinate a zinc ion (Figure 2B). ZF-II is thus smaller than the PARP-1 ZF-II of most other species including humans, which may reflect an altered function. The C-terminal half of the predicted protein of locus Y71F9AL.18 shows several blocks of highly conserved peptides, especially the 'PARP signature' motif where identity is 76% (Figure 2A). The polymerization reaction catalysed by huPARP-1 is dependent on the glutamate residue at position 988 [37] (Figure 2B). This residue is also present in protein Y71F9AL.18 within a very conserved stretch of amino acids composed of LLYNEYIVYD (Figure 2B). We named the gene encoded in the locus Y71F9AL.18 as *pme-1* according to the convention for name designation in *C. elegans*, and the cDNA sequence has been deposited in GenBank® database with accession no. AF499444.

Loci Y71F9AL.18 and AC8.1 are very similar (Figure 1). AC8.1 spans over 10.5 kb on chromosome X and contains 9 exons. The entire exonic sequence of AC8.1 is identical with part of the exonic sequence of Y71F9AL.18. However, the nucleotide sequences of AC8.1 introns diverge significantly (results not shown). AC8.1 encodes a protein highly similar to huPARP-1 with a calculated molecular mass of 82.790 kDa. However, when

the gene product of Y71F9AL.18 was cloned using RT–PCR, with a pair of primers matching sequences in the gene product of AC8.1, the 2900 nucleotide fragment corresponding to the Y71F9AL.18 messenger was consistently amplified but not the predicted fragment of 2184 from AC8.1. Moreover, Northern-blot analysis of *pme-1* gene performed with a probe corresponding to nucleotides 260–1141 of *pme-1*, and also corresponding to nucleotides 260–936 of the AC8.1 predicted cDNA, did not show the 2.2 kbp fragment corresponding to AC8.1 mRNA. We named the gene at locus AC8.1 as *pme-6* (although this cDNA was not isolated at this time). The names *pme-3* and *pme-4* had already been attributed to the PARGs at loci F20C5.1 and H23L24.5 respectively, and locus ZK1005.1 had already been given the name *pme-5*.

The E02H1.4 locus encodes a protein similar to huPARP-2. A complementary DNA that encodes gene product E02H1.4 was cloned using RT–PCR. We named this gene *pme-2* and its cDNA sequence has been deposited in the GenBank® database with accession no. AF500111. The full-length cDNA sequence contains 1902 nucleotides with a 1617-nucleotide open reading frame (ORF): 32 nucleotides in the 5′-UTR and 253 nucleotides of a 3′-untranslated sequence. The ORF extends from the initiation ATG codon, in a *C. elegans* consensus context for translation initiation [(a/g)NN**ATG**T], AAAAAA (nt 1860–1865), and AACAAA (nt 1865–1870), are located 22, 19 and 15 nucleotides upstream from the poly(A)$^+$ tail. The general gene organization in *C. elegans* is reflected well in the primary structure of *pme-2*; it is composed of fewer exons and has shorter introns than its mammalian counterpart [36]. There are only 5 exons coding for PME-2 (Figure 1) compared with 16 exons for huPARP-2 [38]. The ORF encodes a 538-amino-acid protein (Figure 2C), and the deduced amino acid sequence predicts a protein with a pI of 7.1 and a molecular mass of 61.275 kDa. After optimal alignment, the putative protein had 24% sequence identity with huPARP-2. Most of the identities/similarities are located at the C-terminal portion of the PARPs, which houses the PARP signature motif in the catalytic domain (Figure 2D). The PME-2 protein has a glutamate residue in a very conserved stretch of amino acids LLYDEYVMFN (Figure 2D); Glu-509 must thus be a critical residue for PARP activity, as has been shown for PARP-1. PME-2 is also structurally distinct from PME-1 and PARP-1 of other species.

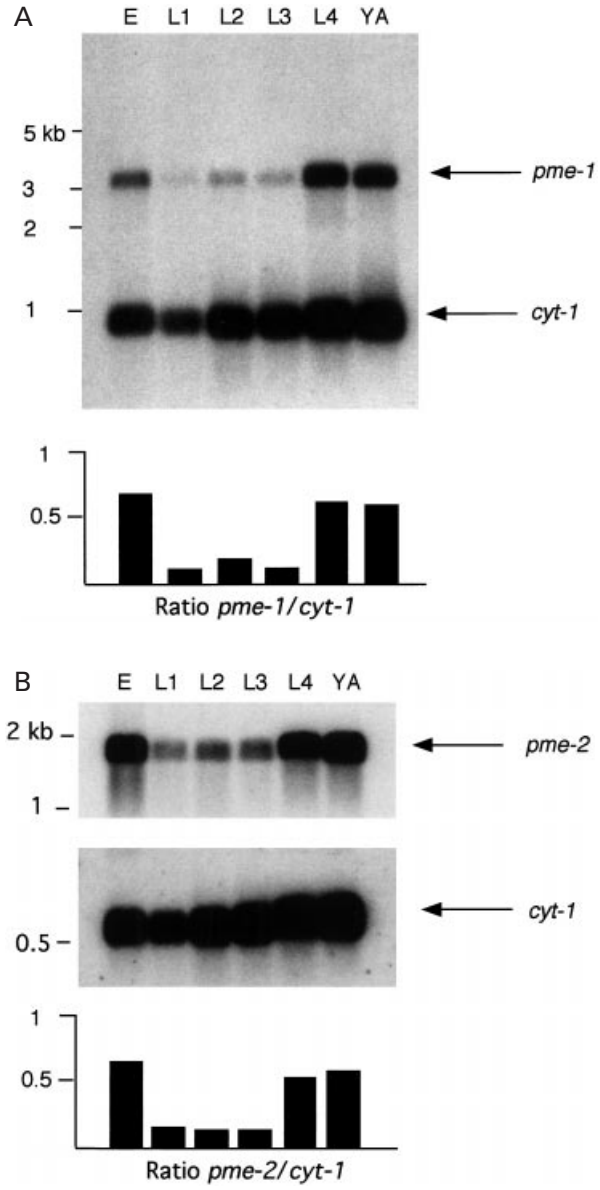

Figure 4 The expression of *pme-1* and *pme-2* genes is developmentally regulated

Total RNAs were extracted from synchronous worm cultures of *C. elegans*. Poly(A)$^+$ RNA from embryos (E), from L1, L2, L3 and L4 larvae and from young adults (YA) was subsequently purified and probed by Northern-blot hybridization analysis using labelled *pme-1* (**A**) or *pme-2* (**B**) as a probe. *cyt-1* was used as an internal control (see the Experimental section).

Structural analysis of *pme-1* and *pme-2* mRNA

The 5′-ends of *pme-1* and *pme-2* mRNAs were analysed by RT–PCR using SL-1 or SL-2 as forward primers and gene-specific primer as reverse primer (Figure 3A). We show that PME-1 mRNA has the SL-1 sequence in its 5′-end (Figures 3A and 3B). The same analysis shows that PME-2 mRNA has SL-2 sequence in its 5′-end (Figures 3A and 3B), suggesting a possible operon-like expression [36]. Moreover, a genomic region (180 bp) upstream of the translation initiation of *pme-2* is composed of 77% A-T, and a potential splicing sequence (UUUCCAAGC; nt −17 to 10) is also present. These structures provide the conditions essential for a trans-splicing reaction. Composition of the site (CUUCCAGA) is also present at nucleotides −26 to −19.

Figure 5 Poly(ADP-ribose) synthesis in *C. elegans* worm extracts and bacterially expressed PME-1 and PME-2

(**A**) A worm mixed-stage culture was extracted with high pressure and assessed for PARP activity with [^{32}P]NAD. Radiolabelled polymers were detached from modified acceptor proteins after alkaline treatment, then selectively removed by dihydroxyboronyl Bio-Rex affinity chromatography and analysed on a polyacrylamide gel. A 30 μl sample was loaded on to the gel. Numbers indicate size of poly(ADP-ribose), XC, xylene cyanol, BB, Bromophenol Blue. (**B**) cDNAs were cloned by RT–PCR and introduced into pQE-30 vector. *E. coli* M15 and XL1-Blue bacteria were transformed with the constructs and induced with IPTG for 4 h at 20 °C (huPARP-1) or 37 °C (PME-1 and PME-2). Equal amounts of bacterial extract were loaded on to a SDS/PAGE gel and stained with Coomassie Blue dye. Each recombinant protein is shown (↑) at the expected molecular mass: 65 kDa (His_6-PME-2), 111 kDa (His_6-PME-1) and 120 kDa (His_6-huPARP-1^{D214A}). (**C**) The bacterial extracts were immediately assessed for PARP activity with [^{32}P]NAD as mentioned above; 10 and 20 μl were analysed on a polyacrylamide gel. 6 × His, His_6.

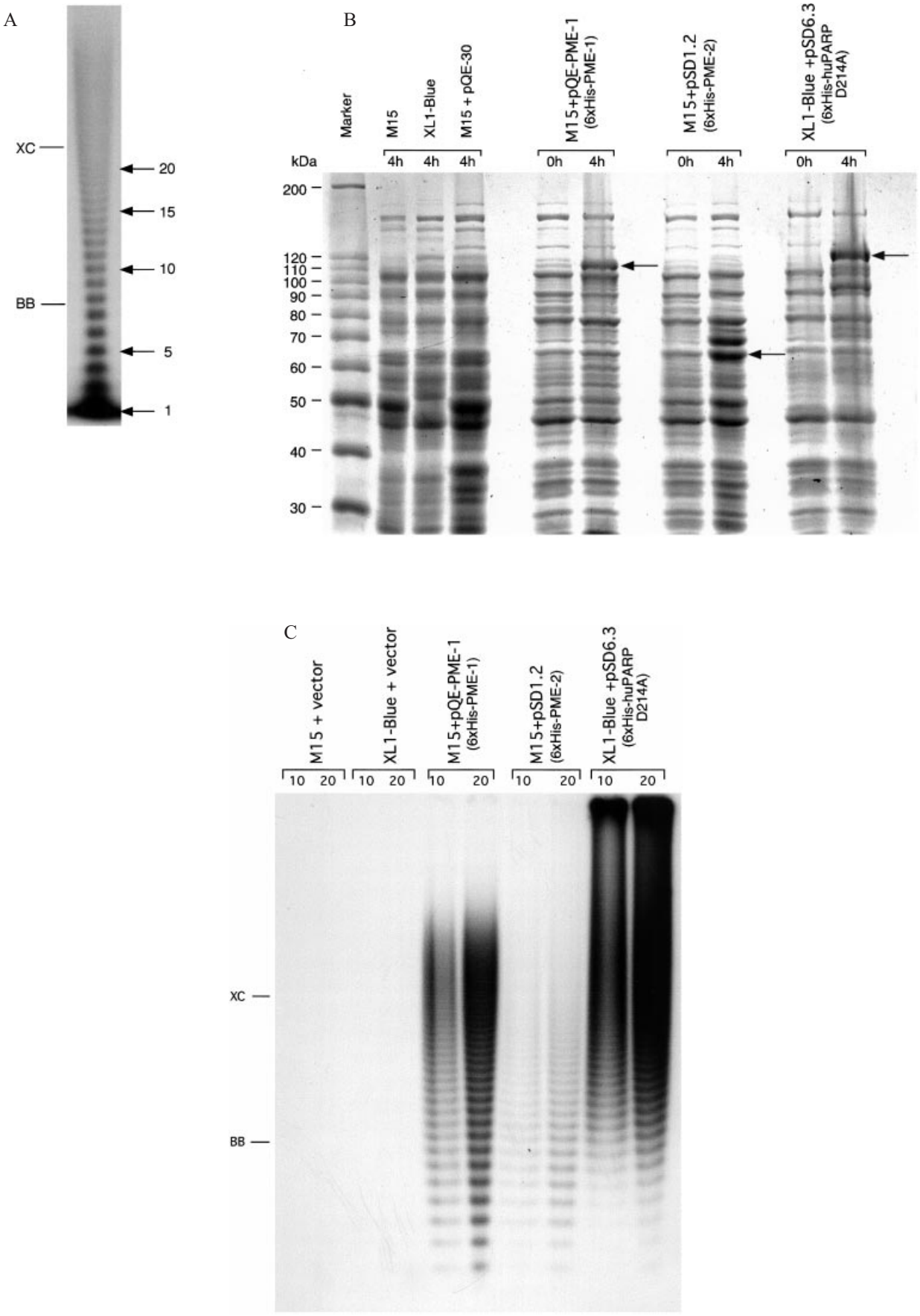

Figure 5 **For legend, see facing page.**

Developmental regulation of expression of *pme-1* and *pme-2* mRNA

Northern blots of PME-1 and PME-2 mRNA expression are shown (see Figure 4). Poly(A)$^+$ RNAs from animals at all developmental stages showed a single transcript at 3.1 kb for PME-1 (Figure 4A) and at 1.8 kb for PME-2 (Figure 4B). To correct for loading variation, the membranes were also analysed with a CYT-1 probe, an mRNA known to be constitutively expressed throughout the life cycle of *C. elegans* [31]. Ratio analysis (Figure 4) showed that both genes are predominantly expressed early, in the embryonic stage, and later, in the L4 and young adult stages. Note, however, that young adult worms used for this analysis could contain embryos.

Poly(ADP-ribose) synthesis in the *C. elegans* worm extract

We conducted qualitative PARP assays on *C. elegans* to gain information on lower-eukaryote PARP status. A worm extract (1.53 mg/ml) was tested for PARP activity. Figure 5(A) shows the distribution of ADP-ribose polymers synthesized by the worm extract. A typical ladder of poly(ADP-ribose) polymers was observed, with short polymers predominating. These results show for the first time the presence of an endogenous source of poly(ADP-ribosyl)ation in *C. elegans*.

Analysis of activity of recombinant PME-1 and PME-2 poly(ADP-ribose)

The full-length PME-1 (His_6-PME-1), PME-2 (His_6-PME-2) and mutated human PARP-1^{D214A} (His_6-huPARP-1^{D214A}) were expressed in *E. coli* as histidine-tagged proteins (Figure 5B). A qualitative PARP activity assay was performed on crude bacterial extracts expressing the recombinant proteins. Radiolabelled poly(ADP-ribose) polymers were synthesized and subsequently removed and purified from bacterial extract expressing His_6-PME-1, His_6-PME-2 and His_6-huPARP-1^{D214A}. PAGE showed the characteristic poly(ADP-ribose) polymer ladders synthesized in the different bacterial extracts (Figure 5C). Extracts expressing His_6-huPARP-1^{D214A} showed a predominantly high level of long polymers. Extracts expressing His_6-PME-1 showed lesser degree of polymer production as well as a majority of short polymers. Extracts expressing His_6-PME-2 show the lowest level of poly(ADP-ribose) synthesis, with smaller polymers than the other two.

DISCUSSION

We have characterized two members of the PARP family in *C. elegans*, namely PME-1 and PME-2, similar to huPARP-1 and huPARP-2 respectively. Although the presence of a short form of PARP in *C. elegans* has been previously reported [26,27], we present here a detailed analysis of both *C. elegans* PME-1 and PME-2.

We found three different loci that might encode PARPs in *C. elegans*. The Y71F9AL.18 locus encodes the gene *pme-1*, the product of which belongs to the PARP-1 subfamily. It is characterized by an N-terminal DNA-binding domain, a central automodification domain and a C-terminal active domain containing the PARP signature motif. The *pme-1* mRNA was shown to be trans-spliced by the SL-1, which is characteristic of genes not localized in clusters [36]. Indeed, the genomic organization in the vicinity of *pme-1* shows both up- and downstream non-coding regions that are several thousand base pairs long. Locus AC8.1, which encodes *pme-6* (an almost identical copy of *pme-1*), does not seem to be active in normally growing mixed-stage worm cultures, as a RT–PCR failed to amplify a product from this gene. Interestingly, pseudogenes of PARP-1 exist in humans and are localized on chromosomes different from that of the active gene [24]. Although it is tempting to label *pme-6* as a pseudogene, its activity has to be investigated in both male and dauer populations. Moreover, in *pme-6* and *pme-1*, intronic sequences diverge significantly, which is characteristic of functional genes.

PME-2 is also closely related to another PARP subfamily member, PARP-3 [6], but alignment of sequences showed a closer link with PARP-2. This would suggest that PARP-3 emerged later than PARP-2. Although in single copy, several lines of evidence point towards *pme-2* as being part of an operon [36]. First, *pme-2* localized within a cluster of four genes including upstream locus E02H1.3 and downstream loci E02H1.5 and E02H1.6 (WormBase, version WS56, http://wormbase.org). Secondly, all genes have the same 5′ to 3′ orientation. Thirdly, they are very closely spaced, separated by only 192, 33 and 156 nucleotides. Finally, *pme-2* mRNA is trans-spliced with SL-2, known to belong to a gene start found downstream of an identified operon [36]. Although the functions of SL are not known, trans-splicing is common in the phylum nematoda, and approx. 70 % of all transcripts in *C. elegans* possess an SL. One interesting aspect of the gene organization of huPARP-2 and mouse PARP-2 is its bidirectional promoter, which drives the expression of PARP-2 and that of a nearby gene, RNase P RNA [38]. Our findings suggest a conserved expression pattern for PARP-2 in which PARP-2 and *pme-2* share common promoter region with other genes throughout evolution. Moreover, locus E02H1.5, immediately downstream of *pme-2*, encodes a protein similar to the RNA-directed RNA polymerase domain of encephalomyocarditis virus (accession no. P17593). This enzyme also belongs to the RNA processing machinery, thereby adding to the similarity with PARP-2.

PARP activity detected in total *C. elegans* worm extract would be partially the result of PME-1 and PME-2 expressed in this worm. Amino-acid sequence alignment revealed that the catalytic domains of PME-1 and -2 are highly similar to the PARP-1 catalytic domain. Moreover, the PARP signature motif is conserved in both enzymes along with a critical glutamate residue in the catalytic domain, both of which are important to enzymic activity. This suggests that PME-1 and -2 can synthesize poly(ADP-ribose); we have shown that recombinant PME-1 and PME-2 can synthesize poly(ADP-ribose) and they display typical PARP activity (Figure 5C). We cannot, however, exclude the possibility that a portion of recombinant PME-2 molecules was not readily available for synthesis. The recombinant products of PME-2 were expressed at a level comparable with recombinant PME-1; nonetheless, poly(ADP-ribose) polymer production was higher in the case of PME-1 (Figures 5A and 5B). This could be related to the finding that PARP-2 is not as strongly stimulated by DNA strand breaks as PARP-1 is, *in vitro* [5]; therefore, overall polymer production in the worm would depend much more on the activity of PME-1. Hence, the enzymic activity ratio PME-1/PME-2 would be conserved in *C. elegans*.

Expression patterns of *pme-1* and *pme-2* mRNAs were studied and both were detected as a single transcript at all developmental states (Figure 4), suggesting continuous expression during the life cycle of the worm. *pme-1* and *pme-2* have a similar expression pattern, with their mRNA being highly expressed in embryos, suggesting a role in the early development of the worm. Interestingly, an increase is also observed at the L4 and young adult stages, which could be related to the increasing number of germ cells and embryos in the hermaphrodite. Therefore *pme-1* and *pme-2* could also play a role in germ line formation. It is

particularly noteworthy that the PARP-2 tissue expression pattern is very high in germ line of mammals [5].

At least two genes, *pme-1* and *pme-2*, encoding highly similar proteins to PARP-1 and PARP-2, take part in poly(ADP-ribose) polymerization in *C. elegans*. The conservation in evolution of these two genes hints towards important functions in multicellular animals. *C. elegans* has long provided a classic genetic and developmental model through which many mammalian gene products have been assigned a role, and we propose to use the worm to understand better the functions of PARP-1 and PARP-2.

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