DNA Polymerases $\alpha$ and $\beta$ Are Required for DNA Repair in an Efficient Nuclear Extract from Xenopus Oocytes

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Xenopus oocytes and an oocyte nuclear extract efficiently repair the bulky DNA lesions cyclobutane pyrimidine dimers, (6-4) photoproducts, and N-acetoxy-2-acetylaminofluorene (AAF) adducts by an excision repair mechanism. Nearly all (>95%) of the input damaged DNA was repaired within 5 h in both injected cells and extracts with no significant incorporation of label into control undamaged DNA. Remarkably, more than 10^{10} cyclobutane pyrimidine dimers or (6-4) photoproducts are repaired/nuclei. The extracts are free from nuclease activity, and repair is independent of exogenous light. Both the high efficiency and DNA polymerase requirements of this system appear to be different from extracts derived from human cells. We demonstrated a requirement for DNA polymerases $\alpha$ and $\beta$ in repair of both photoproducts and AAF by inhibiting repair with several independent antibodies specific to either DNA polymerases $\alpha$ or $\beta$ and then restoring repair by adding the appropriate purified polymerase. Repair is inhibited by aphidicolin at concentrations specific for blocking DNA polymerase $\alpha$ and dideoxynucleotide triphosphates at concentrations specific for inhibiting DNA polymerase $\beta$.

Repair of UV-photoproducts and many other bulky adducts in DNA is generally considered to require the nucleotide excision repair (NER) pathway (1, 2). Cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts are the major products of UV irradiation of DNA (1, 2) while N-acetoxy-2-acetylaminofluorene (AAF) is a widely used reagent to study NER of bulky chemical adducts (3). Whole-cell extracts and permeabilized cell systems from eukaryotes have been employed for identification of many proteins involved in repair even though only <5% of the input damaged DNA is generally repaired and undamaged control DNA supports incorporation of significant amounts of labeled nucleotides (4, 5). Sancar and co-workers (6) demonstrated that the eukaryotic NER pathway excises CPDs and psoralen monoadducts in a manner analogous to the Escherichia coli NER pathway. Proteins involved in eukaryotic NER have recently been identified (4), and the overall reaction has been reconstituted with purified proteins (7).

Xenopus oocytes contain an extensive reservoir of proteins and RNA required for early amphibian development (8). Consequently, injected oocytes or extracts have been used to study a wide variety of biochemical processes including transcription, DNA replication, recombination, and repair. We previously demonstrated the extraordinarily efficient repair of cyclobutane dimers (more than 10^{10} dimers/oocyte) in injected Xenopus laevis oocytes (9). Here, we demonstrate that the Xenopus oocyte extract derived from Xenopus oocytes repairs CPD, the (6-4) photoproduct, and the AAF lesion at an equivalent efficiency to that in injected cells, without significant incorporation of label into undamaged control DNA. Furthermore, both DNA polymerases $\beta$ and $\alpha$ are required for the excision repair pathway of both photoproducts and AAF.

EXPERIMENTAL PROCEDURES

Materials—[32P]Orthophosphoric acid (8500 Ci/mmol), α-[32P]CTP (3000 Ci/mmol) were from Du Pont NEN. Uniformly labeled (~10^6 cpm/μg) pSP65 (Promega) was prepared as described previously (9). Xenopus were from Xenopus, Xenopus Express, and Nasco.

Oocyte Microinjections—20-nl samples were microinjected into the animal pole of mature stage VI oocytes as described by Gurdon (10) followed by incubation at 18 °C for 3 h.

Preparation/Analysis of Damaged DNA—(a) For CPD, form I pSP65 was irradiated with a 15-watt germicidal lamp (254 nm, 9815 Series, Cole Parmer, 5.1 J/m^2) for various times. Irradiation at 10 cm for 25 s produces ~4 CPD/plasmid. Repair was monitored as described previously (9) with T4 UV endonuclease digestion and electrophoresis on alkaline agarose gels. Repaired DNA is resistant to UV endonuclease and migrates as an intact band. Unrepaired uniformly labeled DNA cut with UV endonuclease migrates as a smear of low molecular weight products on alkaline gels. Incorporation of α-[32P]CTP as a consequence of repair was also used to monitor removal of lesions. (b) For (6-4) lesions, 200 μg of pSP65 DNA (0.05 μg/μl) were irradiated (254 nm) 75 s at 10 cm, incubated with 20 μg of E. coli photolyase (Drs. Aziz and Gwendolyn Sancer (University of North Carolina, Chapel Hill) in 50 mM Tris-HCl, pH 7.6, 10 mM NaCl, 1 mM EDTA, 0.1 mg/ml bovine serum albumin, 10 mM DTT, and the reaction mixture was irradiated in borosilicate tubes with filtered 365-nm UV-light for 1 h, at 20 cm, followed by proteinase K, phenol/chloroform extraction, ethanol precipitation, and digestion with 20 μg of E. coli endonuclease III (Dr. Richard Cunningham (SUNY, Albany) for 2 h, 37 °C, 50 mM Hepes, pH 8.0, 100 mM KCl, 1 mM EDTA, 1 mM DTT, and treated with T4 UV endonuclease (Dr. Stephen Lloyd, University of Texas, Galveston) to remove any remaining CPDs. The DNA was again purified by proteinase K, phenol/chloroform extraction, and ethanol precipitation. Form I DNA was isolated by CsCl-EtBr ultracentrifugation, and after dialysis diluted to 0.1 μg/μl. For the analysis of (6-4) lesions, labeled DNA, after extraction and linearization, was photooxidized by irradiation in TE buffer with filtered (Mylar<sup>®</sup>) 500D (Du Pont; cutoff <320 nm) UVB light (FS20 sunlamps, Westinghouse Electric, Somerset, N J) at 10 cm, 4 h. Cleavage at Dewar photoproducts was with 0.4 μM NaOH, pH 13.5 for 20 h, 25 °C in the dark. Neutralized samples were electrophoresed on 1% alkaline agarose gels. (c) For AAF lesions, pUC18 containing 5 AAF...
adducts/plasmid were purchased from Texagen (Plano, TX) or prepared as described previously (11) with AAF purchased from Midwest Research Institute (Kansas City, Mo).

Antibodies—Hybridoma cells for pol β mAbs 10 and 17 Sweregrown at Bio-Molecular Technology, Inc. (Frederick, MD). All antibodies were purified on Perseptive Biosystems Protein G affinity columns (4.6 × 50 mm) equilibrated with 20 mM NaPO₄, pH 8.0, 150 mM NaCl, washed with at least 20 column volumes, eluted with 0.1 M citric acid, pH 3.0; antibodies were dialyzed against 10 mM HEPES, pH 7.4, 20 mM NaCl, and concentrated to ~3 mg/ml. The neutralizing pol β polyclonal antibody was purifed on a pol β affinity column. All antibodies were free of non-specific nuclease activity because undamaged controls did not incorporate label (Fig. 3 and 5), and repair occurred in the dark (not shown). The extract is notably deficient in nonspecific nuclease activity because undamaged control DNA does not incorporate label (Figs. 3 and 5) and the uniformly labeled undamaged DNA became completely resistant to repair rate observed in injected cells (9). Photoreactivation cannot account for this repair because labeled dCMP is incorporated as a consequence of repair (Figs. 3 and 5) and repair occurred in the dark (not shown). The extract is notably deficient in nonspecific nuclease activity because undamaged control DNA does not incorporate label (Figs. 3 and 5) and the uniformly labeled undamaged DNA is not degraded even after 4 h of incubation (Fig. 1).

Preparation of Nuclear Extracts—Oocyte nuclear extracts were prepared as described elsewhere (12) except J-buffer (10 mM HEPES, pH 7.4, 70 mM KCl, 7 mM MgCl₂, 0.1 mM EDTA, 2.5 mM DTT, 1% polyvinylpyrrolidone, and 10% glycerol) (13) was used as the storage buffer. Nuclear extracts are stable for at least 2 years at –80°C.

Repair Reactions—Repair reactions (0.1–0.2 μg of DNA in 30 μl), buffers, and nucleotide concentrations were as described previously (14), with the sole exception that reactions with uniformly labeled substrate contained five times higher [dCTP] (i.e. 50 μM) than used for the label incorporation assays. Each label incorporation assay (30 μl) used J-buffer (70 mM KCl, 7 mM MgCl₂, 0.1 mM EDTA, 2.5 mM DTT, 10% glycerol, 10 mM HEPES, pH 7.4, 1% polyvinylpyrrolidone 360), 0.1–0.2 μg of pSp65 (form I), 2.5 μCi of [α-³²P]dCTP, 50 μM dATP, dGTP, and dTTP each, 10 μM dCTP, and an ATP-regenerating system (6 mM creatine phosphate and creatine kinase (10 units/ml)). Each reaction contained the equivalent of two to five nuclei (~3–7.5 μg of total protein). All repair reactions were incubated at 25°C for the times indicated. Recovered DNA was processed as described previously (9) and linearized with EcoRI before electrophoresis on alkaline agarose gels.

RESULTS AND DISCUSSION

Rapid Repair of CPDs in Nuclear Extracts with Low Background—DNA repair of CPDs was determined by removal of UV endonuclease-sensitive sites as monitored by alkaline agarose gel electrophoresis (Fig. 1). Repair reactions with uniformly labeled DNA (Figs. 1 and 2) or incorporation of α-[³²P]dCTP into irradiated DNA (Figs. 3 and 5). The uniformly labeled DNA became completely resistant to UV-endonuclease within 3 h at ~4 CPDs/plasmid, corresponding to repair of ~5 × 10¹⁰ CPDs/nucleus and correlating with the repair rate observed in injected cells (9). Photoreactivation cannot account for this repair because labeled dCMP is incorporated as a consequence of repair (Figs. 3 and 5), and repair occurred in the dark (not shown). The extract is notably deficient in nonspecific nuclease activity because undamaged control DNA does not incorporate label (Figs. 3 and 5) and the uniformly labeled undamaged DNA is not degraded even after 4 h of incubation (Fig. 1).

Comparison of Repair for CPDs versus (6-4) Lesions in Extracts—The extract can repair up to ~40 lesions/plasmid in 6 h (Fig. 1B). Irradiation conditions producing this level of damage form not only CPDs, but also (6-4) damage and minor photoproducts (15). While both CPD and (6-4) lesions are mutagenic (16), studies with mutant cell lines and extracts indicate that CPD and (6-4) lesions may be repaired differently because extracts from human cell lines showed more repair synthesis for (6-4) lesions than CPDs (17, 18). We produced templates containing either CPD or (6-4) lesions and DNA repair of (6-4) photoproducts was monitored as described previously (15). (6-4) photoproducts were converted into Dewar pyrimidinone photoproducts by photoisomerization and these adducts are labile in mild conditions.

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Aphidicolin and Antibodies to DNA Pols in repair rates for CPD migrate as a smear. We found no significant difference in rose gels, whereas unrepaired plasmid will be cleaved and repaired plasmid migrates as an intact band on alkaline agar-alkali (Fig. 2).

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FIG. 3. Repair of A, CPDs; B, (6-4); and C, AAF lesions is inhibited by aphidicolin and antibodies to DNA Pols α and β in injected oocytes. Aphidicolin was 0.1 mg/ml and antibodies to DNA polymerase α were as described previously (14). Approximately four to five lesions/plasmid; α-AB1 = 1 mg mAb 5315 to calf DNA polymerase α; α-AB2 = 1 mg mAb SJ K132-20 to KB DNA polymerase α; β-AB1, β-AB2, β-AB3 = mAb 10 S (IgG1) and 17 S (IgG2a), and polyclonal anti-β-pol, respectively, to rat DNA polymerase β (30). DNA, label, and inhibitors were injected simultaneously.

Repair of CPDs, (6-4), and AAF Lesions Is Inhibited by Aphidicolin and Antibodies to DNA Pols α and β in Injected Oocytes. Aphidicolin was 0.1 mg/ml and antibodies to DNA polymerase α were as described previously (14). Approximately four to five lesions/plasmid; α-AB1 = 1 mg mAb 5315 to calf DNA polymerase α; α-AB2 = 1 mg mAb SJ K132-20 to KB DNA polymerase α; β-AB1, β-AB2, β-AB3 = mAb 10 S (IgG1) and 17 S (IgG2a), and polyclonal anti-β-pol, respectively, to rat DNA polymerase β (30). DNA, label, and inhibitors were injected simultaneously.

FIG. 4. Antibodies recognize Xenopus DNA Pols a and β. A, immunoblot of DNA pol α from Xenopus oocytes and calf thymus. IgM mAb 5315 to calf DNA polymerase α (28) was used to detect the calf pol α or Xenopus pol α (kindly provided by Dr. R. M. Benbow). ~10-μg protein samples were electrophoresed on a Novex™ 4–12% SDS Tricine gel for staining with Coomassie Brilliant Blue (CBB) and ~1-μg samples on the same gel were immunoblotted (Western) using a Millipore Milli-Electroblotter. Detection was with an Amersham ECL kit utilizing rat IgG, horseradish peroxidase-linked F(ab)2 fragment from sheep as the secondary antibody. Both Xenopus and calf pol α exhibit a major band at 68 kDa (line). B, antibody to pol α recognizes calf pol α but does not recognize Klenow DNA polymerase. Conditions were as in A except ~1 μg of Pharmacia Klenow DNA polymerase was substituted for Xenopus DNA polymerase α. Mark 12th protein standards were from Novex.

and colleagues recently demonstrated a requirement for DNA pol ε, but also showed that repair is influenced by DNA pols α and δ (20). NER extracts from human cells require PCNA and DNA pol δ (21). Yet, DNA pol β was suggested to be involved in UV and bleomycin-induced repair (22). It has been shown to be involved in G: T mismatch repair (23) and uracil-initiated base excision repair in a human cell line extract (24), and a bovine testis nuclear extract (25). DNA β-pol's catalytic activity is consistent with a role in short gap-filling synthesis to restore double-stranded DNA at gapped intermediates produced during excision repair (25, 26).

Specific inhibitors of DNA polymerases are available for DNA pols α and β. Monoclonal antibody SJ K132-20 recognizes a conserved structure found in all known α-pol (27); rat mAb 5315 was raised against bovine pol α (28) and also specifically interacts with Xenopus DNA pol α (Fig. 4). Both of these pol α mAbs were shown to inhibit single-stranded to double-stranded DNA replication in Xenopus oocytes and extracts (14). Antibodies 5315 and SJ K132-20 inhibited CD repair in injected oocytes, and 5315 inhibited both (6-4) and AAF lesion repair in injected oocytes (Fig. 3), indicating a requirement for pol α. In the oocyte nuclear extracts, the DNA pol α antibodies inhibit CPD, (6-4), and AAF lesion repair (Fig. 5).

Inhibition of Repair by Antibodies Can Be Reversed by Adding Purified DNA Pols α and β—The inhibitory effects of the 5315 antibody on repair of all three lesions were reversed by adding purified Xenopus DNA pol α (Fig. 5A), thereby demonstrating a role for DNA pol α in repair. Aphidicolin inhibited repair of CPDs in injected oocytes (9), (6-4), and AAF lesions (Fig. 3, B and C), consistent with a role for one or more of the α-like DNA polymerases.

DNA pol β is specifically recognized by antibodies and also
can be inhibited selectively at appropriate concentrations of dideoxynucleotide triphosphates (29). One of the pol β antibodies used here was shown to specifically recognize Xenopus pol β and inhibit single-stranded to double-stranded replication in oocytes and extracts (14). This pol β antibody, as well as a neutralizing polyclonal antibody (Ref. 25, not shown) and two monoclonal antibodies to pol β (30) inhibit repair in the oocyte system (Figs. 3 and 5). Furthermore, dideoxynucleotides inhibited repair (Fig. 5C) under conditions where Xenopus DNA polymerase α is not inhibited (31). Addition of purified pol DNA polymerase β (32) reversed the inhibitory effects of the pol β antibodies for repair of CPD, (6,4), and AAF lesions (Fig. 5B). The reversals with DNA pol α and β demonstrate that the inhibitions induced with the multiple independent, purified antibodies reflect specific interactions between the antibodies and the DNA pols, rather than nonspecific inhibitory components in the antibody preparations.

The data presented here strengthen the model that pol β functions in a gap-filling role in both extracts and cells. A requirement for DNA pols α and β for CPD, (6,4), and AAF lesions in Xenopus does not eliminate roles for δ and/or ε.

Since the oocyte is a single germ cell arrested in first meiotic prophase (33), perhaps different DNA pols may be recruited for repair at different stages of meiotic and/or somatic cell cycles. Rosenstein and Setlow (34) demonstrated photolyase activity in frog skin. Our experiments with oocytes and extracts demonstrate no significant photolyase activity, because repair occurs in the dark, label is incorporated into covalently closed substrate containing lesions, and no label is incorporated into undamaged template. Thus, frog skin appears to repair CPD damage utilizing different mechanisms from oocytes. The physiological relevance of the extraordinary repair in Xenopus oocytes to Xenopus biology may be that germ cells must maintain undamaged genomes, and that consequently, oocytes efficiently utilize their ~4000 cells worth of housekeeping proteins, including stockpiled DNA polymerases and other repair proteins. It is relevant that, in Xenopus, DNA replicates faster than in E. coli in log phase, during the first few cell cycles after fertilization. Therefore, the extraordinary repair reported here is directly related to the source of the extracts.

There are complex requirements for DNA pols in closely related base excision repair reactions. Pol β is required for normal base excision repair in a bovine testes nuclear extract (25), yet pol δ and proliferating cell nuclear antigen are required to repair a similar lesion, but with a reduced sugar ring, in a Xenopus whole cell extract (35). Embryonic fibroblast cell lines homozygous for a deletion in the gene for pol β exhibit increased sensitivity to monofunctional DNA-alkylating agents, but not to other DNA-damaging agents (36) while wild type levels of pol β are required for resistance to UV damage in an NIH 3T3 cell line (37).

The notably high efficiency and low background synthesis for excision repair reported here, together with our complementation system, should allow identification of other components in the repair pathway not detectable with other systems. This should permit identification of critical residues and domains of the proteins and will be particularly useful for proteins whose three-dimensional structure is known, such as DNA pol β (38).

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