DNA strand interruptions are generated directly by many DNA-damaging agents. In higher, and many lower, eukaryotes the cellular response to such damage involves induction of synthesis of poly(ADP-ribose) from NAD\(^+\) in cell nuclei (for reviews, see Refs. 1–6). Synthesis of this negatively charged polymer enhances repair of DNA strand breaks (7). In proliferating undamaged cells the amount of poly(ADP-ribose) is very low. In contrast, there are about 2–10\(^{10}\) molecules of poly(ADP-ribose) polymerase (NAD\(^+\); protein ADP-ribosyltransferase, pADPR) within DNA replication complexes. The pADPR within DNA replication complexes. The pADPR was purified from calf thymus according to Zahradka and Ebisuzaki (26). A similar stimulatory effect by NAD\(^+\) was observed for repair of ultraviolet-irradiated DNA, and this could be ascribed to the presence of pyrimidine hydrates as minor radiation-induced DNA lesions. No effect was observed on the sealing of \(\gamma\)-irradiated DNA by supplementation of cell extracts with purified mammalian DNA ligase I or DNA ligase II. The results indicate that poly(ADP-ribose) polymerase interferes with base excision-repair processes because bound enzyme molecules block DNA strand interruptions. Release of bound poly(ADP-ribose) polymerase following automodification, or physical removal of the protein from reaction mixtures, facilitates DNA repair.

Rejoining of DNA single-strand breaks generated by treatment of plasmids with \(\gamma\)-rays, neocarzinostatin, or bleomycin was catalyzed inefficiently by human cell extracts. The reaction was strongly promoted by the addition of NAD\(^+\), which was employed for rapid and transient synthesis of poly(ADP-ribose). The DNA rejoining reaction was accompanied by DNA repair replication, apparently due to replacement of damaged residues at termini. Selective depletion of poly(ADP-ribose) polymerase from cell extracts improved the repair of DNA exposed to a variety of DNA-damaging agents by removing NAD\(^+\)-dependent NAD\(^+\) dependence of DNA repair reaction. NAD\(^+\)-promoted DNA repair by soluble cell extracts also occurred with alkylated DNA as substrate and was suppressed by 3-aminobenzamide. A similar stimulatory effect by NAD\(^+\) was observed for repair of ultraviolet-irradiated DNA, and this could be ascribed to the presence of pyrimidine hydrates as minor radiation-induced DNA lesions. No effect was observed on the sealing of \(\gamma\)-irradiated DNA by supplementation of cell extracts with purified mammalian DNA ligase I or DNA ligase II. The results indicate that poly(ADP-ribose) polymerase interferes with base excision-repair processes because bound enzyme molecules block DNA strand interruptions. Release of bound poly(ADP-ribose) polymerase following automodification, or physical removal of the protein from reaction mixtures, facilitates DNA repair.

but the enzyme is present in larger quantities than appears to be required for poly(ADP-ribose) synthesis. A structural involvement of pADPR might explain the observation that cell lines with a 10–15-fold reduced amount of the protein grow very slowly (9, 10).

A human cDNA encoding pADPR has been cloned, sequenced, and expressed in E. coli and yeast (11–16). The pADPR gene has been mapped to chromosome 14q11-q42 (Ref. 14). The 113-kDa enzyme comprises an N-terminal DNA binding domain with two zinc fingers, and a C-terminal NAD\(^+\) binding catalytic domain, that are united by a short region containing several automodification sites (6, 17). The protein binds tightly at DNA strand interruptions (18–20), although Okazaki fragments appear to be inaccessible to pADPR within DNA replication complexes. The pADPR may prevent initiation of spurious transcription at DNA single-strand breaks (21) or generate a cellular signal in response to DNA damage by binding to DNA at strand interruptions, but the exact physiological role of the protein is not understood.

Recently, we developed a cell-free system for studying the role of poly(ADP-ribose) synthesis during repair of \(\gamma\)-ray-induced DNA strand breaks (22). Here, we have used this approach to investigate the NAD\(^+\)-dependent repair response to a variety of different DNA-damaging agents.

MATERIALS AND METHODS

Reagents—Escherichia coli endonuclease IV was isolated from an overexpression strain (23). E. coli Nth protein (endonuclease III) was also purified from an overproducer strain (24). E. coli exonuclease III was purchased from Boehringer Mannheim. Mammalian DNA ligases I and II were purified separately from calf thymus as described (25). pADPR was purified from calf thymus according to Zahradka and Ebisuzaki (26).

Neocarzinostatin was obtained from Kayaku Co. (Tokyo, Japan) and bleomycin from Calbiochem. NAD\(^+\), 3-aminobenzamide, N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and methyl methanesulfonate (MMS) were from Sigma. [\(\alpha\)-\(^{32}\)P]ATP (3000 Ci mmol\(^{-1}\)) and [\(^{32}\)P]NAD\(^+\) (30 Ci mmol\(^{-1}\)) were obtained from Amersham.

Normal human lymphoblastoid cells, GM06315A (from the NIGMS Human Genetic Mutant Cell Repository, Camden, NJ), were grown in RPMI 1640 medium supplemented with 15% fetal bovine serum and antibiotics. HeLa cells were cultured under the same conditions but in 5% serum. Cell extracts were prepared according to Manley et al. (27) and stored in aliquots at \(-80^\circ\)C.

Damaged Plasmids—The 3-kilobase pair Bluescript II KS\(^+\) plasmid was obtained from Stratagene and propagated in E. coli JM109. Plasmids were prepared by standard procedures (28).

For \(\gamma\)-irradiation, supercoiled plasmid DNA (3.4 mg ml\(^{-1}\)) in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (TE buffer) was exposed to 50 grays of \(\gamma\)-rays from a 60Co source (16 grays min\(^{-1}\)) at 0°C. For neocarzinostatin treatment, plasmid DNA (0.17 mg ml\(^{-1}\)) in 50 mM Tris-HCl (pH 8.0), 10 mM 2-mercaptoethanol was incubated with 0.3 unit ml\(^{-1}\) neocarzinostatin for 15 min at 37°C. For bleomycin treatment, plasmid DNA (0.17 mg ml\(^{-1}\)) in 50 mM Tris-HCl (pH 8.5), 0.15 mM Fe([NH\(_4\)]\(_2\)(SO\(_4\))\(_2\)) and 0.3 mM EDTA was incubated with 0.45 unit ml\(^{-1}\) bleomycin for 15 min at 37°C.

After exposure of plasmids to neocarzinostatin or bleomycin, the DNA was ethanol-precipitated and redissolved in TE buffer. The nicked circular DNA generated by

Received for publication, July 31, 1992

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 268, No. 8, Issue of March 15, pp. 5460–5467, 1993
Printed in U.S.A.

NAD\(^+\)-dependent Repair of Damaged DNA by Human Cell Extracts*
exposure to these agents, or γ-rays, was purified by ethidium bromide/CsCl density gradient centrifugation and stored at −80 °C in TE buffer. For MNNG and MMS treatment, plasmid DNA (0.2 mg·ml⁻¹) was incubated with 0.4 mM MNNG or 2 mM MMS in TE buffer for 30 min at 37 °C. The treated plasmid DNA was ethanol-precipitated, redissolved in TE buffer (pH 7.5), and stored at −20 °C. For ultraviolet (UV) irradiation, 2 μCi of [³²P]NAD⁺ and 10 μCi of [³²P]dATP were added to 5 μg of plasmid DNA in TE buffer exposed to 450 J·m⁻² of 254 nm (peak) germicidal UV light at a fluence rate of 0.5 W·m⁻². When indicated, the subpopulation of UV-irradiated plasmids that contained pyrimidine hydrates was removed by endonuclease III treatment followed by recovery of remaining closed circular DNA molecules by ethidium bromide/CsCl density gradient centrifugation (28).

Apurinic (AP) sites were introduced into plasmid DNA (average of 1.5 AP sites/DNA molecule) by incubation in 0.1 M NaCl, 10 mM sodium citrate (pH 5.0) for 10 min. After ethanol precipitation, the DNA was redissolved in TE buffer. For endonuclease III treatment of the partly depurinated DNA, plasmid DNA (0.1 mg·ml⁻¹) was incubated with 1 μg·ml⁻¹ endonuclease III in 40 mM Hepes-KOH (pH 8.0), 0.1 M KCl, 0.5 mM EDTA, 0.05 M dithiothreitol, and 0.2 mg·ml⁻¹ bovine serum albumin for 30 min at 37 °C. After purification of nicked circular plasmids by ethidium bromide/CsCl density gradient centrifugation, the DNA was either used for assays or treated with exonuclease III. Treatment of partly depurinated or endonuclease III-treated plasmid DNA with exonuclease III (2500 units·ml⁻¹) was performed in 0.15 M NaCl, 15 mM sodium citrate (pH 7.0) for 5 min at 37 °C. Under these conditions, the enzyme retains its AP endonuclease function but shows no exonuclease activity (29). Treatment of DNA (0.1 mg·ml⁻¹) with endonuclease IV (0.1 μg·ml⁻¹) was carried out in 0.1 M Tris-HCl (pH 7.5), 5 mM dithiothreitol. Reactions were terminated by addition of SDS to a final concentration of 1% and phenol/chloroform extraction, followed by purification of nicked circular plasmids by ethidium bromide/CsCl density gradient centrifugation.

**Assay Procedures**—Incubations of damaged plasmids with human cell extracts were performed essentially as described previously (22, 28). Reaction mixtures (50 μl) contained 0.3 μg of plasmid DNA, 45 mM Hepes-KOH (pH 7.8), 70 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 0.4 mM EDTA, 2 mM ATP, 20 μCi of [³²P]dATP, [³²P]dGTP, and [³²P]dCTP, 8 μM dATP, 2 μCi of [α-³²P]dATP (when added), 40 mM phosphocreatine, 2.5 μg of creatine phosphokinase, 3% glycerol, 20 μM·ml⁻¹ bovine serum albumin, 0.25 or 2 mM NAD⁺ (when added), and human cell extract (50 μg of protein). Reaction mixtures were incubated for the times indicated at 37 °C and reactions terminated by the addition of SDS (to 0.6%), EDTA (to 20 mM), and proteinase K (240 μg·ml⁻¹). Following digestion for 30 min at 37 °C and phenol/chloroform extraction, carrier TRNA (5 μg) was added and the DNA precipitated with ethanol in the presence of 2 M ammonium acetate. The precipitate was dissolved in 20 μl of TE buffer, treated with 2 μg of ribonuclease A at 37 °C for 10 min, and then fractionated by ethidium bromide/agarose gel electrophoresis. DNA bands were visualized by UV light and photographic negatives analyzed by densitometry (22). When required, gels were dried and further analyzed by autoradiography.

**pADPR-depleted Cell Extracts**—Removal of pADPR from cell extracts was carried out by a procedure slightly modified from that described previously (22). An extract of GM06315A cells was diluted in 0.3 M NaCl, 50 mM Tris-HCl (pH 8.0), 10 mM 2-mercaptoethanol, and 10% glycerol (buffer A) to 2 mg·ml⁻¹ protein, and 1 ml was applied to a double-stranded DNA-cellulose column (Sigma) (1 × 2 cm). The column had been equilibrated with buffer A. After application of the sample, the column was washed with 1 volume of buffer A and 2 volumes of buffer A containing 0.4 M NaCl instead of 0.3 M. Eluted material was pooled, and protein precipitated with ammonium sulfate and diethyl ether as for the original preparation of the cell-free extract (27).

**Measurements of Poly(ADP-ribosyl)ation Activity**—Poly(ADP-ribose)lation reactions were performed at 30 °C in 50 μl of the DNA repair assay reaction mixture containing 0.5 μg of damaged plasmid, 0.25 μCi of [³²P]NAD⁺, and 25 or 200 μl of cell-free extract protein. The reaction was terminated by the addition of 2 μl of 0.1% of bovine serum albumin and 750 μl of ice-cold 25% trichloroacetic acid. The samples were left on ice for 15 min. The trichloroacetic acid precipitates were then collected on Whatman GF/C filters, washed with 10 ml of 0.25% trichloroacetic acid, and twice with 10 ml of ice-cold ethanol. The filters were dried and radioactive material quantified by liquid scintillation counting.

**Determination of NAD⁺ Concentrations**—NAD⁺ levels were assayed essentially as described (30). Briefly, 50 μg of human cell extract and 0.3 μg of γ-irradiated plasmids containing single strand breaks were incubated for various times at 30 °C with 1 μCi of [³²P]NAD⁺ in the standard (50 μl) DNA repair assay reaction mixture containing 0.25 mM NAD⁺. After the incubations, samples were directly applied to glycol ether (25) or directly spotted (20 × 20 cm, 0.25 μCi of ³²P) and developed with 0.3 M LiCl and 0.9 M acetic acid. The position of NAD⁺ was determined by autoradiography. The remaining amount of radioactive NAD⁺ was determined by liquid scintillation counting to evaluate the extent of NAD⁺ degradation.

**RESULTS**

**DNA Repair Assays**—Soluble extracts of HeLa cells and human lymphoblastoid cells can rejoin single-strand breaks in γ-irradiated circular DNA in an NAD⁺-promoted reaction (22). The gently prepared cell extracts employed have been concentrated by ammonium sulfate precipitation and dialysis (27) and do not contain detectable amounts of endogenous NAD⁺ or deoxyxynucleoside triphosphates. The repair of DNA strand interruptions introduced by the anti-cancer drugs neocarzinostatin and bleomycin can be evaluated in the same fashion (Fig. 1). Furthermore, the assay procedure can be extended to DNA-damaging agents that do not generate strand breaks directly, if the cell extract contains DNA repair enzymes that incise at altered sites. In the latter case, incision and subsequent rejoining may be monitored by measurements of the relative proportions of covalently closed versus nicked circular DNA molecules at different times (Fig. 1). Incorporation of radioactive material from an α-[³²P]-labeled deoxyxynucleoside triphosphate into DNA in reaction mixtures also allows for determinations of the amount of DNA repair replication by autoradiography.

**Kinetics of DNA Repair**—The exposure of DNA to γ-rays, neocarzinostatin, or bleomycin introduces strand interruptions by oxygen free radical damage. A variety of end groups are generated, which are usually characterized by oxidation and fragmentation of deoxyribose residues at DNA strand termini (31–33). Here, 50 grays of γ-irradiation, 0.3 unit·ml⁻¹ neocarzinostatin or 0.45 unit·ml⁻¹ bleomycin were used to convert about 20% of the covalently closed circular DNA to a nicked form. The γ-irradiation treatment also generated alkali-labile sites in 10% of the remaining closed circular DNA molecules. Open circular DNA molecules were purified by ethidium bromide/CsCl density gradient centrifugation and employed as substrates. Cell extracts from the human lymphoblastoid line, GM06315A, rejoined the strand interruptions at 30 °C as a function of time in an NAD⁺-promoted reaction (Fig. 2). However, the kinetics of DNA repair were different for damage inflicted by the three agents (Fig. 2, A–C), apparently reflecting unequal rates of processing of distinct DNA end groups. Similar results were obtained with HeLa cell extracts. For γ-irradiated DNA and neocarzinostatin-treated DNA, most of the repair was completed within the first hour of incubation, whereas bleomycin-treated DNA was rejoined more slowly. After a 30-min incubation period, the presence of 2 mM NAD⁺ yielded a 3.3-, 2.9-, or 5.6-fold increase in DNA rejoining of γ-irradiated, neocarzinostatin-treated, or bleomycin-treated DNA, respectively. The reduced rate of repair with γ-irradiated and neocarzinostatin-treated DNA after 60 min did not reflect inactivation of repair enzymes, because similar kinetics of rejoining DNA strand interruptions were obtained with cell extracts preincubated for 2 h at 30 °C. Instead, these data may be explained by end group heterogeneity. Treatment of γ-irradiated DNA with alkaline phosphatase at 56 °C to remove ³²P-phosphate groups at strand breaks prior to incubation did not significantly alter the rate or degree of NAD⁺ stimulation of DNA rejoining.

**DNA Repair Replication**—The DNA termini produced by
NAD+-dependent DNA Repair in Cell Extracts

Fig. 1. Schematic illustration of the assay procedure. For details, see text.

oxygen free radical-induced damage cannot be directly rejoined by DNA ligases. A requirement for DNA excision and repair synthesis during the rejoicing of strand interruptions by cell-free extracts was indicated by the incorporation of radioactively labeled material from [a-32P]dATP into rejoined circular DNA. Thus, little or no conversion of γ-irradiated open circular DNA to a closed circular form occurred (<10% reopening) if deoxynucleoside triphosphates were excluded from reaction mixtures. Moreover, the rate of DNA repair replication of γ-irradiated, neocarzinostatin-treated, or bleomycin-treated DNA, as measured by 32P incorporation into covalently closed circular DNA, was greatly increased by the presence of NAD+ in reaction mixtures (Fig. 3).

The DNA repair synthesis was inhibited (>80% inhibition) by 80 μg·ml⁻¹ aphidicolin, an inhibitor of DNA polymerase α, δ, and ε, whereas the DNA polymerase β inhibitor dideoxyTTP (80 μg·ml⁻¹) caused little or no reduction of incorporation. Repair synthesis in response to DNA damage inflicted in vivo by radiomimetic agents such as bleomycin is complex and involves both aphidicolin-sensitive and -resistant gap-filling processes (34, 35). In the in vitro assay employed here, aphidicolin-sensitive DNA repair replication at oxygen free radical-induced lesions appeared predominant, possibly because long repair patches were generated.

Stimulation of Poly(ADP-ribose) Formation by DNA Single-strand Breaks—Poly(ADP-ribose) formation occurring in association with DNA repair was followed by measurements of the amounts of [32P]NAD⁺ converted to an acid-insoluble form and by boronate affinity chromatography of reaction products released from protein by alkali treatment (36). Both assays yielded similar results. The cell extracts employed here contained ~1.2 μg of pADPRT/mg of protein, as estimated...
FIG. 2. Kinetics of NAD+-promoted rejoicing of damaged nicked plasmid DNA by a human cell extract. Reaction mixtures (50 µl) contained 0.3 µg of plasmid DNA with a single-strand break generated by a DNA-damaging agent, 50 µg of extract protein from GM06315A cells, and either 2 mM (closed symbols) or no added NAD+ (open symbols).

A, γ-irradiated DNA; B, neocarzinostatin-treated DNA; C, bleomycin-treated DNA. D shows primary data obtained with neocarzinostatin-treated DNA. Rejoined DNA (CC) was separated from nicked DNA (OC) by agarose gel electrophoresis in the presence of ethidium bromide.

FIG. 3. NAD+-stimulated DNA repair replication by human cell extract. Reaction mixtures containing damaged or nondamaged plasmids (0.3 µg) and 50 µg of extract protein from GM06315A cells, [α-32P]dATP and either 0 or 2 mM NAD+ were incubated for 30 min, and the amount of radioactive material incorporated into closed circular plasmids was measured by autoradiography of dried agarose gels.

by immunoblotting (data not shown). On incubation of cell extracts with γ-irradiated, neocarzinostatin-treated, or bleomycin-treated DNA and 0.25 mM NAD+, rapid poly(ADP-ribose) synthesis occurred, which reached a peak between 2 and 5 min (Fig. 4, A and B). The poly(ADP-ribose) was attached to pADPRT, and strongly delayed the migration of this protein during SDS-polyacrylamide gel electrophoresis (data not shown). The amount of poly(ADP-ribose) in reaction mixtures was reduced during prolonged incubation due to poly(ADP-ribose) glycohydrolase activity in the cell extracts. Consumption of NAD+ in cell extracts was insignificant in that 80% of the initial NAD+ levels were retained after a 2-h incubation, as determined by thin layer chromatography. Since automodified pADPRT no longer binds tightly to DNA strand breaks (37, 38), the DNA attachment, automodification, and subsequent release of pADPRT mainly occurred during the early part of incubations.

Repair of Incised AP Sites and Single-nucleotide Gaps—The end groups occurring at strand interruptions in DNA generated by ionizing radiation are complex and heterogeneous. In order to evaluate the effect of NAD+ on repair of strand breaks with better defined terminal structures, AP sites were introduced into circular DNA by brief incubation of covalently closed circular DNA at pH 5.0 and 70 °C. An average of 1.5 AP sites/DNA molecule was obtained, as judged by the amount of conversion of closed DNA circles to an alkali-labile form. Such DNA was incubated with an excess of either the major AP endonuclease of E. coli (exonuclease III) under conditions where the accessory exonuclease activity of the enzyme was not expressed or with another AP endonuclease of E. coli, endonuclease IV. Both these enzymes catalyze hydrolytic incisions on the 3′ side of base-free residues (39), resulting in a strand break with a 5′ terminal deoxyribose-phosphate. Alternatively, the DNA containing AP sites was incubated with an excess of E. coli endonuclease III. This enzyme is a DNA glycosylase catalyzing the excision of ring-saturated and ring-fragmented pyrimidine residues, with an accessory AP lyase activity that promotes p-elimination on the 3′-side of a base-free site (40). In this case, a strand break with a 3′-terminal unsaturated aldehyde form of the sugar residue is generated. Finally, consecutive treatment of partly depurinated DNA with endonuclease III, followed by exonuclease III (under conditions where the exonuclease function is not expressed), generates single-nucleotide gaps at base-free sites (41).

Rejoining of DNA with a hydrolytic incision on the 5′ side of an AP site by a human cell extract was effective both in the absence and presence of NAD+. This is consistent with
previous results (42), which demonstrated rapid replacement of the damaged residue with a single nucleotide by human cell extracts. In the present experiments, only a marginal stimulation of DNA rejoining by NAD$^+$ was observed. In contrast, rejoining of strand breaks generated by $\beta$-elimination was less effective and showed a strong NAD$^+$ dependence (data not shown). The results with DNA molecules containing single-nucleotide gaps were intermediate between these two cases. These observations indicate that the local structure of the termini at DNA strand interruptions influences the NAD$^+$ dependence of the DNA repair reaction. Moreover, the results with DNA containing an altered base-free sugar residue on the 3' terminus of a strand break were similar to those obtained with DNA exposed to $\gamma$-rays and radiomimetic agents (Fig. 2). This is in agreement with the presence of remnant structures of deoxyribose at 3' termini after oxygen radical damage (31, 33, 41).

**Fig. 4. Stimulation of poly(ADP-ribose) formation by various types of damaged DNA.** Reactions were performed in the cell-free assay mixture containing 0.3 $\mu$g of damaged plasmids, 0.25 mM of NAD$^+$, and 2 $\mu$Ci of $[^{32}P]NAD^+$ for various times at $30^\circ$C. $\gamma$-Irradiated DNA was incubated with either 50 $\mu$g (A, *) or 25 $\mu$g (A, ○) extract protein. Nontreated plasmid (A, ○), neocarzinostatin-treated (B, □), bleomycin-treated (B, ▵), MNNG-treated (C, △), UV-irradiated (C, ○), and UV-irradiated and endonuclease III-treated DNA (C, ●) were incubated with 25 $\mu$g of extract protein. The reaction was terminated by addition of 5% trichloroacetic acid, and radioactive acid-insoluble material was collected and counted on glass filters.

**Lack of Effect on Poly(ADP-ribose) Synthesis by DNA Ligases**—The results on NAD$^+$ stimulation of repair of various types of incised AP sites indicate that poly(ADP-ribose) synthesis is not required for strand breaks that are joined rapidly, whereas strand breaks with damaged sugar residues at 3' termini are inefficiently repaired and depend on poly(ADP-ribose) synthesis. The data suggest that repair of DNA strand breaks with 3'-hydroxyl and 5'-phosphate termini, which can be directly and efficiently rejoined by DNA ligases, would not be detectably stimulated by NAD$^+$. However, early studies indicated that one possible mode of action of poly(ADP-ribose) synthesis in DNA repair could be stimulation of DNA ligase II activity (43), although this was not confirmed in a separate study (44). In our assay, neither the rate nor the extent of DNA rejoining stimulated by NAD$^+$ was altered when purified mammalian DNA ligase I, DNA ligase II, or phage T4 DNA ligase was added in excess to reaction mixtures containing $\gamma$-irradiated DNA and human cell extract (data not shown). These results indicate that DNA ligases are not the targets for the NAD$^+$ stimulation of the reaction and that the final ligation step is not the rate-limiting event in the excision-repair of oxygen free radical-damaged DNA termini.

**Repair of Alkylation Damage**—The monofunctional alkylating agents MMS and MNNG generate a variety of DNA lesions, in particular N-modified base residues such as 3'-methyladenine and 7-methylguanine. Intracellularly, these lesions are removed at various rates, and their repair involves the intermittent generation of strand breaks. In the overall repair reaction, poly(ADP-ribose) synthesis occurs in vivo and promotes rejoining (5).

In order to investigate the NAD$^+$ dependence of repair of alkylated plasmid DNA by human cell extracts, the amount of closed circular DNA in reaction mixtures was measured as a function of time, as outlined in Fig. 1. Enzymatic incisions occurred in the alkylated DNA during the first 5–10 min, apparently followed by slower incision activity and simultaneous partial rejoining of nicked DNA circles during continued incubation (Fig. 5, A–C). The rejoining activity was stimulated by NAD$^+$, in agreement with the in vivo results (5), whereas NAD$^+$ had no detectable effect on the initial incision reaction. During the first minutes of the reaction with cell extracts, rapid conversion of superhelical DNA molecules to relaxed covalently closed circular DNA by topoisomerases in the extract also occurred (28). In an alternative approach, NAD$^+$ stimulation of repair of alkylated DNA by human cell extracts could be observed by following DNA repair replication (Fig. 5D). These data demonstrated the effect of NAD$^+$ on the repair reaction, especially during the short incubation times of 5–30 min. Synthesis of poly(ADP-ribose) in reaction mixtures was also triggered by the damaged DNA substrate (Fig. 4C). The NAD$^+$-dependent
repair replication of alkylated closed circular DNA was strongly suppressed by 0.2–1 mM 3-aminobenzamide, a known inhibitor of poly(ADP-ribose) synthesis (4, 5, 7). It was observed previously (22) that 3-aminobenzamide inhibits the NAD+-dependent rejoining of γ-irradiated DNA in vitro. The present data implicate pADPRT activity as being required for efficient DNA repair replication of alkylated DNA and are in general agreement with the original in vivo observations by Durkacz et al. (7), although a number of recent in vivo investigations on the effect of 3-aminobenzamide on DNA repair of alkylated damage have yielded more complex and apparently contradictory results (reviewed in (5)).

Selective Removal of pADPRT from Cell Extracts—Since pADPRT binds very tightly to strand breaks in DNA, chromatography of cell extracts on DNA-cellulose in the presence of 0.4 M NaCl depleted extracts selectively of pADPRT. All detectable amounts (>98%) of pADPRT had been removed from such depleted extracts, as determined by removal of this 113-kDa protein with capacity for automodification in the presence of [32P]NAD+ and nicked DNA and by immunoblotting with antibodies against pADPRT (data not shown). As observed previously with γ-irradiated DNA (22) and here with neocarzinostatin-treated DNA (data not shown), pADPRT-depleted cell extracts repaired DNA strand breaks efficiently even in the absence of NAD+, and addition of NAD+ to reaction mixtures did not stimulate the reaction. Supplementation of cell extracts with purified pADPRT caused a reversion to the original situation by 3-4-fold suppression of rejoining of neocarzinostatin-treated DNA in the absence of added NAD+. Similarly, in experiments with MNNG-treated DNA under the conditions used in Fig. 5A, pADPRT-depleted cell extracts exhibited the same amount of rejoining in the absence or presence of NAD+, whereas regular cell extracts, or pADPRT-depleted extracts supplemented with 1.7 μg/ml purified pADPRT, were equivalent and showed markedly reduced rejoining in the absence of NAD+. These data demonstrate an inhibitory effect of pADPRT on DNA repair, independent of the DNA-damaging agent used to introduce strand breaks, which could be alleviated by automodification of pADPRT or by physical removal of the protein from reaction mixtures.

Repair of UV Damage—Exposure of DNA to UV light generates cyclobutane pyrimidine dimers and 6-4 pyrimidine-pyrimidone adducts as major lesions and also pyrimidine hydrates as minor lesions. Repair of the major lesions by human cells, or cell extracts, occurs by a nucleotide excision-repair process and is associated with a slow and rate-limiting incision step (45–47). Consequently, the amount of DNA strand breaks at dimers at any specific time after incubation is very low, and the repair reaction would not be expected to induce poly(ADP-ribose) synthesis effectively. Nevertheless, UV induction of such polymer synthesis is observed in vivo (45). This could be due to generation of DNA strand breaks during the removal of pyrimidine hydrates by a base excision-repair process. In the present experiments on repair of damaged plasmid DNA molecules added to cell-free extracts, a marked difference was reproducibly observed between the UV-irradiated DNA from which pyrimidine hydrates had been specifically removed by treatment with E. coli endonuclease III followed by repurification versus unprocessed UV-irradiated DNA (Fig. 6, A–D). A small but significant amount of NAD+-promoted DNA repair was observed with UV-irradiated DNA, but this was not the case for DNA depleted of pyrimidine hydrates (Fig. 6, A and B). Similarly, poly(ADP-ribose) formation was clearly induced in cell extracts by UV-irradiated DNA, with maximal formation after 5 min, whereas UV-irradiated DNA from which pyrimidine hydrates had been removed failed to activate poly(ADP-ribose) synthesis (Fig. 4C). Furthermore, UV-induced DNA repair replication in rejoined covalently closed circular DNA molecules was always higher after incubation in NAD+ containing reaction mixtures than in the absence of NAD+, but this difference was no longer apparent with DNA substrates depleted of pyrimidine hydrates (Fig. 6, C and D). A smaller amount of NAD+-independent DNA repair replication was also observed. This may be ascribed to nucleotide excision-repair in vitro of 6-4 pyrimidine-pyrimidone adducts and cyclobutane pyrimidine dimers (46, 47). The present data were obtained with circular DNA molecules containing on average 12 pyrimidine dimers and 0.9 pyrimidine hydrates/molecule after UV irradiation.

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**Fig. 5. NAD+ promoted DNA repair of alkylated DNA.** Covalently closed circular DNA (0.3 pg) treated with MNNG (A), MMS (B), or nontreated (C) was incubated at 30°C with GM06315A cell extract (50 μg) under standard assay conditions in the presence (closed symbols) or absence (open symbols) of 2 mM NAD+. At various times, the relative proportions of closed circular DNA (CC) and open circular DNA (OC) were determined by agarose gel electrophoresis in the presence of ethidium bromide, and densitometry. Data are shown as mean and standard deviation. The mean and standard deviation data are shown in A.
The results indicate that the NAD$^+$ stimulation of repair of UV-irradiated DNA observed in vivo and in vitro is due to the presence of pyrimidine hydrates in the DNA and not to pyrimidine dimers.

**DISCUSSION**

Activation of poly(ADP-ribose) synthesis from NAD$^+$ by purified pADPRT requires DNA containing single- or double-strand breaks as cofactor, whereas covalently closed circular DNA or single-stranded DNA are ineffective (19). Such data, in combination with the sensitization of cells treated with the pADPRT inhibitor 3-amino benzamide to agents generating strand breaks in DNA (7) have implied a role for NAD$^+$ consumption and poly(ADP-ribose) synthesis in DNA repair. Studies with permeabilized cell ghosts (18) also indicated a dependence on NAD$^+$ for repair of strand breaks in damaged endogenous DNA. The development of a cell-free system for NAD$^+$ dependent repair of strand interruptions in DNA (22) has allowed for more detailed experimentation. This assay employs an exogenous plasmid DNA substrate, which can be more readily manipulated than endogenous cellular DNA, together with a gently prepared essentially DNA-free and NAD$^+$-free soluble cell extract. By this experimental approach, plasmids containing a specific type of damage can be employed as substrates, and the effects of charged or macromolecular reagents to which cells would be impermeable can be directly investigated. Moreover, specific depletion of histones from cell extracts did not affect the results (22), although histones have been proposed as possible targets of poly(ADP-ribose)lation during DNA excision repair (48).

The three DNA-damaging agents neocarzinostatin, bleomycin, and γ-rays introduce DNA strand breaks with different termini. However, since the main targets are deoxyribose residues there is a partial overlap of lesions. The non-protein chromophore of neocarzinostatin can be converted into a radical form that attacks the deoxyribose of TMP residues, generating as the major lesion a chain break with a thymidine 5'-aldehyde on the 5' and a phosphate on the 3' side (32). In contrast, bleomycin in the presence of oxygen and iron causes the formation of strand scissions with a phosphoglycolate at the 3' end (33), and such lesions account for >95% of the strand breaks (49). Ionizing radiation generates strand breaks of a more heterogeneous nature, including about 50% lesions with 3'-terminal phosphoglycolates, and also lesions with 3'-phosphate termini. Although excision enzymes that remove 3'-terminal phosphoglycolate residues from DNA strand breaks have been described (49,50), it would appear from the repair kinetics observed here (Fig. 2) that single-strand scissions with 3'-terminal phosphoglycolates were particularly poorly repaired by extracts from lymphoblastoid cells and HeLa cells and show a strong dependence on addition of NAD$^+$ for rejoicing. On the other hand, breaks with 3'-phosphate residues were more effectively rejoined. A strong potentiation of the cell killing effect of bleomycin by inhibitors of poly(ADP-ribose) synthesis has been observed in vivo (51). These data emphasize the possible role of 3'-terminal phosphoglycolate residues at DNA single-strand interruptions as potential cytotoxic lesions after cellular exposure to bleomycin and ionizing radiation, since this form of damage is much more frequent than DNA double-strand breaks. In the latter regard, little or no recircularization of plasmid DNA linearized by HindIII treatment was observed under the conditions used here, even in the presence of NAD$^+$ (data not shown).

Exposure of cells to UV light induces poly(ADP-ribose) synthesis in cell nuclei, although the response is weaker than that to ionizing radiation or alkylating agents (5). In apparent agreement, DNA repair replication with UV-irradiated plasmids added to cell extracts showed a marked and completely reproducible stimulation by NAD$^+$ in the present study (Fig. 6). However, when the UV-irradiated DNA was pretreated with E. coli endonuclease III (a DNA glycosylase/AP lyase specific for pyrimidine hydrates) and re-purified, the DNA failed to elicit significant NAD$^+$-dependent DNA repair replication. Similarly, endonuclease III treatment of UV-irradiated DNA suppressed the ability of the damaged DNA to induce poly(ADP-ribose) formation in the cell-free system (Fig. 4C). The remaining DNA had been freed of pyrimidine hydrates but still contained UV-induced cyclobutane pyrimidine dimers and pyrimidine-pyrimidone 6-4 adducts (52).
repair of these latter lesions requires a multi-protein complex including several DNA replication factors (46, 47). One function of this nucleotide excision-repair complex may be the protection of DNA from repair of strand breaks adjacent to pyrimidine dimers, to prevent binding of pADPRT to the pyrimidine residues also occurs with γ-irradiated DNA (for review, see Ref. 53).

The sensitization of mammalian cells to DNA-damaging agents by inhibitors of poly(ADP-ribose) synthesis has usually been interpreted to mean that pADPRT plays an important but undefined accessory role in DNA repair. However, there is no direct evidence available to support this notion. The experiments with a cell-free system described here instead indicate only an incidental and negative role of pADPRT in DNA excision-repair, and it seems unclear whether further studies on poly(ADP-ribose) will contribute to the elucidation of basic mechanisms of DNA repair in higher eukaryotes. In recent investigations of reduction of pADPRT levels in HeLa cells by antisense RNA expression, the cellular chromatin protection of newly synthesized Okazaki fragments from the often protection of DNA with single-strand breaks adjacent to light to induce poly(ADP-ribose)ent protection of DNA from the reduction of pADPRT levels in HeLa cells suggests a structural role for pADPRT-depleted cells implies a structural role for pADPRT (9, 46, 47).

acknowledgments—We thank Rick Wood for many discussions, Danièle Poirier for assistance, and Grigory Dianov, Chris Althaus, F. R., and Richter, C. recent investigations of reduction of pADPRT levels in HeLa cells by antisense RNA expression, the cellular chromatin protection of newly synthesized Okazaki fragments from the reduction of pADPRT levels in HeLa cells suggests a structural role for pADPRT-depleted cells implies a structural role for pADPRT (9, 46, 47).

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