Dual Function for Poly(ADP-ribose) Synthesis in Response to DNA Strand Breakage

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Received February 16, 1994; Revised Manuscript Received April 15, 1994®

ABSTRACT: Soluble extracts of human cells repair γ-ray-induced single-strand breaks in DNA. Accompanying NAD-dependent automodification of poly(ADP-ribose) polymerase is required for effective DNA rejoining. The kinetics of poly(ADP-ribose) synthesis by this polymerase, and subsequent polymer degradation by poly(ADP-ribose) glycohydrolase, have been compared with the rate of DNA repair. The results agree with previous *in vivo* data. In response to addition of γ-irradiated plasmid DNA, rapid and heavy automodification of poly(ADP-ribose) polymerase occurred in NAD-containing human cell extracts. After 2 min at 30 °C, when very little DNA rejoining had yet occurred, synthesis of long polymers essentially ceased, although only a minor fraction of the NAD had been consumed. Poly(ADP-ribose) chains were then reduced to oligomer size by poly(ADP-ribose) glycohydrolase. These short chains were present for longer times and were sufficient to permit DNA repair. Thus, most but not all poly(ADP-ribose) synthesis could be suppressed without marked inhibition of DNA repair, and prolonged occurrence of long poly(ADP-ribose) chains in consequence to glycohydrolase inhibition did not improve DNA repair. The temporary presence of short poly(ADP-ribose) chains on poly(ADP-ribose) polymerase avoids inhibition of excision-repair by that protein, but the initial very transient formation of long and branched chains of poly(ADP-ribose) in response to DNA damage apparently serves an entirely different purpose. Local poly(ADP-ribose) synthesis in the vicinity of a DNA strand interruption causes negative charge repulsion, and this may function to prevent accidental homologous recombination events within tandem repeat DNA sequences.

An immediate stress response in mammalian cells exposed to ionizing radiation or alkylating agents is the synthesis of poly(ADP-ribose) from NAD in cell nuclei. The poly(ADP-ribose) polymerase is a 113-kDa protein that binds tightly to DNA but requires DNA strand breaks for activation (Benjamin & Gill, 1980; Ménissier-de Murcia et al., 1989). Newly synthesized polymers remain covalently attached to poly(ADP-ribose) polymerase, and the automodified enzyme no longer binds DNA (Zahradka & Ebisuzaki, 1982; Ferro & Olivera, 1982). Poly(ADP-ribose) chains are labile *in vivo* because they are degraded by a nuclear poly(ADP-ribose) glycohydrolase that preferentially attacks long polymers (Hatakeyama et al., 1986; Jonsson et al., 1988; Ménard et al., 1990; Maruta et al., 1991). Nevertheless, the transient synthesis of poly(ADP-ribose) improves the cellular capacity for DNA repair after exposure to DNA-damaging agents, and inhibition of polymer synthesis by 3-aminobenzamide sensitizes cells to DNA damage (Shall, 1984; Cleaver & Morgan, 1991).

Poly(ADP-ribose) polymerase initially competes with DNA repair enzymes at strand breaks, as indicated by studies with DNA repair-competent human cell extracts depleted of poly(ADP-ribose) polymerase (Satoh & Lindahl, 1992) and by expression of a subcloned DNA-binding domain of the enzyme *in vivo* (Molinete et al., 1993). Automodification of poly(ADP-ribose) polymerase is necessary to relieve this inhibitory effect, accounting for the apparent enhancement of DNA repair through poly(ADP-ribose) synthesis (Satoh et al., 1993). Suppression of poly(ADP-ribose) synthesis by 3-aminobenzamide consequently delays the rejoining of DNA strand interruptions (Ahnström & Ljungman, 1988). Such delayed DNA rejoining in turn leads to other cellular changes, e.g., prolonged accumulation of p53 in cell nuclei (Lu & Lane, 1993).

In the present work, we have investigated the kinetics of poly(ADP-ribose) synthesis and degradation and simultaneous DNA repair in a human cell-free system. Many related studies have been performed with viable or permeabilized mammalian cells [reviewed by Cleaver and Morgan (1991)], but the use of soluble cell extracts offers some distinct advantages. Thus, specific proteins [e.g., poly(ADP-ribose) polymerase, poly(ADP-ribose) glycohydrolase, or relevant antibodies] can be added to, or depleted from, the system, and charged low molecular weight compounds which would not enter cells can be included, that is, radioactively labeled NAD as the direct precursor of poly(ADP-ribose) synthesis, or a recently introduced specific inhibitor of poly(ADP-ribose) glycohydrolase, ADP-dihydroxypyrollidine (Slama et al., 1992). Moreover, it is not necessary to prepare the extract from cells exposed to DNA-damaging agents. Instead, a DNA-free cell extract from normal cells can be supplemented with a DNA substrate containing a precalibrated amount of DNA damage to trigger poly(ADP-ribose) synthesis and DNA repair. Here, plasmid DNA containing one γ-ray-induced single-strand break per plasmid molecule has been employed; such breaks cannot be directly rejoined by DNA ligase, and the conversion into repaired, covalently closed circular molecules has been followed in parallel with the poly(ADP-ribosyl)ation reaction.

MATERIALS AND METHODS

Reagents. The poly(ADP-ribose) polymerase inhibitors 3-aminobenzamide and 2-nitro-6(5*H*)-phenanthridinone were

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® Abstract published in *Advance ACS Abstracts,* June 1, 1994.

0006-2960/94/0433-7099$04.50/0

obtained from Sigma and Aldrich. The poly(ADP-ribose) glycohydrolase inhibitor ADP-dihydroxypyrrolidine was a kind gift from Dr J. Slama, University of Toledo, Toledo, OH. [^{32}P]NAD (800 Ci·mmol^{-1}) was purchased from New England Nuclear.

Poly(ADP-ribose) polymerase was purified from calf thymus according to Zahradka and Ebisuzaki (1984). Poly(ADP-ribose) glycohydrolase was purified from calf thymus as described (Thomassin et al., 1990). A monoclonal antibody and a rabbit polyclonal antibody against poly(ADP-ribose) polymerase, C-2–10 and 318, respectively, have been described elsewhere (Lamarre et al., 1988). ^{125}I-Labeled anti-mouse IgG was obtained from ICN.

Cell-Free Assay System. The human lymphoblastoid cell line GM06315A, derived from a normal individual, was obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ) and propagated in suspension culture in RPMI 1640 medium supplemented with 15% fetal bovine serum and antibiotics. The following simple procedure was employed to obtain cells of >90% viability, as judged from the Trypan-blue exclusion test: Proliferating cell cultures (1.8 L, $\sim 6 \times 10^5$ cells/mL) were harvested by low-speed centrifugation, and the cells were suspended in 10 mL of serum-free RPMI 1640 medium ($\sim 10^8$ cells/mL). The cell suspension was mixed with 10 mL of Ficoll-Paque (Pharmacia), and the mixture was layered on top of 10 mL of Ficoll-Paque in a 50-mL Falcon culture tube. After centrifugation at 1600 rpm for 30 min at room temperature in an IEC Centra-4X swing-out rotor, most of the top phase was removed, and the living cells were recovered from the interphase. Dead cells were pelleted at the bottom of the tube and were discarded. The living cells were washed twice with 50 mL of a buffer containing 126 mM NaCl, 15 mM Tris-HCl (pH 7.6), 0.5 mM KCl, 0.1 mM $MgCl_2$, 5 μM $CaCl_2$, and 0.5% glucose. Cell extracts were prepared according to Manley et al. (1983). When required, poly(ADP-ribose) polymerase was removed from cell extracts by double-stranded DNA–cellulose chromatography in 0.4 M NaCl (Satoh et al., 1993).

DNA repair assays were performed as described previously (Satoh & Lindahl, 1992; Satoh et al., 1993). Briefly, a reaction mixture (50 μL) contained 45 mM Hepes·KOH (pH 7.8), 70 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.4 mM EDTA, 0.25 mM NAD, 2 mM ATP, 20 μM each of dATP, dCTP, dGTP, and TTP, 40 mM phosphocreatine, 2.5 μg of creatine phosphokinase, 1 μg of bovine serum albumin, 3% glycerol, 0.3 μg of nicked plasmid DNA (Bluescript II KS$^+$ plasmid, Stratagene) obtained after exposure of plasmids to 50 Gy of γ-irradiation in 50 mM Tris-HCl, pH 7.8 (20% nicking), followed by purification of open circular molecules by ethidium bromide/CsCl density gradient centrifugation, and human cell extract (50 μg of protein, when not otherwise stated). Incubations were at 30 °C, for 30 min if not otherwise stated. Reactions were terminated by addition of SDS, excess EDTA, and proteinase K, and the plasmid DNA was analyzed by ethidium bromide/agarose gel electrophoresis. DNA bands were visualized with ultraviolet light and photographic negatives analyzed by densitometry to estimate the degree of conversion of nicked to covalently closed circular DNA.

Synthesis of poly(ADP-ribose) was followed in the same reaction mixture supplemented with 2 μCi of [^{32}P]NAD. Reactions were carried out at 30 °C and terminated by the addition of 500 μL of ice-cold 20% trichloroacetic acid, precipitated material was collected on glass filters, washed, and dried, and radioactive material was determined by liquid scintillation counting (Satoh et al., 1993). For size analysis of the synthesized poly(ADP-ribose), reaction mixtures contained 20 μCi of [^{32}P]NAD. Reactions were stopped by addition of 500 μL of ice-cold 20% trichloroacetic acid and collection of precipitated material by centrifugation; the poly(ADP-ribose) was dissolved and liberated from protein by alkali treatment (1 M KOH/50 mM EDTA at 37 °C for 2 h) followed by purification by boronate chromatography as described previously (Huletsky et al., 1989). The polymers were fractionated by SDS–polyacrylamide (20%) gel electrophoresis and visualized by autoradiography according to Panzeter and Althaus (1990).

Analysis of Automodified Poly(ADP-ribose) Polymerase. Poly(ADP-ribosyl)ation reactions were performed in the DNA repair assay reaction mixture supplemented with 10 μCi of [^{32}P]NAD. Reactions were carried out at 30 °C and terminated at various times by addition of 55 μL of ice-cold acetone, and insoluble protein was collected by centrifugation. The samples were analyzed by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) with a 3% stacking and a 10% separation gel. Alternatively, samples were dissolved in 10 μL of 30 mM phosphate (pH 6.0), 4.5 M urea, 0.2% SDS, 5 mM dithiothreitol, and 0.01% bromophenol blue, incubated at 65 °C for 10 min, and analyzed in an agarose–polyacrylamide composite gel as described previously (Huletsky et al., 1989). The gels were stained with Coomassie brilliant blue R-250, dried, and autoradiographed.

For immunoblotting and activity blots, incubations and termination of reactions were the same, but proteins were fractionated by SDS–polyacrylamide gel electrophoresis with a 3% stacking and a 7.5% separation gel. Proteins were then transferred from the gel onto a nitrocellulose membrane, either overnight for immunoblotting or 1 h for activity blots. Under the conditions used, >90% of automodified poly(ADP-ribose) polymerase molecules with apparent molecular mass of up to 200 kDa were transferred. For immunoblotting, the membrane was incubated in skimmed milk and a 1:1000 dilution of C-2–10 monoclonal antibody overnight at 4 °C. After being washed, the membrane was incubated with 1 μCi·mL^{-1} anti-mouse IgG ^{125}I-labeled antibody for 1 h at room temperature, washed, and autoradiographed. Purified bovine poly(ADP-ribose) polymerase (23 ng) was used as reference. For activity blots, the poly(ADP-ribose) polymerase on the nitrocellulose membrane was renatured and then incubated with DNase I-treated calf thymus DNA and [^{32}P]NAD as described by Simonin et al. (1990). Poly(ADP-ribose) polymerase activity was detected by autoradiography.

Immunoprecipitation of Poly(ADP-ribose) Polymerase. Poly(ADP-ribosyl)ation reactions were carried out in the standard DNA repair assay reaction mixture for 2 min at 30 °C, except for reduction of the NAD concentration to 32 nM (2 μCi of [^{32}P]NAD) instead of 0.25 mM. Reactions were terminated by addition of 55 μL of ice-cold acetone followed by centrifugation to collect precipitated materials. The pellets were solubilized in 250 μL of 175 mM sodium phosphate (pH 7.5), 100 mM NaCl, 1% Triton-X 100, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mM EDTA (buffer A), and protease inhibitors (0.17 μg·mL^{-1} phenylmethanesulfonyl fluoride, 27 μg·mL^{-1} aprotinin, and 0.5 μg·mL^{-1} each of leupeptin, pepstatin, chymostatin, and tosylphenylalanine chloromethyl ketone). After incubation at 4 °C for 30 min, 5 μL of polyclonal antibody 318 was added, and the incubation was continued overnight at 4 °C. Protein A–Sepharose (Pharmacia) was added to a final concentration of 2% and the incubation continued for 1 h at 4 °C. The protein A–Sepharose antigen–antibody complexes were collected by centrifugation,

washed 6 times with buffer A, and analyzed by SDS–polyacrylamide gel electrophoresis with a 3% stacking and a 10% separation gel. The gels were stained with Coomassie blue, dried, and subjected to autoradiography.

Poly(ADP-ribose) Glycohydrolase Assays. This enzyme activity was measured essentially as described previously (Thomassin et al., 1990), using 10 μM ^{32}P-labeled poly(ADP-ribose) as substrate. The enzyme assay was performed in the same reaction mixture as the DNA repair and poly(ADP-ribosyl)ation assays, with incubations at 30 °C for 10 min.

DNA Repair Assay with Suppression of Simultaneous Turnover of Poly(ADP-ribose). Repair assays were performed with cell extracts depleted of poly(ADP-ribose) polymerase but supplemented with previously automodified poly(ADP-ribose) polymerase, which carried a defined number of ADP-ribose residues, in the presence of both 3-aminobenzamide (3AB) and ADP-dihydroxypyrrolidine. The whole procedure was performed in one tube for each reaction mixture to reduce technical errors. The initial automodification reactions were carried out in a 5-μL reaction mixture containing 50 mM Tris·HCl (pH 8.0), 5 mM $MgCl_2$, 5 mM dithiothreitol, 200 μM NAD, 1.25 μCi of [^{32}P]NAD, 0.3 μg of γ-irradiated plasmid DNA, and 0.16 μg of calf thymus poly(ADP-ribose) polymerase for 5 min at 30 °C. The reaction was terminated by addition of 10 μL of 10 mM 3AB followed by addition of 0.05 unit (4 μL) of poly(ADP-ribose) glycohydrolase. This mixture was incubated for 0–20 min at 30 °C to gradually remove ADP-ribose residues. The glycohydrolase reaction was terminated and the repair of DNA strand interruptions initiated by addition of 31 μL of DNA repair assay reaction mixture containing 97 μM ADP-dihydroxypyrrolidine as well as 73 mM Hepes·KOH (pH 7.8), 110 mM KCl, 8 mM $MgCl_2$, 1.6 mM EDTA, 3.2 mM ATP, 32 μM each of dATP, dCTP, dGTP, and TTP, 65 mM phosphocreatine, 4 μg of creatine phosphokinase, 4.8% glycerol, 32 $\mu g \cdot mL^{-1}$ bovine serum albumin, and cell-free extract (50 μg of protein) previously depleted of poly(ADP-ribose) polymerase. The mixture was incubated for 30 min at 30 °C to allow repair of DNA breaks. The reactions were terminated, and the DNA was analyzed as described for the standard DNA repair assay. In the final 50-μL reaction mixture, the concentrations of NAD, 3-aminobenzamide, and ADP-dihydroxypyrrolidine were 20 μM, 2 mM, and 60 μM, respectively.

RESULTS

Size of Poly(ADP-ribose) Synthesized in Response to DNA Damage. The NAD-promoted rejoining of γ-ray-induced single-strand breaks in DNA by human cell extracts was described previously (Satoh & Lindahl, 1992; Satoh et al., 1993). The accompanying poly(ADP-ribose) formation has now been investigated. Rapid synthesis of poly(ADP-ribose) occurred in response to addition of γ-irradiated, open circular DNA to DNA-free cell extracts (Figures 1–3). In contrast, unirradiated covalently closed circular DNA had no effect (Satoh & Lindahl, 1992), in agreement with data of Benjamin and Gill (1980). Poly(ADP-ribose) remained covalently attached to poly(ADP-ribose) polymerase, and polymer–enzyme complexes could be readily detected by employing reaction mixtures containing [^{32}P]NAD. At very low NAD concentration, only monomers and short oligomers of $(ADP\text{-}ribose)_n$ were made (data not shown), so the automodified, radioactively labeled enzyme remained as a band of ~113 kDa on analysis by SDS–polyacrylamide gel electrophoresis (Figure 1). When poly(ADP-ribose) polymerase was depleted

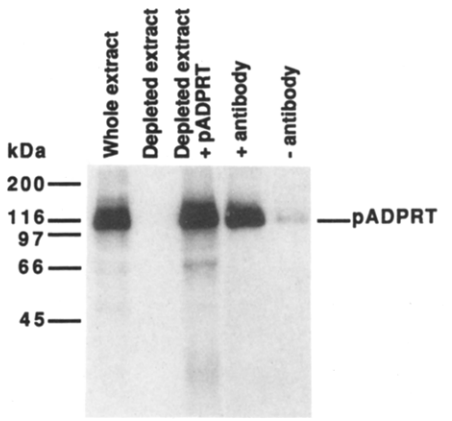

FIGURE 1: Identity of poly(ADP-ribosyl)ated protein in the cell-free system. Human cell extracts (50 μg of protein) were incubated for 2 min at 30 °C in the standard reaction mixture (50 μL) containing 0.3 μg of γ-irradiated plasmid but only 32 nM [^{32}P]NAD. Reactions were terminated by addition of acetone and protein precipitates analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography. Lane 1, whole cell extract; lane 2, extract depleted of poly(ADP-ribose) polymerase by double-stranded DNA–cellulose chromatography in 0.4 M NaCl; lane 3, depleted extract supplemented with 0.16 μg of purified bovine poly(ADP-ribose) polymerase. In a separate experiment, reactions were stopped by immunoprecipitation using either polyclonal antibody 318 against poly(ADP-ribose) polymerase (lane 4) or preimmune serum (lane 5); precipitates were analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography. The migration position of unmodified poly(ADP-ribose) polymerase (pADPRT) is indicated.

from cell extracts by DNA–cellulose chromatography (Figure 1, lane 2) or by immunoprecipitation (Figure 1, lane 4), ^{32}P-labeled ADP-ribose residues were also removed. This demonstrated that all detectable polymer synthesis in the cell extracts was due to automodification of poly(ADP-ribose) polymerase. Traces of apparent polymer acceptors of lower molecular weight than intact poly(ADP-ribose) polymerase could be ascribed to active proteolytic fragments of the enzyme itself (Figure 1, lanes 1 and 3).

By immunoblotting, the amount of poly(ADP-ribose) polymerase in a 50-μL reaction mixture containing 50 μg of extract protein was estimated to be ~0.1 μg by comparison with a known amount of the protein (Figure 1). Thus, reaction mixtures contained an approximately 5-fold higher amount of poly(ADP-ribose) polymerase molecules than DNA strand interruptions in plasmids. Since poly(ADP-ribose) polymerase is a very abundant enzyme in nuclei, the cellular response to nonlethal ionizing radiation would involve an excess of the enzyme over DNA strand interruptions *in vivo*.

Within the first 2 min of incubation of reaction mixtures at an NAD concentration (0.25 mM) similar to the intracellular one, heavily automodified poly(ADP-ribose) polymerase containing polymers with >20 ADP-ribose residues was produced. Such automodification could be detected either by the retarding effect on the electrophoretic migration of poly(ADP-ribose) polymerase (Figure 2A,B) or directly by size determination of the newly synthesized polymers (Figure 3A). The specific poly(ADP-ribose) polymerase activity was 1.1 nmol of ADP-ribose residue incorporated min^{-1} (mg of extract protein)$^{-1}$. After 10 s at 30 °C, only a small amount of poly(ADP-ribose) had yet been synthesized, but at 30 s and 2 min heavily automodified protein molecules were frequent. To improve resolution, composite agarose–polyacrylamide gels (Figure 2B) were employed in addition to standard polyacrylamide gels (Figure 2A). Highly automodified poly(ADP-ribose) polymerase molecules were not recognized by antibodies raised against the enzyme (Lamarre et al., 1988), so

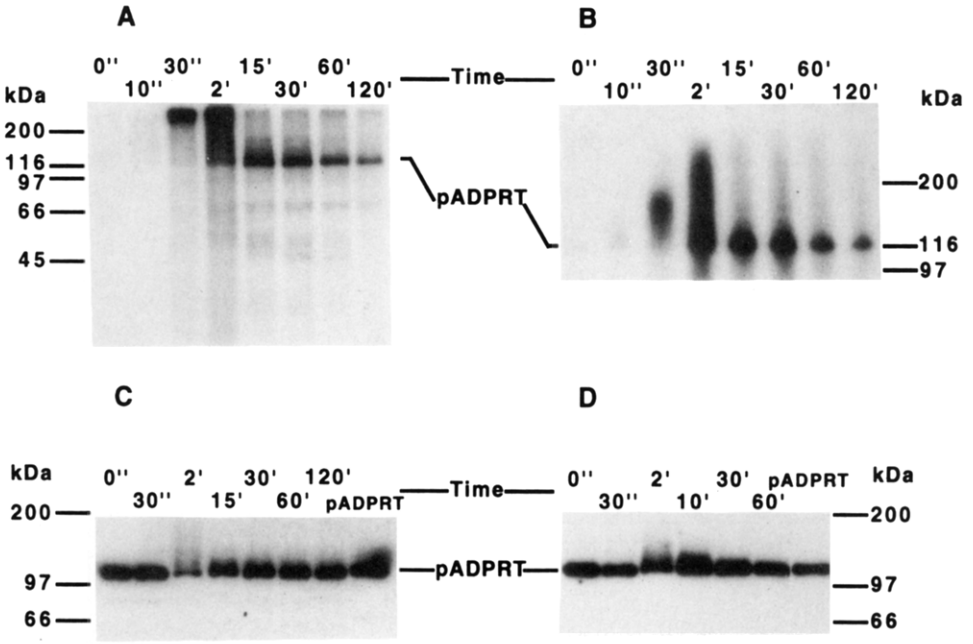

FIGURE 2: Kinetics of poly(ADP-ribose) polymerase automodification. Reaction mixtures containing γ-irradiated plasmid, human cell extract, and 0.25 mM NAD were incubated at 30 °C for various times. The reactions were terminated by precipitation of proteins with ice-cold acetone followed by gel electrophoresis in the presence of SDS. For analysis of poly(ADP-ribose) acceptors (frames A and B), 10 μCi of [^{32}P]NAD was also included in reaction mixtures. Precipitates (25 μg of protein each) were dissolved and electrophoresed on SDS–10% polyacrylamide gels (frame A) or composite agarose–polyacrylamide gels (frame B), followed by autoradiography. For immunoblotting (frame C) and activity blots (frame D), 12.5 μg of extract protein was fractionated on an SDS–7.5% polyacrylamide gel, followed by transfer to a nitrocellulose membrane. In immunoblotting experiments, membranes were incubated with C-2–10 monoclonal antibody against poly(ADP-ribose) polymerase followed by ^{125}I-labeled anti-mouse IgG, and the amount of immunoreactive poly(ADP-ribose) polymerase was visualized by autoradiography. For activity blots, proteins on the nitrocellulose membrane were exposed to a renaturation procedure followed by incubation with [^{32}P]NAD and DNase I-treated calf thymus DNA, and poly(ADP-ribose) polymerase activity was detected by autoradiography. Purified poly(ADP-ribose) polymerase (23 ng) was included as reference in frames C and D. The molecular mass markers were from Bio-Rad (frames A and B) and Amersham (^{14}C-labeled material, frames C and D).

about 80% of the enzyme molecules could not be detected by immunoblotting after 2 min of incubation (Figure 2C). Similar results were obtained by activity blots (Figure 2D). The poly(ADP-ribose) chains were heterogeneous in size. After 30 s, most chains appeared to be 20–60 residues long (Figure 3A), with an even broader size distribution after 2 min (Figures 2B and 3A) including branched polymers that did not enter the gel. The synthesis of such long chains of poly(ADP-ribose) seemed restricted to the first 1–2 min of the reaction, in agreement with *in vivo* data (Alvarez-Gonzalez & Jacobson, 1987). The cessation of poly(ADP-ribose) synthesis was not due to exhaustion of the NAD precursor, because direct determinations of NAD levels by thin-layer chromatography of aliquots of reaction mixtures showed that <20% of the NAD had been consumed (Satoh et al., 1993). At longer incubation times, the poly(ADP-ribose) chains were strongly reduced in size by glycohydrolase activity [0.5 nmol of ADP-ribose released min^{-1} (mg of protein)$^{-1}$] in the extract (Figure 3A, see also Figure 2A,B). The glycohydrolase mainly degrades polymers from free termini (Miwa et al., 1974; Hatakeyama et al., 1986). The polymer degradation could be ascribed to the distinct poly(ADP-ribose) glycohydrolase rather than nonspecific phosphodiesterase activity, because specific inhibition of the glycohydrolase with the "transition-state" inhibitor ADP-dihydroxypyrrolidine resulted in prolonged occurrence of long polymers, whereas supplementation of extracts with purified glycohydrolase accelerated polymer degradation (Figure 3B).

DNA Repair Capacity at Different Levels of Poly(ADP-ribose) Formation. The formation of poly(ADP-ribose) and the amount of rejoining of DNA strand interruptions were compared in reaction mixtures supplemented with different amounts of inhibitors of poly(ADP-ribose) synthesis (Figure 4). Most of the poly(ADP-ribose) synthesis occurs during early incubation times, whereas DNA excision-repair at strand breaks takes place gradually over a 40–60-min period (Satoh et al., 1993). In the presence of the widely used inhibitor 3-aminobenzamide, relatively low concentrations (100–200 μM) caused 80–90% inhibition of initial poly(ADP-ribose) synthesis (Figure 4B). However, a small amount of polymer synthesis (5–10% of controls) remained in the presence of larger amounts of inhibitor (see also below, Figure 5, lane 4). The results for DNA rejoining were different in that no strong inhibition of DNA repair was observed at low 3-aminobenzamide concentration (Figure 4A). In the standard reaction mixture employed here, most of the NAD-promoted DNA repair capacity was retained even in the presence of 1 mM 3-aminobenzamide, although somewhat more efficient inhibition occurred at lower cell extract concentrations (Figure 4A) in agreement with earlier data (Satoh & Lindahl, 1992). Similar results (Figure 4C,D) were obtained with a different inhibitor of poly(ADP-ribose) polymerase, 2-nitro-6(5*H*)-phenanthridinone, which is active at lower concentrations (Banasanik et al., 1992). These data demonstrate that there is no quantitative relationship between the extent of suppression of poly(ADP-ribose) synthesis and DNA repair by inhibitors of poly(ADP-ribose) polymerase, although such a correlation has sometimes been assumed for *in vivo* studies. Instead, the results suggest that weak modification of poly(ADP-ribose) polymerase is sufficient for dissociation of the protein from DNA strand breaks, and for subsequent efficient DNA rejoining.

For evaluation of the role of poly(ADP-ribose) glycohydrolase in DNA repair, reaction mixtures were supplemented either with the glycohydrolase inhibitor ADP-dihydroxypyrrolidine or with an 80-fold excess of purified glycohydrolase

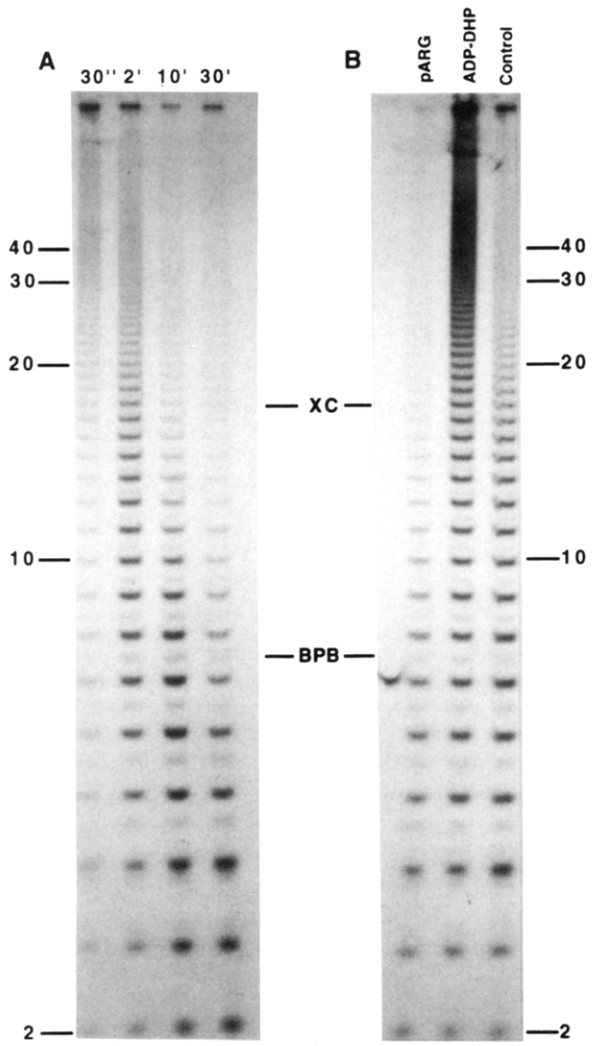

FIGURE 3: Size determination of poly(ADP-ribose) . Reaction mixtures containing γ-irradiated plasmid, human cell extract, and [^{32}P]NAD (20 μCi, 0.25 mM) were incubated at 30 °C for the times indicated (frame A) or incubated for 2 min in the presence of ADP-dihydroxypyrrolidine (ADP-DHP, 60 μM) or excess poly(ADP-ribose) glycohydrolase (pARG, 2 units) (frame B). Reactions were stopped with trichloroacetic acid, and poly(ADP-ribose) chains were released from protein by alkali treatment and purified by boronate chromatography. These polymer chains were fractionated by SDS–20% polyacrylamide gel electrophoresis and visualized by autoradiography. Polymer lengths in ADP-ribose residues are indicated, as well as the migration positions of xylene cyanol (XC) and bromophenol blue (BPB) markers.

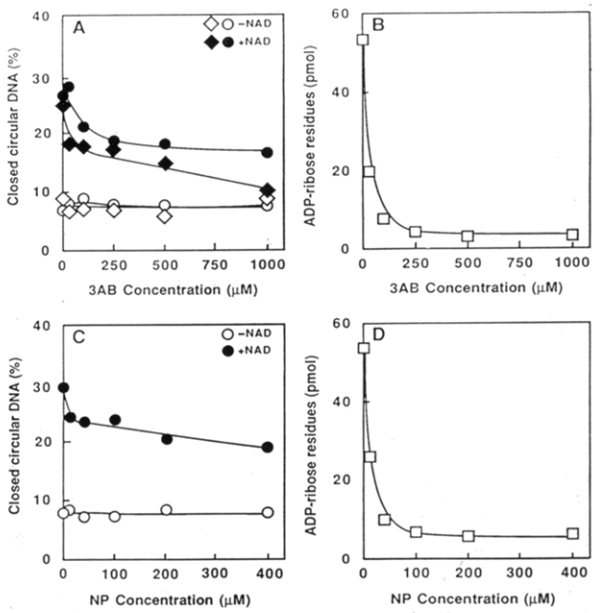

FIGURE 4: Effects of inhibitors of poly(ADP-ribose) polymerase on DNA strand rejoining and poly(ADP-ribose) formation. Reaction mixtures containing γ-irradiated plasmid and human cell extract were incubated at 30 °C in the presence of different amounts of either 3-aminobenzamide (3AB, frames A and B) or 2-nitro-6(5*H*)-phenanthridinone (NP, frames C and D). For DNA repair assays (frames A and C), reaction mixtures contained either 50 μg (circles) or 25 μg (diamonds) of extract protein and were incubated for 30 min in the presence (closed symbols) or absence (open symbols) of 0.25 mM NAD. Because of the limited incubation time and end-group heterogeneity in the γ-irradiated open circular DNA, only 30% were rejoined in the presence of NAD and absence of inhibitors. For poly(ADP-ribosyl)ation assays (frames B and D), standard NAD-containing reaction mixtures (50 μg of extract protein each) were supplemented with 2 μCi of [^{32}P]NAD and incubated for 2 min at 30 °C, followed by measurement of acid-precipitable material.

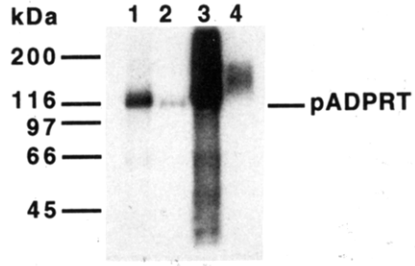

FIGURE 5: Poly(ADP-ribose) formation in the presence of various enzyme inhibitors. Standard DNA repair reaction mixtures containing γ-irradiated plasmid, human cell extract (50 μg of protein), 0.25 mM NAD, and 2 μCi of [^{32}P]NAD were incubated for 30 min at 30 °C, and automodified poly(ADP-ribose) polymerase was visualized after electrophoresis on an SDS–10% polyacrylamide gel. Lane 1, standard reaction without inhibitors; lane 2, reaction mixture containing 1 mM 3-aminobenzamide; lane 3, reaction mixture containing 40 μM ADP-dihydroxypyrrolidine; lane 4, both inhibitors present. Molecular mass markers (Bio-Rad) are shown on the left.

over that present in the cell extract. Addition of the inhibitor resulted in accumulation of very high amounts of poly(ADP-ribose) (Figures 5 and 6), and these polymers were of larger size than usually detected (Figure 3B). Thus, the effect of this inhibitor seemed opposite to that of 3-aminobenzamide (Figure 5). Only small perturbations of DNA repair capacity were observed in the presence of either ADP-dihydroxypyrrolidine or excess poly(ADP-ribose) glycohydrolase (Figure 6A), although very different levels of poly(ADP-ribose) were present under these conditions (Figure 6B). These data indicate that most of the processing of poly(ADP-ribose) by the glycohydrolase took place after the release of automodified poly(ADP-ribose) polymerase molecules from DNA strand interruptions. The polymers occurring in the presence of a large excess of glycohydrolase were, not surprisingly, much shorter than those normally observed (Figure 3B). A small amount of poly(ADP-ribose) synthesis was required for efficient DNA repair, however, since the absence of NAD suppressed the repair reaction (Figures 4A,C and 6A). Taken together, these data indicate that short polymers are sufficient for DNA base excision-repair, and consequently that longer polymers are nonessential to this reaction.

Minimal Amount of Poly(ADP-ribose) Required for DNA Repair. To investigate the relationship between DNA repair capacity and degree of poly(ADP-ribose) polymerase automodification, cell-free assays were carried out in the presence of both 3-aminobenzamide and ADP-dihydroxypyrrolidine to suppress poly(ADP-ribose) turnover. In addition, cell extracts depleted of endogenous poly(ADP-ribose) polymerase but supplemented with purified poly(ADP-ribose) polymerase containing a defined number of ADP-ribose residues were employed.

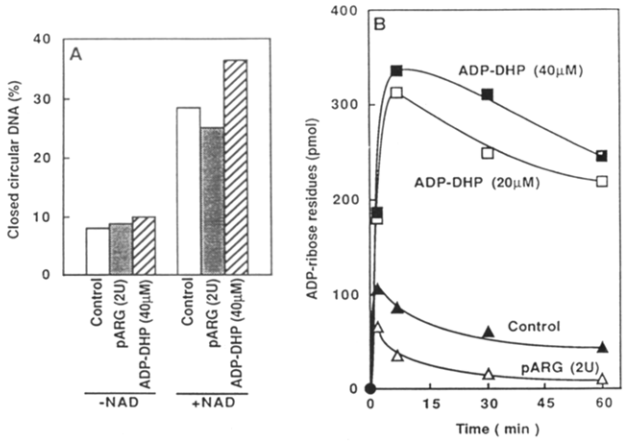

FIGURE 6: Effect of poly(ADP-ribose) glycohydrolase on DNA repair and poly(ADP-ribose) levels. Standard DNA repair reaction mixtures, with or without NAD, were supplemented with either 2 units of purified poly(ADP-ribose) glycohydrolase (pARG) or 40 μM ADP-dihydroxypyrrolidine (ADP-DHP), and DNA joining was measured as described under Materials and Methods (frame A). The amounts of acid-precipitable poly(ADP-ribose) present at various times were analyzed in reaction mixtures containing 0.25 mM NAD and 2 μCi of [^{32}P]NAD, supplemented with either purified glycohydrolase or ADP-dihydroxypyrrolidine as indicated.

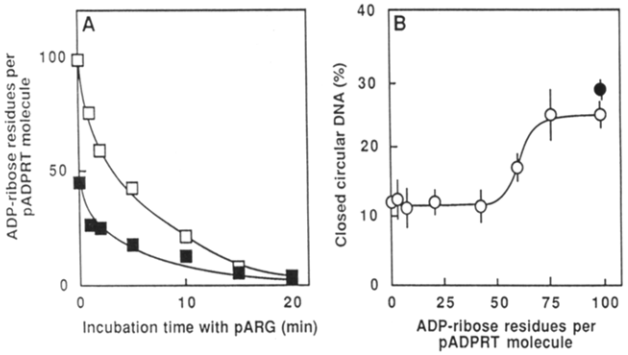

FIGURE 7: Degree of automodification of poly(ADP-ribose) polymerase necessary for efficient DNA repair. Automodification of poly(ADP-ribose) polymerase in the presence of [^{32}P]NAD was carried out, and then the poly(ADP-ribose) was partially degraded by purified glycohydrolase (pARG; frame A). Such automodified poly(ADP-ribose) polymerase molecules carrying different amounts of poly(ADP-ribose) were supplemented with standard DNA repair reaction mixtures containing polymerase-depleted cell extract and both 3-aminobenzamide and ADP-dihydroxypyrrolidine to prevent polymer turnover. Frame A shows numbers of ADP-ribose residues attached to poly(ADP-ribose) polymerase at the start (open symbols) and end (closed symbols) of the 30-min DNA repair reaction. Frame B shows the amount of repair of γ-irradiated plasmid DNA in the presence of different amounts of poly(ADP-ribose). The filled symbol in frame B shows the standard DNA repair reaction in the absence of enzyme inhibitors, and experimental standard deviations are also shown in frame B.

Purified poly(ADP-ribose) polymerase was automodified in a reaction mixture containing 1.2 pmol (0.14 μg) of the enzyme and 0.17 pmol (0.3 μg of open circular plasmid) of γ-ray-induced DNA single-strand breaks. The polymerization reaction was terminated by addition of 10 mM 3-aminobenzamide. Purified poly(ADP-ribose) glycohydrolase was then added, and reactions were stopped at various times with ADP-dihydroxypyrrolidine. The incubation with glycohydrolase resulted in decreasing sizes of the poly(ADP-ribose) chains attached to the automodified polymerase (Figure 7A, see also Figure 3B). When such modified poly(ADP-ribose) polymerase molecules were included in the standard DNA repair assay, molecules initially carrying 80–100 ADP-ribose residues allowed for DNA rejoining similar to that observed in a standard NAD-containing reaction mixture without enzyme inhibitors (Figure 7B). In contrast, poly(ADP-ribose) polymerase molecules with <50 ADP-ribose residues interfered with DNA repair. In spite of the presence of ADP-dihydroxypyrrolidine in reaction mixtures, some polymer degradation occurred during the 30-min incubation period for DNA repair, with an approximately 2-fold decrease in the number of ADP-ribose residues at the end of the incubation period (Figure 7A). Since each poly(ADP-ribose) polymerase molecule has 18–28 automodification sites (Desmarais et al., 1991), these data indicate that short chains of poly(ADP-ribose) on multiple modification sites would be sufficient for dissociation of the polymerase from DNA strand breaks, and also adequate for preventing poly(ADP-ribose) polymerase from binding to strand breaks and interfering with DNA repair. However, modification by mono(ADP-ribosyl)ation at each glutamic acid acceptor site of poly(ADP-ribose) polymerase, which is a weakly basic protein in its unmodified form (Cherney et al., 1987), would be insufficient for this purpose.

DISCUSSION

The physiological roles of the abundant nuclear enzyme poly(ADP-ribose) polymerase have remained unresolved in spite of many investigations. A stimulatory effect on DNA repair by poly(ADP-ribose) synthesis (Durkacz et al., 1980), therefore, has received much attention. Recent studies have established that this effect is restricted to the base excision-repair pathway, including repair of DNA chain breaks generated by ionizing radiation, whereas the different nucleotide excision-repair pathway is not affected and does not trigger poly(ADP-ribose) synthesis (Satoh et al., 1993; Molinete et al., 1993). The involvement of poly(ADP-ribose) polymerase with base excision-repair appears to be an incidental and mainly negative one, in that the enzyme binds tightly to newly generated DNA strand breaks and competes with excision-repair enzymes (Satoh & Lindahl, 1992). Automodification of poly(ADP-ribose) polymerase leads to liberation of the protein from DNA due to charge repulsion between the newly synthesized poly(ADP-ribose) and DNA (Zahradka & Ebisuzaki, 1982; Ferro & Olivera, 1982), so one important role of poly(ADP-ribose) formation is to provide a release mechanism for the enzyme in order to make a DNA strand interruption accessible for DNA repair. As shown in the present work, however, short chains of poly(ADP-ribose) are sufficient for this purpose, so the initial transient formation of long, branched chains of poly(ADP-ribose) plays a different role. In this regard, the enzymatic properties of poly(ADP-ribose) glycohydrolase seem excellently suited to prevent interference with joining of DNA strand interruptions. The critical role of the glycohydrolase in allowing for efficient DNA repair has been largely overlooked. Thus, the glycohydrolase degrades long chains of poly(ADP-ribose) very rapidly, but the enzyme exhibits an increasingly high K_m and decreasing V_{max} for polymer chains smaller than 20 residues (Hatakeyama et al., 1986; Jonsson et al., 1988). This means that short chains of poly(ADP-ribose) will persist on poly(ADP-ribose) polymerase molecules for an extended period after release of the enzyme from DNA strand breaks, preventing immediate reattachment of the enzyme and creating a window in time for productive DNA strand rejoining. A scheme outlining these events is shown in Figure 8.

What is the function of the early transient synthesis of long chains of poly(ADP-ribose)? The evolutionary conservation of such polymer synthesis strongly indicates that it serves a useful and specific role. One possibility is that after exposure

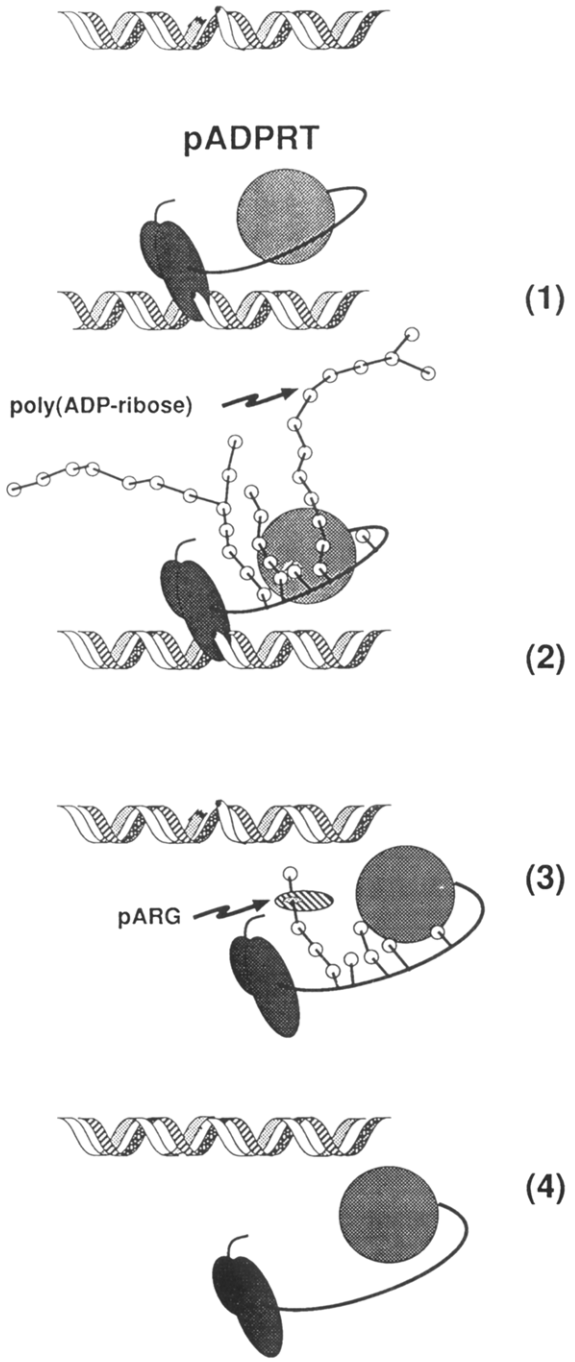

FIGURE 8: Illustration of poly(ADP-ribose) turnover and DNA repair at strand interruptions in DNA. After introduction of a DNA strand break by ionizing radiation or in consequence to alkylation damage, poly(ADP-ribose) polymerase (pADPRT) binds rapidly to the strand break (1). The enzyme is schematically shown as having a DNA-binding and a catalytic domain linked by a region containing automodification sites. Rapid synthesis of long, branched chains of poly(ADP-ribose) is triggered by the attachment of the enzyme to a strand break (2). These long polymers are strongly negatively charged. Following release of the automodified poly(ADP-ribose) polymerase from DNA, most of these chains are rapidly degraded by poly(ADP-ribose) glycohydrolase (3). However, short poly(ADP-ribose) chains persist for some time on the automodified polymerase because of the low affinity of the glycohydrolase for oligo(ADP-ribose). These chains are sufficient to prevent the reattachment of poly(ADP-ribose) polymerase to a DNA strand interruption, allowing for DNA rejoining by excision-repair enzymes (4). Finally, the remaining short poly(ADP-ribose) chains are slowly removed from the automodified polymerase by the glycohydrolase.

of cells to very high lethal concentrations of DNA-damaging agents that cause strand breaks, the resulting massive and futile poly(ADP-ribose) synthesis may deplete the cellular NAD pool. Together with extensive DNA fragmentation, this might contribute to cell death (Berger, 1985; Zhang et al., 1994). However, for moderate doses of DNA damage resulting in partial survival of the cellular population, such a mechanism is unlikely to explain the need for poly(ADP-ribose) synthesis. Another model for early polymer synthesis proposed by Althaus and co-workers (Realini & Althaus, 1992; Althaus, 1992; Panzeter et al., 1993) suggests a "histone shuttle" mechanism, the basic idea being that the negatively charged poly(ADP-ribose) might bind histones and promote temporary unfolding and relaxation of chromatin in the vicinity of a damaged site to allow for more efficient DNA repair. Using chromatin reconstituted from γ-irradiated plasmid DNA and core histones as a substrate in our cell-free assay system, we have been unable to detect any marked effect on DNA repair that could be ascribed to histone shuttling—a 2-fold overall decrease in DNA rejoining efficiency occurred in comparison with naked DNA as substrate, but the 3–4-fold stimulatory effect of including NAD in reaction mixtures was the same for repair of γ-irradiated plasmid DNA with or without histones (M.S.S., unpublished data). However, such observations do not address the possibility that poly(ADP-ribose) formation might serve to remove histone H1 from condensed chromatin in regions of DNA damage. It should be noted in this context that base excision-repair, in contrast to nucleotide excision-repair, occurs equally efficiently with transcribed and nontranscribed DNA *in vivo* (Scicchitano & Hanawalt, 1989).

Poly(ADP-ribose) polymerase always is present together with poly(ADP-ribose) glycohydrolase in different organisms to allow for polymer turnover. Both enzymes are apparently lacking in *Saccharomyces cerevisiae*, so expression of a human cDNA encoding poly(ADP-ribose) polymerase in budding yeast nuclei leads to an unphysiological situation with growth arrest due to massive synthesis of stable poly(ADP-ribose) chains (Kaiser et al., 1992), apparently analogous to that observed in human cell-free extracts under conditions of glycohydrolase inhibition (Figure 5, lane 3). An important lead in deducing a function for long chains of poly(ADP-ribose) comes from the dinoflagellate *Crypthecodinium cohnii*, which has both a poly(ADP-ribose) polymerase and a glycohydrolase, although histones are absent, and the cellular DNA is organized as in prokaryotes with little association of basic proteins (Werner et al., 1984). Clearly, in this organism at least, poly(ADP-ribose) synthesis is not employed for displacing histones from DNA. The dinoflagellate, which belongs to the algae, is a typical eukaryote with a distinct cell nucleus and a primitive mitotic apparatus, and interestingly it resembles higher eukaryotes in that it possesses large amounts of interspersed repeated DNA sequences (Allen et al., 1975).

We propose that a main function for the long chains of poly(ADP-ribose) synthesized transiently in response to DNA strand interruptions in most eukaryotes may be the prevention of accidental recombination events in regions of tandem repeat sequences. Since DNA is tightly packed and folded in nuclei, such DNA sequences may often be in close vicinity of each other, and a single-strand interruption would be a "hot spot" for initiating an intrachromosomal homologous recombination event. However, if the abundant poly(ADP-ribose) polymerase binds first to the lesion and generates extended and branched chains of poly(ADP-ribose) by automodification, a nucleic acid-free zone will be created adjacent to the DNA strand break, because of electrostatic repulsion by the highly negatively charged (two charges per monomer) poly(ADP-ribose) chains. This would serve to reduce the potential

problem of rearrangements occurring within repeated DNA sequences. Indeed, prevention of poly(ADP-ribose) synthesis in mammalian cells by 3-aminobenzamide or 3-methoxybenzamide treatment leads to an increase in intrachromosomal homologous recombination (Waldman & Waldman, 1991) and sister chromatid exchange events (Oikawa et al., 1980; Natarajan et al., 1981). Moreover, poly(ADP-ribose) formation may be involved in chromosome maintenance since transfected oncogenes are frequently lost from NIH 3T3 cells grown for long times in the presence of inhibitors of poly(ADP-ribose) polymerase (Nakayasu et al., 1988). While such observations are consistent with an anti-recombinogenic role of the polymer, the results are inconclusive, because binding of poly(ADP-ribose) polymerase at DNA strand interruptions delays their rejoining if poly(ADP-ribose) synthesis is prevented (Satoh & Lindahl, 1992). A direct prediction is, however, that transgenic mice deficient in poly(ADP-ribose) polymerase should exhibit genomic instability due to increased expansion or loss of complex DNA repeat sequences not readily corrected by mismatch repair.

ACKNOWLEDGMENT

We thank Dr. James Slama for providing ADP-dihydroxypyrrolidine and Danièle Poirier for assistance.

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