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Molecular Cloning and Expression of Human Poly (ADP-ribose) Synthetase cDNA in *E. coli*

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The present study was performed to clone and express human poly (ADP-ribose) synthetase (PARS) cDNA in *E coli*. For these purposes, the cDNA for human poly (ADP-ribose) synthetase, encoding the entire protein, was cloned into pGEM-7Zf(+). The resulting recombinant plasmid pPARS6.1 was restriction enzyme mapped and its identity was confirmed by Southern blot analysis. The pPARS6.1 contained full-length cDNA of human PARS and the nucleotide sequences were identical with those reported previously. The recombinant protein which migrated as a unique 120 kDa band on 10% SDS-polyacrylamide gels, was identified as PARS by Southwestern blots using nick-translated DNA probes and by activity gels and activity blots using ^{32}P-NAD as a substrate for poly (ADP-ribose) synthetase (PARS). The signals corresponding to 120 and 98 kDa proteins were obtained following IPTG (0.4 mM) induction of the PARS cDNA cloned into Xba I-digested pGEM-7Zf(+) vector. Nonspecific signals corresponding to 45 and 38 kDa proteins were also shown in both IPTG-induced and noninduced cells. The nonspecific proteins may be products of incomplete translation or proteolytic products of intact PARS.

KEY WORDS: Human Poly (ADP-ribose) Synthetase cDNA, Expression, *E. coli*, Activity Blot, Southwestern Blot

The enzyme poly (ADP-ribose) polymerase (PARS) catalyzes the poly(ADP-ribosylation) reaction in the presence of DNA and the substrate NAD and modifies specific nuclear proteins such as histones and topoisomerases located adjacent to DNA strand breaks (Thraves *et al.*, 1985). PARS also undergoes extensive automodification with the attachment of ADP-ribose polymers via an ester linkage to glutamic acid residues (Kawaichi *et al.*, 1981).

The various activities of PARS are organized into separate functional domains (Kameshita, *et al.*, 1986). These domains comprise an amino-terminal DNA-binding domain, a central hydrophilic automodification domain containing 15 glutamic acid residues, and a carboxyl-terminal NAD binding domain. Satoh and Lindahl (1992) have described an *in vitro* DNA repair system in which UV-irradiated closed circular DNA was reacted with extracts of human cell (Manley *et al.*, 1983). DNA repair in this system is stimulated by NAD. Removal of PARS from the extract increases the basal level of DNA repair and abolishes NAD sensitivity; the basal level of repair is reduced and NAD sensitivity restored on addition of purified PARS to the reaction (Satoh

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and Lindahl, 1992).

Proteins of human PARS have previously been expressed in a number of *E. coli* expression systems (Herzog *et al.*, 1989; Gradwohl, *et al.*, 1990; Simonin *et al.*, 1990). Recently, Ikejima *et al.* (1990) expressed full-length human PARS in *E. coli*. However, the relative yield of expressed products appeared low and a majority of these products appeared as proteins that are shorter than full-length. In most cases of these experiments, a conspicuous fraction of the synthesised products appeared as shorter polypeptides not only due to proteolytic degradation in *E. coli* but also as a consequence of initiation of translation at signals located within the coding region of PARS (Herzog *et al.*, 1989). Recently, we have cloned human PARS cDNA (designated as pPARS6.1) and expressed it in *E. coli* in order to purify PARS and to produce its antibody for an investigation of biochemical and physiological roles of PARS. The present study was performed to identify the activity of the recombinant pPARS6.1 by Southwestern blots using nick-translated DNA as a probes and by activity gels and activity blots using [^{32}P]-NAD.

Materials and Methods

Chemicals and reagents

All culture supplies were purchased from Difco Laboratories. Radioactive materials [α-^{32}P] dCTP (sp. act. 3,000 Ci/mmol), [^{32}P] NAD (sp. act. 1000 Ci/mmol) and [^{35}S] dATP (sp. act. 1,000 Ci/mmol) were obtained from Amersham International Inc. All restriction enzymes and DNA modifying enzymes including T4 DNA ligase, DNA polymerase I Klenow fragment were obtained from Boehringer Mannheim and Promega. Random Primed DNA Labeling Kit, Sequenase Version 2.0 DNA Sequencing Kit and Gene Clean II Kit were from Boehringer Mannheim, United States Biochemical and Bio 101 Inc., respectively.

Construction of recombinant plasmid, pPARS6.1

Plasmid prepared by alkaline lysis method and insert DNA liberated from pPARS5.8 (Rho *et al*, 1995) were purified using GeneClean II Kit. To construct recombinant pPARS6.1, Xba I-digested pGEM-7Zf(+) DNA and the insert PARS3.1 cDNA digested with Xba I were ligated as described by Rusche and Howard-Flanders (1985). The ligation of sticky end DNA was carried out in 20 μl of reaction mixture containing 2 μl of 10X ligase buffer, 1 U of T4 DNA ligase, 50 ng of vector DNA and 200 ng of insert DNA for 16 hrs at 16°C. After the ligation reaction the mixture was used for transformation.

Preparation of the DNA probe by random priming

DNA probe for Southern analysis was prepared by a random primed DNA labeling method (Feinberg and Vogelstein, 1984). The complementary strand was synthesized from the 3′ OH termini of the random hexanucleotide primer using Klenow enzyme. The labeling reaction was carried out in 20 μl of the standard random priming buffer containing 50 ng of insert DNA, 30 μCi of [α-^{32}P] dCTP, dATP, dGTP, dTTP and 2 units of Klenow enzyme for 1 hr at 37°C. The reaction was terminated by heating at 95-100°C for 2 minutes and subsequent chilling on ice and adding EDTA to 20 mM. The probe DNA was given a specific activity of approximately 2×10^9 cpm/μg.

Preparation of the DNA probe by nick-translation

DNA probe for Southwestern blot analysis was prepared by a nick-translated DNA labeling method. The complementary strand was synthesized from nicked DNA using polymerase I. The labeling reaction was carried out in 30 μl of the standard nick-translating buffer containing 200 ng of insert DNA, 30 μCi of [α-^{32}P] dCTP, 200 μM each of dATP, dGTP and dTTP, diluted DNase and 1 U of polymerase I for 2 hrs at 15°C. The reaction was terminated by adding 0.1 volume of 2% SDS and 250 mM EDTA.

Southern hybridization

Southern blot hybridization was carried out as described by Southern (1975) with minor modifications. The recombinant pPARS6.1 DNA

was digested with various enzymes and analysed on a 0.7% agarose gel. The gel was blotted onto a nylon membrane with 0.4 M NaOH by downward alkaline Southern blotting (Chomezynski, 1992). The membrane was briefly washed with 2X SSC (175.3 g NaCl, 88.2 g $Na_3C_6H_5O_7$ per 1 L) and prehybridized in prehybridization buffer (1% SDS, 2X SSC, 10% dextran sulphate, 50% deionized formamide) for at least 1 hr at 42°C, followed by hybridization with the radiolabeled pPARS3.1 probe (10^8 cpm/ml) for overnight at 42°C. The membrane was briefly washed at room temperature in 2X SSC and washed twice in 2X SSC and 1% SDS for 20min at 68°C for low stringency condition, or twice in 0.2X SSC, 0.1% SDS for 20 min at 68°C for high stringency condition. After excessive liquid removed from membrane, the membrane was wrapped and exposed to X-ray film for 1-24 hrs at −70°C.

Southwestern hybridization

The recombinant pPARS6.1 crude extracts were separated on 7 × 8 cm SDS/polyacrylamide minigels (10%) according to the protocol of Laemmli (1970). Electrotransfer of proteins onto nitrocellulose sheets was performed with a Semi-Dry Transfer equipment (Bio-Rad). The blots were washed for 30 min in TBS Nonidet-p40 buffer. Preincubation of the blots was performed for 30 min in the DNA binding buffer. The blots were then incubated for 4 hours in a sealable plastic bag with 10 ml of binding buffer containing [^{32}P] labelled nick-translated DNA. After three changes of the binding buffer, the blots were dried and autoradiographed.

Activity blot

Proteins transferred onto nitrocellulose sheets were first incubated for 1 hr at room temperature in 20 ml of 50 mM Tris-HCl, pH 8, 100 mM NaCl, 1 mM DTT, 0.3%(v/v) Tween 20 (renaturation buffer) containing DNase I activated DNA (2 μg/ml), 20 mM $ZnCl_2$ and 2 mM $MgCl_2$. The blots were then soaked for overnight in the same solution (5 ml) containing 1 to 2 μCi [α-^{32}P] NAD+ (sp. act. 1000 Ci/mmol) /ml and washed with 20 ml of renaturation buffer three times. The blots were dried and analyzed by autoradiography.

Activity gel experiment

The samples were separated on 10% SDS-polyacrylamide mini gels containing 100 μg/ml of sonicated calf thymus DNA (added immediately before gel polymerization). Renaturation was accomplished at room temperature by washing the gel for 1 hr with 50 ml of renaturation buffer (50 mM Tris-HCl, pH 8, 30 mM 2-mercaptoethanol). The gels were then treated with renaturation buffer containing 6 M guanidine HCl for 1 hr at room temperature, followed by several changes of renaturation buffer, preincubating the intact gel in a plastic Petri dish at 37°C for 15 min with 10 ml reaction mixture containing 100 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 1 μM NAD and 1 mM DTT then by incubation with 2 ml of the same reaction mixture containing 10 μCi/ml [^{32}P] NAD for overnight, washed with several changes (50 ml each) of cold 5% trichloroacetic acid (TCA). The gels were dried and analyzed by autoradiography.

Results

The purpose of this study is to clone the cDNA of HeLa poly (ADP-ribose) synthetase (pPARS3.1) into expression vectors and to express it in *E. coli* in order to purify PARS and to produce its antibody for the investigation of biochemical and physiological roles of PARS.

The Xba I-digested pPARS3.1 was introduced to Xba I site of pGEM-7Zf(+) vector and the resulting recombinant plasmid was designated as pPARS6.1 (Fig. 1).

In order to confirm the orientation of the insert by restriction mapping of the recombinant plasmid pPARS6.1, the plasmid DNA was digested with various restriction enzymes which cut the polycloning sites of pGEM-7Zf(+) and the restricted DNA fragments were subjected to an 0.7% agarose gel electrophoresis (Fig. 2). The control DNAs, such as pGEM-7Zf(+) and pPARS3.1 were also run for comparison with the digested products. On the basis of results from the above digestion experiment, the restriction map of pPARS6.1 was constructed as shown in figure 3.

In the 3.1 kb insert of pPARS6.1, various restriction sites are found as follows: one site each

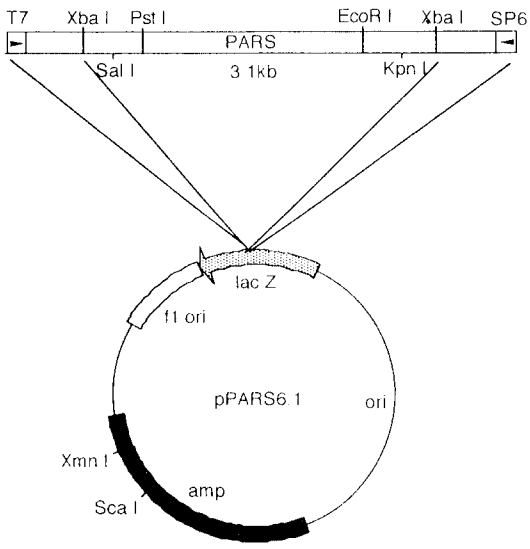

Fig. 1. Subcloning strategy for a putative PARS cDNA clone. PARS3.1 cDNA was digested with Xba I and the resulting fragment was eluted and ligated with pGEM-7Zf(+) digested with Xba I. The resulting recombinant plasmid was designated as pPARS6.1.

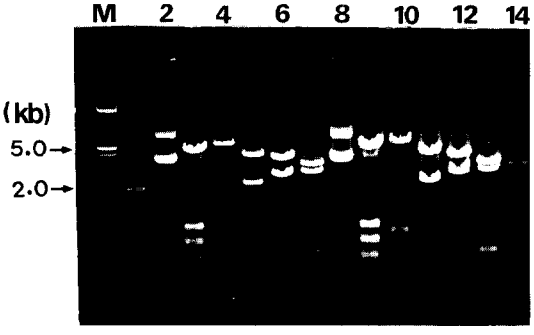

Fig. 2. Restriction enzyme digestion patterns of a recombinant plasmid, pPARS6.1. The recombinant plasmid was digested with various restriction enzymes (Lanes: 3 and 9, BamH I; 4 and 10, Cla I; 5 and 11, EcoR I; 6 and 12, Hind III; 7 and 13, Kpn I) and electrophoresed on a 0.7% agarose gel. pGEM-7Zf(+) (lane 1), pPARS6.1 (lanes 2 and 8) and PARS3.1 (lane 14) that were non-digested were also run. M denotes λ/Hind III and EcoR I marker DNA.

of EcoR I and Hind III, two sites of Cla I, and three sites of BamH I. In order to confirm that pPARS6.1 contains the pPARS3.1 cDNA, Southern blot analysis was performed (Fig. 4).

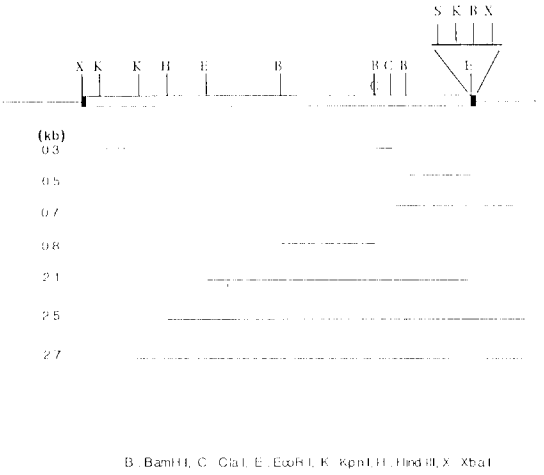

Fig. 3. A restriction map of the recombinant plasmid pPARS6.1. The plasmid pPARS6.1 contains a 3.1 kb fragment of human PARS cDNA. Various restriction enzyme recognition sites were indicated.

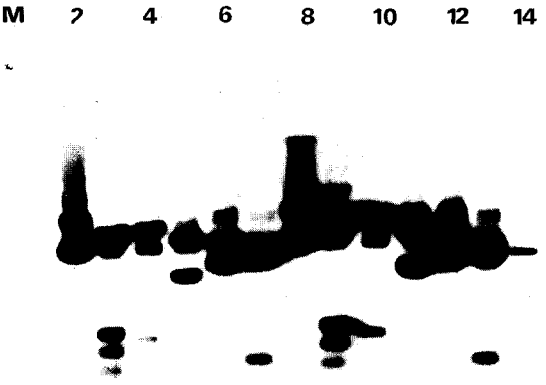

Fig. 4. Southern blot analysis of recombinant pPARS6.1 DNA. Recombinant DNA was digested with various restriction enzymes. The digested samples were electrophoresed on a 0.7% agarose gel and transferred onto a nylon membrane. The membrane blotted was hybridized with human PARS3.1 cDNA as probes, washed under a high stringency condition and autoradiographed. λ DNA digested with Hind III and EcoR I were used as a marker.

The recombinant plasmid DNA (pPARS6.1) was digested with various restriction enzymes, electrophoresed on a 0.7% agarose gel, and then transferred onto a nylon membrane. The hybridization signals are confined only to PARS cDNA fragments and not to vector DNA

fragments (compare Fig. 2 with Fig. 4). The subcloned recombinant colonies (white colonies) were selected on MacConkey agar (Difco) plates containing 50 μg/ml of ampicillin. The nucleotide sequence of the subcloned PARS cDNA was analysed by the dideoxy chain-termination method (Sanger *et al.*, 1977) using Sequenase version 2.0 DNA sequencing Kit (USB).

Figure 5 represents the nucleotide sequences of about 200 nucleotides beginning at translational start codon (ATG) of PARS cDNA. The nucleotide sequence of pPARS6.1 was compared with that of human poly(ADP-ribose) synthetase cDNA using DNasis program (Fig. 6). The determined nucleotide sequence is identical with that of human poly(ADP-ribose) synthetase.

In order to determine whether PARS protein is induced by 0.4 mM of IPTG (isopropyl-thio-β-D-galactoside) or not in pPARS6.1-harboring cells, Coomassie staining and Southwestern blot analysis were performed (Fig. 7 and Fig. 8). After cells were added with 0.4 mM IPTG for various time periods, the cell extracts were subjected to 10% SDS-PAGE, and the gels were either subjected to Coomassie staining (Fig. 7) or Southwest blot analysis using [^{32}P] nick translated DNA (3.1 Kb) as a probe (Fig. 8).

These results show that only signals corresponding to 120 and 98 kDa proteins were obtained following IPTG (0.4 mM) induction. Nonspecific signals corresponding to 45 and 38 kDa proteins were also shown in both IPTG-induced and noninduced cells. These nonspecific proteins may be DNA binding proteins in *E. coli* or products of incomplete translation or proteolysis products of intact PARS.

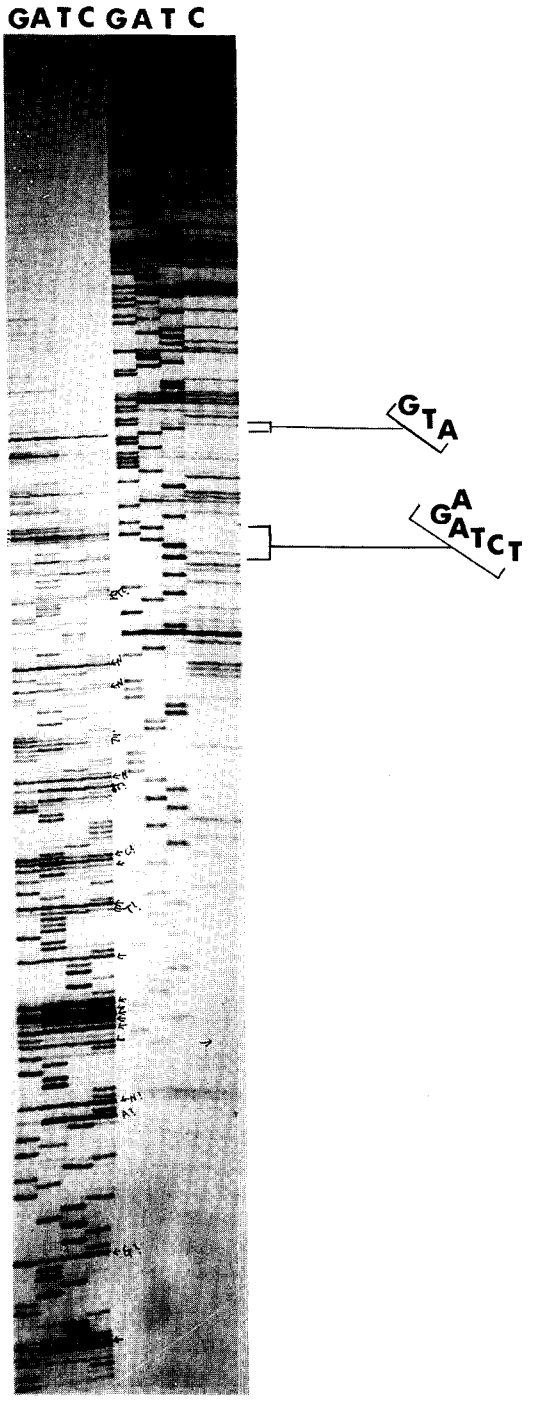

Fig. 5. Autoradiogram showing the sequence of about 200 nucleotides beginning at translational start codon of PARS cDNA.

Discussion

PARS activity is triggered after recognition of DNA strand breaks. It synthesizes poly(ADP-ribose) and attaches it to many nuclear proteins including itself. This auto-poly (ADP-ribosylation) cause dissociation of the PARS molecule from DNA and down-regulation of its activity (Althaus and Richter, 1987). The structures responsible for these multiple functions are expected to be

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*** HOMOLOGY REGIONS REFERENCE ***

NO. TARGET FILE  DEFINITION                                    Match% Over. INIT   OPT
  1 SY3.SEQ     >                                               85.0  167   284   578
85.0% identity in 167 bp overlap, initial score:  284  optimized score:  578
NORMAL key sequence

                    10         20         30         40         50         60
HHIPARS.SEQ    ATGGCGGAGTCTTCGGATAAGCTCTATCGAGTCGAGTACGCCAAGAGCGAGCGCGCCTCT
               :::::::::::::::::::::::::::::::::::::::::::::::::. .....:::::
SY3.SEQ        ATGGCGGAGTCTTCGGATAAGCTCTATCGAGTCGAGTACNCCAAGAGCNGNNNNNCCTCT
               20         30         40         50         60         70
                    70         80         90        100        110        120
HHIPARS.SEQ    TGCAAGAAATGCAGCGAGAGCATCCCCAAGGACTCGCTCCGGATGGCCATCATGGTGCAG
               :.:::::::::::::::.: ::::::::::::::.:::::::.:::::::::::::.:::
SY3.SEQ        TNCAAGAAATNCAGCGANA-CATCCCCAAGGACTCNCTCCGGATNGCCATCATGGTNCAG
               80         90         100        110        120        130
                       130        140        150        160
HHIPARS.SEQ    TCGCCCATGTTTGATGGAAAAGTCCCACACTGGTACCACTTCTCCTG
               ::.:::::::::::::::::::.:.:::::::: ::::::: .. ..
SY3.SEQ        TCNCCCATGTTTGATGGAAAANTNCCACACTGG-ACCACTTANNANN
               140        150        160        170        180
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Fig. 6. Nucleotide sequence of pPARS6.1 compared with that of human poly(ADP-ribose) synthetase cDNA.

conserved in PARS of various species. Mitsuko *et al.* (1994) has isolated *Sarcophaga* PARS gene and also shown that the expressed enzyme was functional upon expression in *E. coli*. Comparison of the primary amino acid sequence of *Sarcophaga* PARS with that of the human enzyme revealed high conservation of two zinc-finger motifs and the whole NAD-binding domain over the evolutionary distance between insect and mammals.

We have cloned human PARS cDNA into pGEM-7Zf(+) vector. The 120 kDa PARS protein was detected in induced recombinant plasmid and various animal tissues. The expression of full-length human PARS in *E. coli* has been reported (Ikejima *et al.*, 1990). Although the enzyme overexpressed in *E. coli* had similar biochemical characteristics to those of PARS purified from mammalian tissues, a large fraction of the expressed products appeared as proteins that were shorter than full-length.

In Southwestern experiments, only the signals corresponding to 120 and 98 kDa proteins were obtained following IPTG (0.4 mM) induction of the PARS cDNA. Nonspecific signals corresponding to 45 and 38 kDa protein were also shown in both IPTG-induced and non-induced cells. It has been reported that the zinc binding sites are totally included in the DNA binding domain, but not in the catalytic part of the protein like other NAD enzymes such as dehydrogenases (Pettersson, 1987). A similar approach was performed for the determination of the zinc and double-stranded RNA binding sites in the Reovirus outer capsid protein σ3 (Schiff *et al.*, 1988).

Using EDTA as a metal chealator it was reported that DNA binding activity detected on the 29 kDa fragment was inhibited by zinc depletion and subsequently restored by adding back the metal to the transferred protein. However, the DNA binding observed for the 116 kDa enzyme as well as the 66 kDa polypeptide after EDTA treatment suggest the existence of the second zinc independent DNA binding site, which could be located in the C-terminal part of the 46 kDa fragment. Indeed Buki and Kun (1988) using plasmin degradation, recently detected a 36 kDa DNA binding fragment and the 54 kDa NAD binding domain.

In activity blot and activity gel experiments, clear results have not yet obtained in extracts of cells harboring induced recombinant plasmids. Lindahl (1995) suggested that poly (ADP-ribose) is a short-lived polymer *in vivo*, and the chains synthesized in response to DNA damage are largely degraded within 1-2 min. This is because the two enzymes,

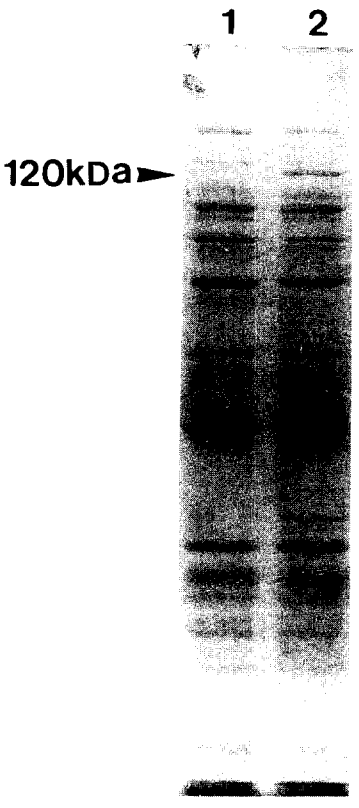

Fig. 7. Polyacrylamide gel electrophoresis of recombinant PARS proteins in *E. coli*. Cells harboring the pPARS6.1 recombinant plasmids were added with 0.4 mM IPTG for 3 hrs (lane 1) or not (lane 1). The samples were boiled and subjected to 10% SDS-PAGE. Gels were visualized by staining with Coomassie brilliant blue.

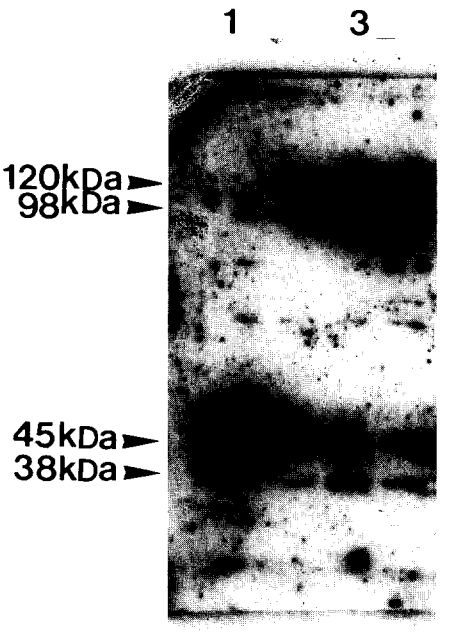

Fig. 8. Southwestern blot analysis of pPARS6.1 plasmid. Induction of pPARS6.1 in *E. coli* cells containing the appropriate plasmids were performed by addition of 0.4 mM IPTG for 0, 1, 2 and 3 hrs (lanes 1 - 4, respectively). The cell extracts were subjected to 10% SDS-PAGE, transferred onto nitrocellulose filter and then incubated with nick-translated DNA (3.1kb) as a probe. Arrows indicate induced proteins bound to the probe DNA.

PARS and poly (ADP-ribose) glycohydrolase, always act simultaneously. Thus, expression of PARS cDNA in cells, such as *Saccharomyces cerevisiae* and *E. coli* that normally are known to lack the two-enzyme system is a lethal event, presumably because gradual accumulation of stable poly (ADP-ribose) chains ultimately causes intolerable interference with DNA replication and transcription. The possible absence of PARS from yeast and bacteria is associated with the unfortunate side effect that a convenient genetic system for investigation of physiological roles of PARS has not been available.

In the present study, we have suggested that the induction of PARS in a low level as well as appearance of nonspecific proteins in cells harboring recombinant PARS plasmid may be due to the secondary structure of the mRNA, the protein stability or the toxicity of the product to the cell. These factors have been often implicated in expression of genes with relatively long open reading frames (Shelby and Kimmel, 1987). Further studies are necessary for the optimization of conditions for expression of genes with long open reading frames.

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인간 Poly(ADP-ribose) Synthetase cDNA의 클로닝 및 대장균에서의 발현

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본 연구의 목적은 인간의 poly(ADP-ribose) synthetase(PARS)의 cDNA를 클로닝하여 발현시키기 위해 수행하였다. 먼저, 인간의 PARS cDNA 전체를 포함한 pPARS3.1을 pGEM-7Zf(+) 등의 발현 벡터에 클로닝하였다. 이 결과로 생성된 재조합 플라스미드 pPARS6.1이 인간의 PARS cDNA 전체를 포함하고, 올바른 방향으로 삽입되었는지를 확인하기 위해 제한효소 지도를 작성하였고, random primed DNA probe을 이용한 Southern blot 분석에 의해서 PARS가 클로닝되었다는 것을 확인하였다. 또한, 염기서열 분석 결과, 단백질 합성이 시작되는 유전 암호가 정확한 순서로 위치하고 있음을 확인하였다. 재조합된 pPARS6.1의 발현을 위해 배양시 0.4 mM IPTG로 처리하여 만들어진 인간의 PARS 단백질이 10% SDS-PAGE에서 120 kDa 위치에 이동하였다는 것을 nick-translation된 DNA를 probe로 이용하여 확인하였고, Southwestern blot 실험 결과 120 kDa과 98 kDa에 위치하는 단백질이 DNA와 결합함을 알 수 있었다.