Expression and Mutagenesis of Human Poly(ADP-Ribose) Polymerase as a Ubiquitin Fusion Protein from *Escherichia coli*†

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ABSTRACT: The cDNA of human poly(ADP-ribose) polymerase (pADPRP), encoding the entire protein, was subcloned into the *Escherichia coli* expression plasmid pYUb. In this expression system, the carboxyl terminus of ubiquitin is fused to the amino terminus of a target protein, in this case pADPRP, stabilizing the accumulation of the cloned gene product. Following induction of the transformed cells, the sonicated extract contained a unique protein immunoreactive with both pADPRP and ubiquitin antibodies and corresponding to the predicted mobility of the fusion protein in SDS-PAGE. Fusion of ubiquitin to pADPRP increased the yield of pADPRP approximately 10-fold compared to that of the unfused enzyme. The resulting recombinant fusion protein had catalytic properties which were nearly identical to those of native pADPRP obtained from mammalian tissues. These properties included specific activity, K_m for NAD, response to DNA strand breaks, response to Mg^{2+}, inhibition by 3-aminobenzamide, and activity in activity gel analysis. An initial analysis by deletion mutagenesis of pADPRP's functional domains revealed that deletions in the NAD binding domain eliminated all activity; however, partial polymerase activity resulted from deletion in the DNA binding or automodification domains. The activities were not enhanced by breaks in DNA. We further report a colony filter screening procedure designed to identify functional polymerase molecules which will facilitate structure/function studies of the polymerase.

The posttranslational modification of nuclear proteins by the synthesis of poly(ADP-ribose) plays prominent roles in the modulation of chromatin structures adjacent to regions of DNA replication, recombination, and repair [for a review, see Ueda and Hayaishi (1985)]. The enzyme poly(ADP-ribose) polymerase (pADPRP) catalyzes this reaction, in concert with DNA and substrate NAD, to modify specific nuclear proteins adjacent to DNA strand breaks (Thraves & Smulson, 1982). Although the physiological functions of this protein modifying system remain speculative, the biochemical characterization of pADPRP has provided important insights into the enzyme's possible roles in biological processes. These studies have shown that in addition to ADP-ribosylating various nuclear proteins the enzyme also carries out an extensive automodification via an ester linkage most probably through glutamic acid residues (Kawaichi et al., 1981). pADPRP is a DNA binding protein which binds to single-strand, double-strand, and closed circular DNA (Ohgushi et al., 1980). However, the catalytic activity of the purified enzyme is stringently dependent upon the presence of DNA strand breaks (Benjamin & Gill, 1980). This recognition and activation by DNA strand breaks appears to be independent of DNA sequence (Menissier et al., 1989). The various activities of the enzyme are organized into separate functional domains which include an amino-terminal DNA binding region, a central automodification sequence, and a carboxyl-terminal nucleotide binding domain (Kameshita et al., 1986).

With the cloning and sequencing of pADPRP, achieved in our laboratory as well as those of others (Cherney et al., 1987; Kurosake et al., 1987; Huppi et al., 1989; Saito et al., 1990), detailed information concerning these domains is now available. The sequence analysis has revealed that the human and mouse enzymes are polypeptides of 1014 and 1013 amino acids, respectively, with 92% identity; the DNA binding domain contains two potential zinc fingers, having no sequence identity to existing zinc finger classes, linked through a flexible glycine-rich region; a hydrophilic automodification domain containing 15 glutamic acid residues; and a nucleotide binding domain containing significant identity with the catalytic site of ricin A chain, an enzyme which like pADPRP also catalyzes cleavage of a purine-based-ribose glycosidic bond (Endo & Tsurug, 1987).

In order to examine the biochemical properties and structural/functional relationships of pADPRP, we wish to characterize the molecular details of this enzyme by site-directed mutagenesis. With this in mind, we have expressed the human gene, or portions thereof, in *Escherichia coli* under the control of the heat-inducible λ P_L promoter. Portions of human pADPRP 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 pADPRP in *E. coli*; however, the relative yield of pADPRP-expressed products appeared low, with a majority of these products appearing as proteins that are shorter than full-length. A unique feature of this expression system, developed in one of our laboratories, utilizes the fusion of ubiquitin to the amino-terminal end of pADPRP. Previously it was shown that ubiquitin, in phase with the amino terminus of target proteins, augments the accumulation of these proteins in *E. coli* from undetectable amounts to as much as 20% of the total cellular protein (Butt et al., 1989). Although portions of human pADPRP fusion proteins have also

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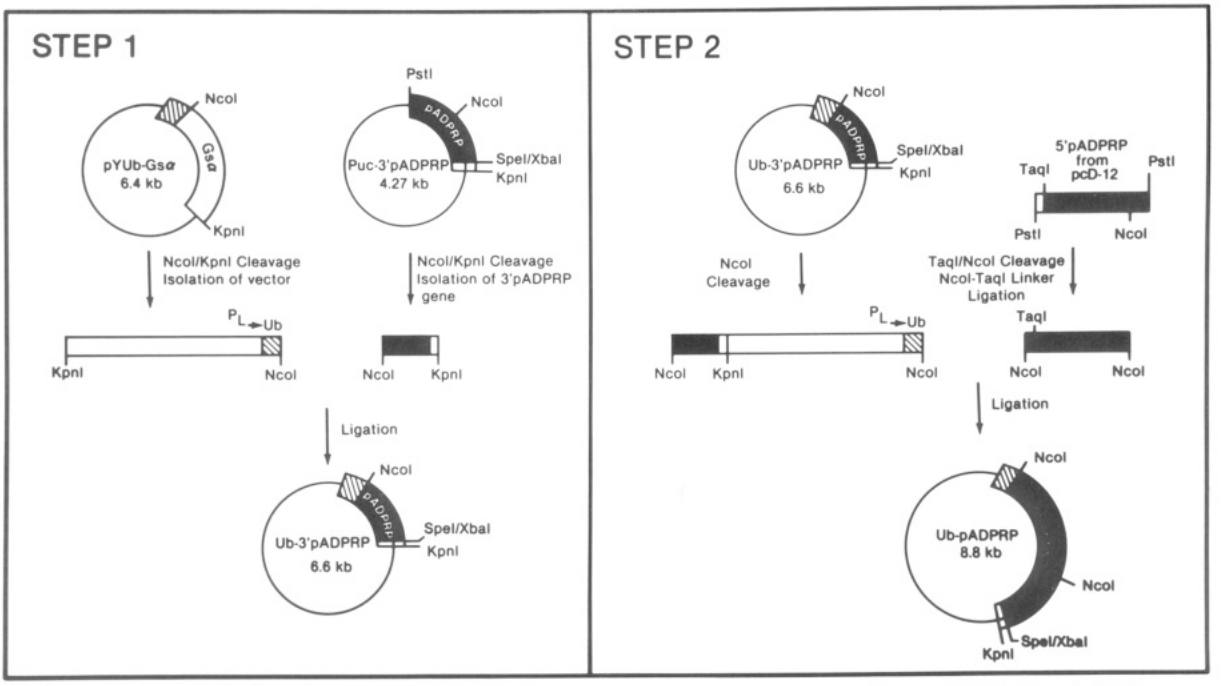


FIGURE 1: Flow diagram of Ub–pADPRP vector construction. Solid black represents DNA encoding pADPRP; the stippled box represents DNA encoding ubiquitin. Pertinent restriction sites, vector sizes in kilobases and the location of the λ P_L promoter are indicated. See text under Material and Methods for details.

been expressed in *E. coli* (Herzog et al., 1989), a salient feature of the ubiquitin fusion protein expression system is the availability of a protease activity in reticulocyte lysates which specifically cleaves the linkage between ubiquitin and the target protein yielding protein free of ubiquitin (Butt et al., 1989).

In part, the accumulation of ubiquitin fusion protein reflects an increased stabilization of the fusion product (Butt et al., 1989). In this stabilization process, ubiquitin may facilitate the proper folding of the fused protein, thereby preventing the formation of randomly folded proteins. Thus, ubiquitin is thought to maintain the biochemical activity of the fused protein. In previous experiments the biochemical activity of the target proteins was difficult to measure. Here we report the use of this system for maintaining biological activity of the target protein. In the present communication we measured the expression of a catalytically functional 113-kDa pADPRP in *E. coli*. In this system, the gene product was biochemically indistinguishable from the native enzyme and is an appropriate source for functional studies of pADPRP. We further demonstrated a novel utility of this expression system for the selection and screening of functional pADPRP mutants for this nuclear protein modifying enzyme.

Materials and Methods

Construction of Expression Vectors. The strategy used to construct Ub–pADPRP, an expression vector of human pADPRP in *E. coli*, involved several steps which are summarized in Figure 1. The starting material for this construction was pYUb–Gsα (Butt et al., 1989), an *E. coli* expression vector which contains the human α subunit of adenylate cyclase stimulatory GTP binding protein (Gsα) in fusion with the carboxyl terminus of ubiquitin. The first step involved substituting the human Gsα protein with the carboxyl-terminal portion of pADPRP. This produced a plasmid, termed Ub-3′pADPRP, which expresses ubiquitin in frame with the NAD binding domain of pADPRP. The source of the pADPRP sequence was pUC–pADPRP, a plasmid which contains a 1.9-kb *Pst*I–*Spe*I fragment encoding the carboxyl-terminal portion of pADPRP (Alkhatib et al., 1987) inserted into the *Pst*I–*Xba*I site of pUC-18. In order to construct Ub–3′pADPRP, a 1.7-kb *Nco*I–*Kpn*I fragment was isolated from pUC–pADPRP and ligated to *Nco*I–*Kpn*I-digested Ub vector. In the second step of Ub–pADPRP construction, a 1.9-kb *Pst*I fragment encoding the amino-terminal end of pADPRP was digested with *Taq*I removing the 5′ untranslated sequence as well as the sequence encoding the first 13 amino acid residues of pADPRP. An *Nco*I site was introduced, and the coding sequence of the missing amino acids was restored by utilizing an *Nco*I–*Taq*I synthetic DNA linker ligated into the *Taq*I site. Subsequently, the *Nco*I fragment, encoding the amino-terminal half of pADPRP, was ligated into the *Nco*I restricted plasmid Ub–3′pADPRP containing ubiquitin fused to the carboxyl-terminal portion of pADPRP. The resulting plasmid, Ub–pADPRP, contained the entire pADPRP coding sequence fused to the carboxyl terminal of ubiquitin. This fusion protein was under the control of the heat-inducible λ P_L promoter.

The expression of pADPRP without fusion to ubiquitin was achieved by digesting Ub–pADPRP with *Nde*I–*Nco*I, thereby removing the DNA sequence encoding ubiquitin. The resulting fragment was blunted and recircularized. This plasmid, designated V–pADPRP, contains pADPRP downstream of the λ P_L promoter with the ribosome binding site unaltered with respect to the distance to the translation start site.

In order to determine the effects of deletion in the various domains of pADPRP, we constructed deletion mutants of Ub–pADPRP. Figure 2 illustrates pADPRP's functional domains and the deletions constructed. PM-1 was constructed by digesting Ub–pADPRP with *Cla*I. The resulting 8.6-kb fragment was isolated, ligated, and used to transform *E. coli* AR58 cells. Similarly, PM-4 was constructed by deletion of a 762-bp *Bam*HI fragment, PM-5 by deletion of a 320-bp *Kpn*I fragment, and PM-6 by deletion of a 440-bp *Aat*II fragment.

Expression of Recombinant Proteins. E. coli AR58 cells transformed with the Ub expression vectors were grown at 32 °C to an absorbance of 0.5–0.6 unit at 650 nm in 10 mL of LB media containing 100 μg/mL ampicillin. Gene transcription was heat-induced by addition of 10 mL of LB media

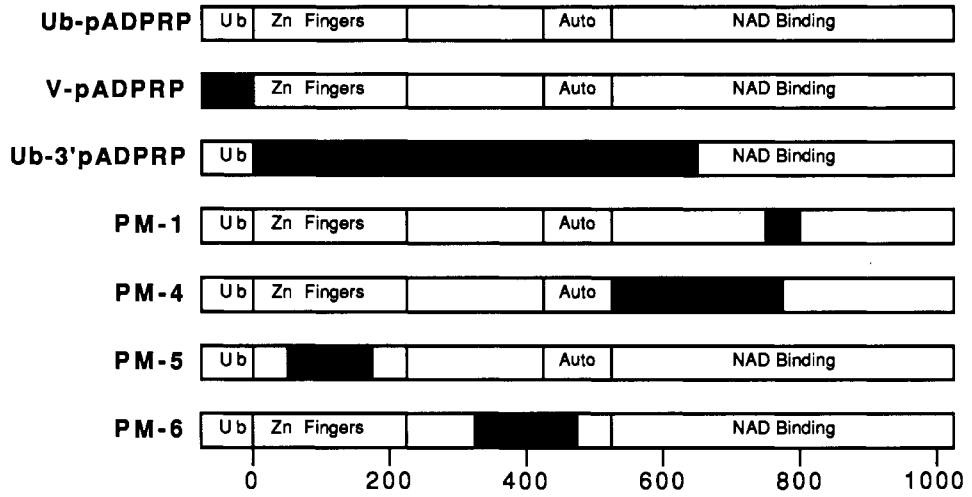


FIGURE 2: Schematic diagram of plasmid constructs expressing pADPRP. Ub–pADPRP is the ubiquitin full-length pADPRP fusion construct. Ubiquitin is composed of 76 amino acids. pADPRP contains 1014 amino acids (note scale on bottom). On the basis of biochemical (Kameshita, et al., 1986) and sequence analysis (Cherney et al., 1987) the approximate locations within pADPRP of the Zn^{2+} fingers, automodification domain (Auto), and NAD binding domain are illustrated. Black areas represent the approximate location of the deletions. None of the deletions alter the reading frame of the remaining sequence. In V–pADPRP, the entire ubiquitin coding region is deleted. The various vectors are altered as follows: Ub–3′pADPRP, pADPRP residues 1–685 are deleted; PM-1, pADPRP residues 791–835 are deleted; PM-4, residues 535–788 are deleted; PM-5, residues 52–157 are deleted; and PM-6, residues 315–460 are deleted.

at 65 °C. The culture was then incubated at 42 °C in an incubator shaking at 350 rpm. Samples were removed at desired time points and centrifuged, and the resulting cellular pellet was frozen in an ethanol/dry ice bath. The cell pellets were resuspended in polymerase buffer (0.1 M NaCl, 50 mM Tris, pH 8.0, 12% glycerol, 2 mM $MgCl_2$, and 0.1 mM PMSF) and sonicated on ice in a Branson sonifier at 50 mA three times for 6 s with a 1-min interval between pulses. Sonicates were either assayed for pADPRP activity or placed in 1% SDS sample buffer and boiled for 5 min for polyacrylamide gel electrophoresis (PAGE). For Western blots, proteins were electrophoretically transferred to nylon filters (Bio-Rad), and the blots were probed with antibodies against ubiquitin (Butt et al., 1989) or pADPRP (Alkhatib et al., 1987) using Bio-Rad's ProtoBlot AP system. The antibodies had been preexposed to *E. coli* protein extract to eliminate antibodies present in the antiserum which are reactive to *E. coli* proteins.

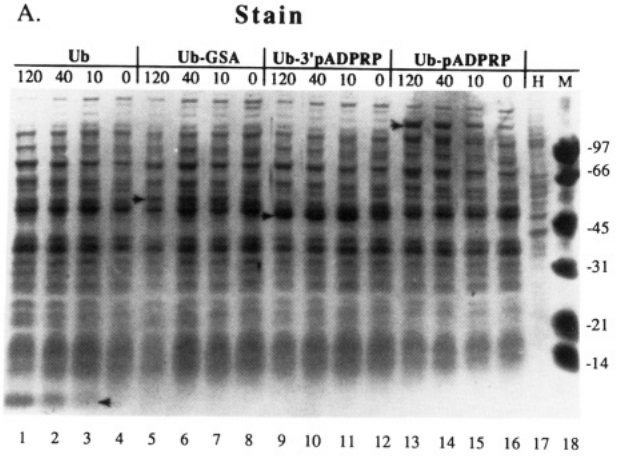
Activity Measurements. pADPRP activity was assayed as described previously (Cherney et al., 1985). Briefly, cell extracts (5–20 μg of protein) were incubated with 100 μM [^{32}P]NAD (specific activity 2 Ci/mmol) in 50 mM Tris, pH 8.0/25 mM $MgCl_2$/2 mM DTT for 45 s. The reaction was terminated by addition of ice-cold 20% TCA. Acid precipitable material was collected on Whatman 3C glass filters, and the radioactive incorporation was quantitated by liquid scintillation counting. For Lineweaver–Burk analysis, Ub–pADPRP cells were induced for 1 h and sonicates were assayed for polymerase activity with 10 μg/mL sonicated salmon sperm DNA and 25–900 μM NAD. Activity was linear with respect to time and the amount of protein. Activity gel analysis of pADPRP in SDS–PAGE was performed exactly as described by Scovassi et al. (1984) using [^{32}P]NAD (specific activity 1000 Ci/mmol). Equal aliquots of protein (8 μg) were loaded per lane. Autoradiography was performed overnight without intensifying screens.

Colony Filter Assay. *E. coli* AR58 cells harboring the respective plasmids were grown overnight at 30 °C on LB/ampicillin plates (100 μg/mL). Colonies were transferred to nylon filters and incubated at 42 °C for 2 h. Filters were floated on a solution of fresh lysozyme (2.0 μg/mL) for 20 min at 25 °C, air dried for 20 min, and incubated at 30 °C for 20 min with buffer containing 50 mM Tris, pH 8.0, 25 mM $MgCl_2$, 1 mM DTT, 10 μM [^{32}P]NAD (specific activity 2 Ci/mmol), and 5 μg/mL sonicated salmon sperm DNA. After 30 min, the filters were gently rinsed with ice-cold 20% trichloroacetic acid until the radioactivity in the wash fell to <500 cpm/mL. Filters were subjected to autoradiography with Kodak AR film.

RESULTS

Expression of the Recombinant Proteins. *E. coli* AR58 cells transfected with the respective plasmids were tested for their ability to express the appropriate-sized protein upon heat induction of the λ P_L promoter. The fidelity of induction was monitored by two criteria: Coomassie staining (Figure 3A) and immunostaining with antibodies specific to either pADPRP or ubiquitin (Butt et al., 1989) (Figure 3B). As a control, cells were transformed with plasmids tested previously containing ubiquitin alone or ubiquitin in fusion with GTP binding protein. *E. coli* AR58 cells, transformed with the Ub expression vectors containing either the entire coding sequence (Ub–pADPRP) or the NAD binding domain of pADPRP (Ub–3′pADPRP), were transferred to 42 °C and incubated for various times, and samples were analyzed for protein expression as described above (Figure 3A). Analysis of the protein samples from Ub–pAdPRP showed induction and accumulation of a 121-kDa protein corresponding to the predicted molecular weight of a full-length Ub–pADPRP fusion protein (Figure 3A, lanes 13–16). This protein, detected within a minute of induction (Figure 3A, lane 16), accumulated significantly (approximately 5–10% of total protein) by 120 min (lane 13). A similar high level of protein accumulation was also observed with Ub–3′pADPRP transformants (Figure 3A, lanes 9–12). Both Ub–Gsα (Figure 3A, lanes 5–8) and Ub (Figure 3A, lanes 1–4) clones expressed proteins at the appropriate MW.

To confirm the identity of induced proteins, Western blot analysis was performed using rabbit antibody to human pADPRP (Figure 3B). Analysis of the stained blots revealed pADPRP reactive bands only in induced samples transformed with the pADPRP vectors (Figure 3B, lanes 9–12 and 13–16). No immunoreactive bands were observed in extracts of cells transformed with the Ub or Ub–Gsα vectors (Figure 3B, lanes 1–8). The Ub–pADPRP sample displayed a prominent im-



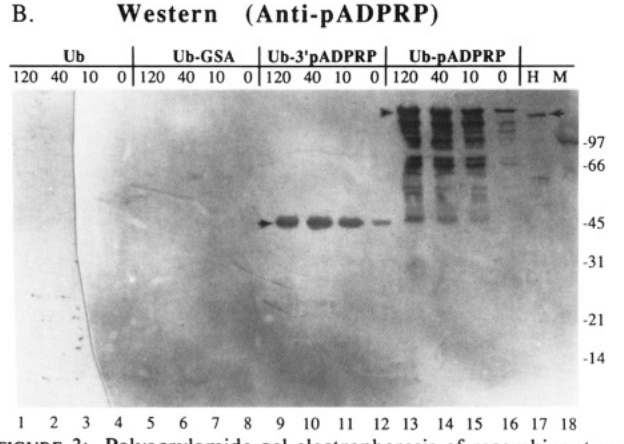
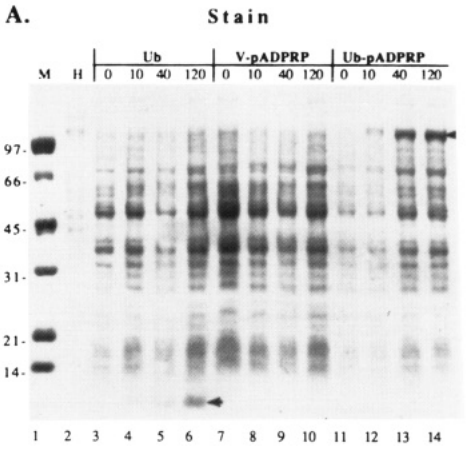
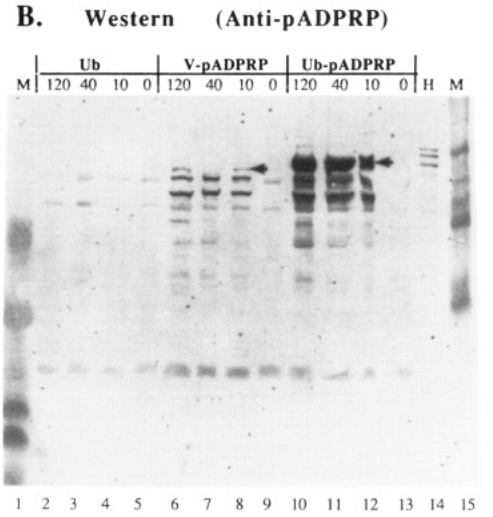


FIGURE 3: Polyacrylamide gel electrophoresis of recombinant eukaryotic proteins in *E. coli*. Cells harboring the indicated recombinant plasmids were heat-induced at 42 °C, aliquots were removed at 1–120 min, as indicated, and were sonicated and subjected to SDS–PAGE on 7.5% gels. Gels were visualized by staining with Coomassie brilliant blue (A) and by immunoblot analysis with polyclonal antibodies against pADPRP (B). Lanes: 1–4, extracts from Ub transformed cells; 5–8, extract from Ub–Gsα transformed cells; 9–12, extract from Ub–3′pADPRP transformed cells; 13–16, extract from Ub–pADPRP transformed *E. coli* AR58; 17, 3.5 M KCl extract of HeLa cells; 18, MW standards. Arrows in panels A and B identify the major inducible protein bands or HeLa pADPRP (lane 17B). Note: 0 time indicated in the figure actually represents 1 min.

munoreactive reactive band at 121 kDa, the correct molecular mass for the fusion protein, as well as bands corresponding to lower molecular mass fragments of pADPRP (Figure 3B, lanes 13–16). Although these smaller bands appeared almost instantaneously with induction (late 16), the most prominent band corresponded to the Ub–full-length pADPRP fusion product. In contrast, the vector containing ubiquitin in fusion with the 3′ region of pADPRP cDNA displayed induction of a single immunoreactive product (Figure 3B, lanes 9–12) with no indication of smaller polymerase products. The appearance of smaller polymerase related expression products has been noted before and has been attributed to protein translation from internal start signals located within the pADPRP cDNA (Herzog et al., 1990; Ikejima et al., 1990). However, antibodies to both ubiquitin and pADPRP identify similar high molecular weight products (see Figure 4B,C), indicating that many amino-terminal ends are similar in these protein fragments. This suggests that both premature termination and alternative start sites are responsible for these smaller products.

In order to determine whether ubiquitin was required for the overexpression of recombinant pADPRP, we analyzed the induction and accumulation of pADPRP without fusion to





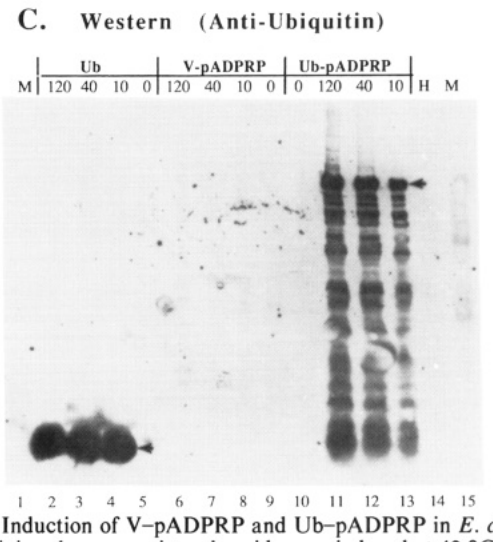


FIGURE 4: Induction of V–pADPRP and Ub–pADPRP in *E. coli*. Cells containing the appropriate plasmids were induced at 42 °C for 0, 10, 40, and 120 min and subjected to SDS–PAGE. Gels were visualized by staining with Coomassie brilliant blue (A) or by immunoblot analysis with polyclonal antibodies against pADPRP (B) or ubiquitin (C). Arrows indicate induced protein bands. M, marker proteins (sizes at left in kilodaltons); H, 3.5 M KCl extract of nuclei from HeLa cells. Lanes: 3–6, extracts from Ub; 7–10, extracts from V–pADPRP; 11–14, extracts from Ub–pADPRP.

ubiquitin (V–pADPRP). As a control, cells containing Ub and Ub–pADPRP vectors were also induced. Analysis of protein expression by SDS–PAGE and Coomassie stain (Figure 4A) showed induction of the Ub–pADPRP fusion protein (Figure 4A, lanes 12–14). No expression of the fusion

protein was detected in uninduced AR58 cells containing the Ub–pADPRP vector (Figure 4A, lane 11). In protein samples from the vector V–pADPRP, no accumulation of pADPRP was noted (Figure 4A, lanes 7–10). Cells transformed with the Ub expression vector when induced accumulated a prominent protein band at approximately 8000 kDa, corresponding to the migration of ubiquitin in SDS–PAGE (Figure 4A, lanes 4–6).

To determine if any pADPRP related proteins were expressed in cells containing the pADPRP expression vector lacking Ub. Western blot analysis was performed on these samples using rabbit antibodies to ubiquitin and pADPRP. Analysis with anti-pADPRP antibodies revealed prominent full-length polymerase as well as smaller polymerase related bands in the induced pADPRP fusion vector (Figure 4B, lanes 10–12); however, in the absence of ubiquitin only a slight immunostaining at 113-kDa (Figure 4B, lanes 6–8) was observed. In these latter samples smaller polymerase fragments appeared to be the major product. No polymerase related products were noted in cells transformed with the Ub expression vector, although *E. coli* related bands were detected in this immunoblot (Figure 4B, lanes 2–5). This reflects incomplete saturation of antibodies to *E. coli* protein indigenous to rabbit anti-serum. Analysis with anti-ubiquitin antibody revealed prominent ubiquitin related products in the induced Ub–pADPRP samples (Figure 4C, lanes 11–13) and a single product in the induced Ub samples (Figure 4C, lanes 2–4). No ubiquitin related products were observed in induced cells containing the vector V–pADPRP (Figure 4C, lanes 6–8).

These data demonstrate that the unfused pADPRP protein was either translated at a lower rate or digested soon after its translation and confirm earlier observations utilizing Ub–Gsα fusion expression that ubiquitin augments and stabilizes the expression of cloned gene products in *E. coli* (Butt et al., 1989).

Catalytic Activity of Bacterially Expressed Eukaryotic Polymerase. With a view toward clarifying the biological role of pADPRP and simultaneously to test ubiquitin's ability to permit proper protein folding, it was important to determine if the bacterially expressed pADPRP fusion protein was enzymatically similar to the native enzyme. Activity measurements for pADPRP, however, are experimentally difficult to assess due to the complex nature of the enzyme's catalytic activities. For example, pADPRP catalyzes the initiation, elongation, and branching of poly(ADP–ribose) on various acceptor molecules which may or may not be accessible for modification (Ueda et al., 1982). The enzyme also undergoes extensive automodification and possesses considerable NADase activity distinct from its ADP-ribosylation activity. Additionally, these differing enzyme activities are strictly dependent on DNA for activity. Thus, pADPRP activity increases proportionally to the number of strand breaks within a given DNA template. Accordingly, we analyzed pADPRP activity using several different enzyme assays.

In the first assay, endogenous sonicated *E. coli* chromosomal DNA together with a high level of Mg^{2+}, which promotes the automodification reaction (Ferro & Olivera, 1982), was used under otherwise standard conditions to monitor polymerase activity. Using this assay, a high level of pADPRP activity was revealed in induced samples containing the Ub–pADPRP vector (Table I). The observed incorporation of [^{32}P]-(ADP–ribose) into *E. coli* extracts was inhibited by the addition of 3-aminobenzamide (3AB), an established pADPRP inhibitor (Table I). No pADPRP activity was detected in uninduced Ub–pADPRP cells or in induced cells lacking a pADPRP vector. In V–pADPRP induced samples, where ubiquitin was not fused to the enzyme, pADPRP activity was detected, but at 10% of the level of the Ub–pADPRP fusion protein (Table I).

Table I: Measurement of pADPRP Activity in *E. coli* Extracts Containing pADPRP Vectors[a]

cells	pmol/(min·mg of protein)
uninduced Ub–pADPRP	4.0
induced Ub	8.0
induced Ub–pADPRP	3200
induced Ub–pADPRP + 3AB	228
induced V–pADPRP	330

[a] *E. coli* AR58 cells harboring the indicated plasmids were grown to an OD_{260} of 0.5, aliquots were removed, and the remaining cells were induced at 42 °C for 20 min. After sonication, cell extracts were assayed for pADPRP activity as described under Materials and Methods.

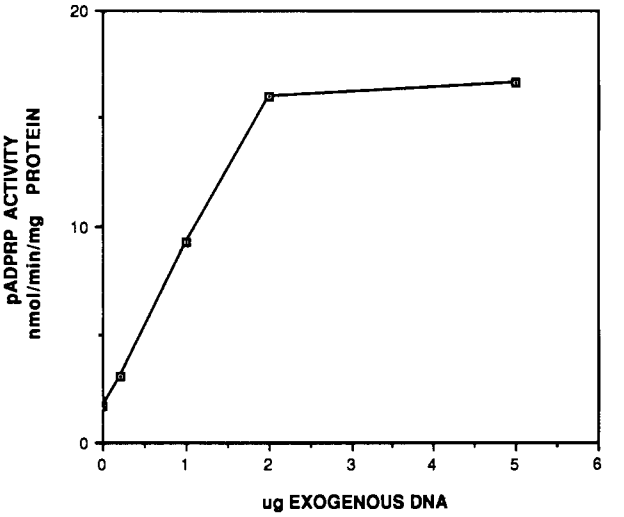


FIGURE 5: The effect of exogenous sonicated DNA on the rate of poly(ADP–ribose) synthesis. *E. coli* AR58 cells containing vector Ub–pADPRP were induced for 1 h, sonicated, and assayed in triplicate for pADPRP activity at various concentrations of sonicated salmon sperm DNA. Reaction conditions were as described under Materials and Methods.

To confirm that the incorporation of ^{32}P-labeled TCA-precipitable material represented authentic pADPRP activity, several further biochemical characterizations were performed. Since pADPRP activity is coordinated with the number of strand breaks in the DNA template, the polymerase activity in the bacterially expressed protein was measured in samples containing increasing amounts of fragmented DNA (Figure 5). The data demonstrate that the activity was stimulated (10–15-fold) by the addition of 5 μg of exogenous sonicated DNA. At higher concentrations of sonicated DNA, no change in polymerase activity was observed. The apparent K_m for NAD of the *E. coli* expressed pADPRP fusion protein was determined to be 100 μM NAD, which is consistent with the K_m for NAD of the enzyme purified from a variety of eukaryotic sources (Ueda & Hayaishi, 1985).

To clarify that the 120-kDa *E. coli* expressed protein was authentic pADPRP, proteins from induced cells were assayed by activity gel analysis. In this procedure, induced *E. coli* extracts were subjected to SDS–PAGE in the presence of fragmented DNA, and polymerase activity was determined directly in the gel by utilizing [^{32}P]NAD (Scovassi et al., 1983) (Figure 6). pADPRP activity, migrating at approximately 121 kDa, was detected in induced cells containing the Ub–pADPRP vector (Figure 6, lane 3). Weak signals were also observed at lower molecular weights, indicating that polymerase fragments contained significant activity. No activity was observed in untransformed AR58 cells.

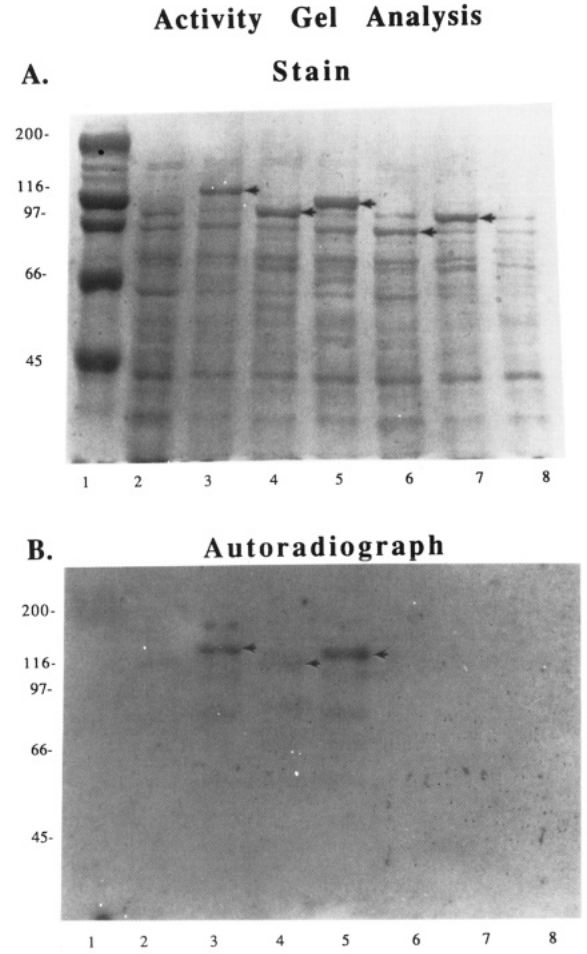


FIGURE 6: Activity gel analysis of pADPRP expressed in *E. coli*. *E. coli* extracts were subjected to SDS–PAGE and either Coomassie stained (A) or processed for activity analysis (B) as described under Materials and Methods. Lanes: 1, marker proteins (sizes at left in kilodaltons); 2, extract from untransformed, induced *E. coli* AR58; 3, extract from induced Ub–pADPRP; 4, extract from PM-6; 5, extract from PM-5; 6, extract from PM-4; 7, extract from PM-3 (a spontaneous pADPRP deletion mutant), 8, extract from PM-1. All inductions were for 1 h.

In order to determine the measurable units of pADPRP activity produced in the *E. coli* expression system, samples were analyzed for in vitro activity at various times after induction (Figure 7). The data displayed increasing specific activity of pADPRP up to 33 units/mg of protein at 120 min after induction. Two hours after induction, the activity levels decreased, possibly reflecting protein degradation and/or cell death. Previously, Ito et al. (1979) had shown that the V_{max} of pADPRP in homogeneous preparations of this enzyme was 1400 units/mg of protein. In Ub–pADPRP transformed cells, induced for synthesis of the enzyme for 2 h, V_{max} was 71 units/mg of protein or 5% of the purified native enzyme. Since in these cells pADPRP comprises 5–10% of the total protein content, recombinant pADPRP molecules appear to be as active as purified native enzyme preparations. These data demonstrate that ubiquitin allows the accumulation of biologically active protein which, as the measured parameters indicate, is essentially indistinguishable from native enzyme preparations.

pADPRP Mutants. In an initial characterization of pADPRP mutants we have deleted portions of the polymerase's functional domains. These deletion mutants (Figure 2) express the expected polymerases as judged by Coomassie staining (Figure 6A) and immunostaining with antibody against pADPRP (data not shown). In order to determine the effects

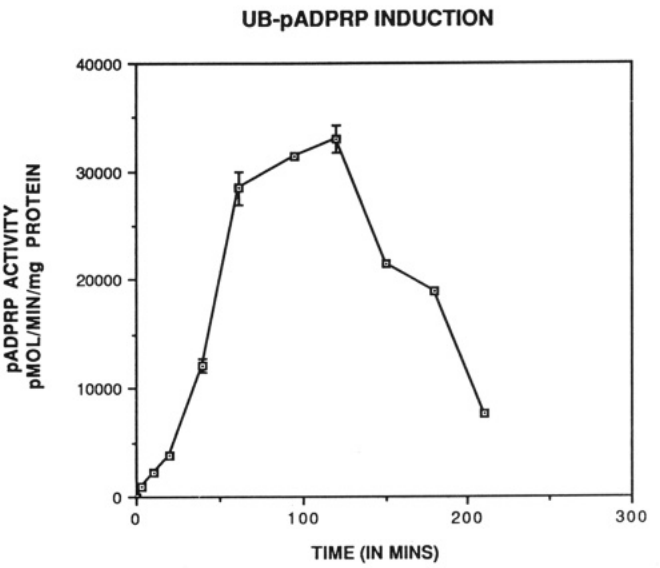


FIGURE 7: pADPRP activity following induction of Ub–pADPRP cells. AR58 cells containing the plasmid Ub–pADPRP were induced at 42 °C, and aliquots were removed at 0–200 min, placed on ice, and assayed for pADPRP activity as described under Materials and Methods.

Table II: Deletion of Various pADPRP Protein Domains on Enzyme Activity[a]

cells	mutation region	+DNA [pmol/(min·mg of protein)]	−DNA [pmol/(min·mg of protein)]
Ub–pADPRP	full-length	25900 ± 2500	5340 ± 30
PM-4	(NAD-binding)	0	0
PM-5	(zinc finger region)	3000 ± 200	2850 ± 500
PM-6	(automodification)	3410 ± 400	2700 ± 350

[a] *E. coli* AR58 cells harboring the plasmids indicated were induced at 42 °C for 1 h and sonicated, and 20-μg protein aliquots of the cell extracts were assayed for pADPRP activity as described under Materials and Methods. pADPRP activity is expressed as picomoles of ADP–ribose incorporated per minute per milligram of protein.

of deletion in the various domains of the polymerase on pADPRP activity, we induced the appropriate cells and assayed for polymerase activity by both the sonication assay and activity gel analysis. Relative to Ub–pADPRP transformed cells, a deletion in the NAD binding domain (PM-4) eliminates polymerase activity (Table II). Deletion of a portion of the DNA binding domain (PM-5) or the automodification domain (PM-6) reduces polymerase activity by 80%. Both of these mutants are nonresponsive to addition of DNA (Table II). In activity gel analysis a strong signal was observed for the DNA binding mutant (Figure 6B, lane 5), whereas a weak signal was obtained for the automodification mutant (Figure 6B, lane 4). Polymerase mutants with no detectable activity by the sonication assay also revealed no activity in the activity gel analysis (Figure 6B, lanes 6, 7, and 8).

Colony Filter Assay for pADPRP Activity. A powerful approach for clarifying the biochemical functions of this enzyme is through mutagenesis. The ability to express an enzymatically normal pADPRP in *E. coli* suggests that this protein is an appropriate source for further structure/function studies of pADPRP. Currently we have constructed a number of deletion mutants of pADPRP; however, increased resolution of pADPRP's functional domains is required for a detailed understanding of its biochemical function. A rapid and efficient method of generating subtle polymerase mutants is through saturation cassette mutagenesis (Reidhaar-Olson & Sauer, 1988). In this procedure areas of particular interest

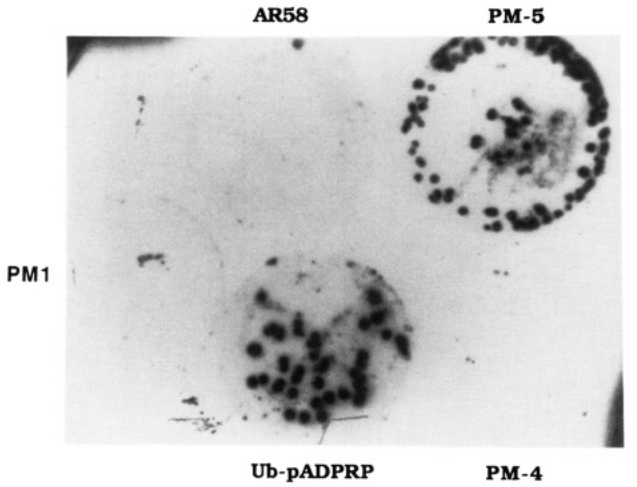


FIGURE 8: Colony filter analysis of pADPRP expressed in *E. coli*. Filters containing between 40 and 100 colonies with the various Ub expression vectors were induced and assayed for pADPRP activity as described under Materials and Methods. Filters were subjected to autoradiography for 4 h with Kodak AR film without the use of intensifying screens.

(i.e., DNA binding or NAD binding) are saturated with various point mutations. With this method we can determine the spectrum of functionally acceptable substitutions at residue positions near the site of interest. By extrapolation we can identify residues in the polymerase sequence which contribute to enzyme function.

For this approach a screening procedure which would identify functional polymerase molecules is essential. Accordingly, we have developed a colony filter assay in which individual clones can be assayed for polymerase activity. This assay involves transfer of bacterial colonies containing pADPRP to nylon filters, lysis, incubation of cells with [^{32}P]NAD, precipitation of long-chain poly(ADP–ribose) by TCA, and detection of radioactive material by autoradiography.

In order to illustrate the feasibility of such a system, *E. coli* cells harboring polymerase expression vectors which synthesize enzymatically functional, partially functional, and nonfunctional polymerase molecules were plated and grown at 32 °C. After colony formation, cells were transferred to nitrocellulose, incubated at 42 °C for 2 h, and treated and assayed as described. All colonies containing the pADPRP vectors expressed the polymerase protein as judged by immunoblot analysis (data not shown). Figure 8 shows the colony assays for eukaryotic pADPRP activity from these filters. All colonies that contained the Ub–pADPRP vector showed poly(ADP-ribosylation) activity as indicated by intense ^{32}P incorporation of radioactive material into TCA-precipitable material. Background radioactive signals were observed with cells expressing a nonfunctional polymerase (i.e., PM-1 and PM-4), while strong incorporation of NAD was noted in cells expressing a polymerase with 10% of normal enzymatic activity (PM-5). This expression system thus provides a method for screening pADPRP mutants, which either express inactive pADPRP or express pADPRP with low levels of activity. Currently, we are using this system to map functional residues within the DNA binding domain.

Discussion

The hypothesis that ubiquitin added at the amino terminus of target proteins stabilizes their expression has been extended to include the stabilization of full-length human pADPRP. We have further demonstrated that the biological activity of this overexpressed protein is maintained. Importantly, we found that the specific activity of the recombinant enzyme obtained from *E. coli* was similar to that of native enzyme preparations. This enzyme's response to Mg^{2+} and to strand breaks in DNA, its apparent K_m for NAD, and its extensive automodification and behavior in activity gels all suggest that the recombinant protein resembles the native enzyme. In contrast to the Ub–pADPRP fusion described here, we have found that thermal induction of the human estrogen receptor, as a ubiquitin fusion product, produced largely inactive protein. However, chemical induction by the addition of nalidixic acid produced a protein which behaved identically to the wild-type receptor in ligand and DNA binding properties (Wittliff et al., 1990). These results indicate that relatively heat-stable proteins (such as pADPRP) can be very efficiently expressed as a Ub fusion product while maintaining biological activity, but this method may not be applicable for heat sensitive proteins.

In experiments described here, ubiquitin is still attached to the polymerase, suggesting that ubiquitin fusion to pADPRP does not interfere with the biochemical activity of this enzyme. However, a salient feature of the Ub expression system is the ability to specifically cleave the junction between C-terminal glycine of ubiquitin and the fused protein. This is achieved with the enzyme NH–ubiquitin protein endoprotease. Thus, ubiquitin may in specific constructs easily be released from pADPRP resulting in the authentic target protein. In the expression vectors described, for convenience, an *Nco*I site was used to place the polymerase in frame with ubiquitin. This change introduced a C-terminal alanine which is not efficiently cleaved by the endoprotease. We are now attempting to reintroduce glycine at the junction between ubiquitin and the polymerase.

The expression of full-length human pADPRP in *E. coli* has been reported (Ikejima et al., 1990). Although this bacterially expressed protein had similar biochemical characteristics to those of pADPRP purified from mammalian tissues, a large fraction of the expressed products appeared as proteins that were shorter than full-length. Although different promoters are used in the respective expression systems, our ability to efficiently express full-length pADPRP probably reflects the fusion of ubiquitin to the amino-terminal end of pADPRP. Although ubiquitin in fusion with target proteins can dramatically increase the half-life of these proteins (Butt et al., 1989), data here suggest a prominent role for ubiquitin in efficient protein translation. Without ubiquitin, translation of pADPRP appears to start primarily at internal start signals within pADPRP producing truncated proteins [Figure 4 and Ikejima et al. (1990)]. Since full-length fusion protein is the major product with most truncated proteins also containing ubiquitin (Figure 4B,C), protein translation appears to initiate primarily at ubiquitin's start site. This suggests that the bacterial translation system preferentially recognizes ubiquitin's start site. Two important consequences emerge from these differences in the respective expression systems. First, purification of the full-length pADPRP is not complicated by the abundance of incomplete polymerase proteins which could copurify with authentic full-length protein. Second, the sensitivity of detecting pADPRP activity is enhanced due to the huge accumulation of the complete protein. Hence, we are able to detect significant polymerase activity in mutants (PM-5) reported to have no detectable activity in activity gel analysis (Ikejima et al., 1990).

An initial characterization of pADPRP by deletion mutagenesis indicates that all these polymerase mutants are expressed to similar high levels, suggesting that the Ub expression

system is advantageous for overexpressing mutants of pADPRP. Although variations of the level of expression are observed, these variations occur within the same expression construct. For example, in data reported here, PM-4 is only slightly expressed (Figure 4A, lane 8) but is usually expressed at levels similar to that of the full-length polymerase.

Biochemical characterization of these deletion mutants demonstrates the importance of an intact NAD binding domain for polymerase activity. In contrast, deletion of zinc finger 2 and the region between fingers (PM-5) eliminates most but not all of the enzymatic activity. Since Gradwohl et al. (1990) have demonstrated that this deleted portion of pADPRP plays a key role in the recognition of single-strand breaks in DNA, it is not surprising that the polymerase's activation by DNA strand breaks is eliminated in this mutant. This residual activity is not due to the presence of the first zinc finger since deletion of both fingers results in the same low level of activity. One interpretation of these results is that in the unbound state the zinc finger domain shields the catalytic domain of the polymerase and the enzyme is totally inactive. When bound to DNA this protein undergoes a major conformation change such that the catalytic site is now open and in an active state. Elimination of the zinc fingers exposes the active site but in a suboptimal conformation. In a similar manner, deletion of a portion of the automodification domain (PM-6), although leaving the zinc finger domain intact, may likewise prevent the structural transformations which occur in the DNA-dependent enzymatic activation of pADPRP.

The ability to produce functional, purified pADPRP in large quantities will facilitate biochemical structure/function studies of pADPRP as well as help elucidate polymerase interactions with other proteins. We are currently using the screening and selection method described here to map functional amino acid residues within defined domains of the polymerase. Such studies should provide new insights into this important but perplexing molecule.

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