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Excision Repair of DNA in Nuclear Extracts from the Yeast *Saccharomyces cerevisiae*†

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ABSTRACT: Excision repair of DNA is an important cellular response to DNA damage caused by a broad spectrum of physical and chemical agents. We have established a cell-free system in which damage-specific DNA repair synthesis can be demonstrated in vitro with nuclear extracts from the yeast *Saccharomyces cerevisiae*. Repair synthesis of UV-irradiated plasmid DNA was observed in a radiation dose-dependent manner and was unaffected by mutations in the *RAD1*, *RAD2*, *RAD3*, *RAD4*, *RAD10*, or *APN1* genes. DNA damaged with *cis*-platin was not recognized as a substrate for repair synthesis. Further examination of the repair synthesis observed with UV-irradiated DNA revealed that it is dependent on the presence of endonuclease III-sensitive lesions in DNA, but not pyrimidine dimers. These observations suggest that the repair synthesis observed in yeast nuclear extracts reflects base excision repair of DNA. Our data indicate that the patch size of this repair synthesis is at least seven nucleotides. This system is expected to facilitate the identification of specific gene products which participate in base excision repair in yeast.

Ultraviolet (UV) light, ionizing radiation, and a variety of chemical agents can cause chemical alterations in the nitrogenous bases of DNA (Friedberg, 1985). Base excision repair and nucleotide excision repair are two important cellular responses to such genetic insults. Base excision repair is initiated by the specific recognition of a variety of chemically altered or inappropriate (such as uracil or hypoxanthine) bases by DNA glycosylases. These enzymes catalyze the hydrolysis of the *N*-glycosyl bond linking the bases to the sugar-phosphate backbone, resulting in their excision as free bases. The resulting sites of base loss are then attacked by an apurinic/

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Table I: Yeast Strains Used

strain	genotype	source
BJ2168	*MATa RAD leu2 trp1 ura3-52 pep4-3 prb1-1122 prc1-407*	Yeast Genetic Stock Center
LN200	*MATa RAD pep4-3 his4 trp1 leu2 ura3-52 GAL*	L. Naumovski, this laboratory
SF657-2D	*MATa RAD gal2 pep4-3 his4-580 leu2-1,112 ura3-52*	S. Fields, SUNY, Stonybrook
SF657-2D *rad1Δ*	*MATa rad1Δ gal2 pep4-3 his4-580 leu2-1,112, ura3-52*	E. Yang, this laboratory
BJ2168 *RAD2::URA3*	*MATa RAD2::URA3 leu2 trp1 ura3-52 pep4-3 prb1-1122 prc1-407*	C. Nicolet, this laboratory
LN203-2	*MATa rad3-2 pep4-3 ade2 lys2 ura3-52*	L. Naumovski, this laboratory
SX46A *RAD4::URA3*	*MATa RAD4::URA3 ade2 his3-532 ura3-52 trp1-289*	R. Fleer, this laboratory
BJ2168 *rad10-Δ*	*MATa rad10-Δ::URA3 leu2 trp1 ura3-52 pep4-3 prb1-1122 prc1-407*	B. Weiss, this laboratory
DRY370	*MATa apn1-Δ1:: HIS3 ura3 leu2 trp1 his3*	B. Demple, Harvard

apyrimidinic (AP) endonuclease. In *Escherichia coli*, there are indications that the terminal deoxyribose–phosphate residues generated by endonucleolytic cleavage 5′ to sites of base loss are excised by a specific enzyme which has been designated as DNA deoxyribophosphodiesterase (Franklin & Lindahl, 1988). The resulting single nucleotide gap can then be filled in by repair synthesis and base excision repair completed by DNA ligation. Other possible modes for the completion of base excision repair following initial 5′-endonucleolytic cleavage of DNA in vivo have not been ruled out. These may include 5′ → 3′ exonucleolytic degradation by one or more exonucleases. Additionally, endonucleolytic incision may be initiated 3′ to sites of base loss.

Nucleotide excision repair of DNA involves the recognition and incision of DNA at or near sites of base damage as a *primary* event, followed by excision of oligonucleotide fragments containing the damaged bases, repair synthesis, and DNA ligation (Friedberg, 1985). In *E. coli*, the products of at least three genes designated *uvrA*, *uvrB*, and *uvrC* are required for damage-specific recognition and incision of DNA, and the products of several other genes are required for the excision and repair synthesis events [see Van Houten (1990) for a recent review].

Considerably less information is available about the biochemistry of base excision and nucleotide excision repair in eukaryotic cells. Such studies would be greatly facilitated by the availability of cell-free systems which reflect enzymatic events specifically associated with excision repair. To this end, Wood and his colleagues (Wood et al., 1988) have successfully adapted a mammalian cell-free system which supports transcription in vitro, for the demonstration of nucleotide excision repair of DNA. This cell-free system measured damage-specific repair synthesis in plasmid DNA and has been shown to recognize base damage caused by UV radiation, *trans*- or *cis*-platin, psoralen, and (*N*-acetoxyacetylamino)fluorene (Wood et al., 1988; Hansson & Wood, 1989; Hansson et al., 1989; Sibghat-Ullah et al., 1989; Reardon et al., 1991). Using this cell-free system, these investigators have demonstrated defective nucleotide excision repair in extracts of human cells from various genetic complementation groups of the hereditary disease xeroderma pigmentosum (XP), and the restoration of normal levels of repair synthesis by complementation of extracts (Wood et al., 1988). The ability to complement extracts of mutant cells has facilitated the purification of the XPA protein (Robins et al., 1991).

The yeast *Saccharomyces cerevisiae* has been shown to be an informative model system for investigating the molecular biology and biochemistry of both base and nucleotide excision repair in eukaryotes. The genetic versatility of yeast has resulted in the isolation and characterization of a large number of mutants, many of which have been successfully exploited for functional cloning of DNA repair genes (Friedberg et al., 1991). In this report, we present the results of studies on excision repair in yeast cell-free extracts. We show that nuclear extracts from *S. cerevisiae* support repair synthesis of plasmid DNA which is strictly dependent on the presence of damage caused by UV radiation or osmium tetroxide. Detailed analysis of this repair synthesis suggests that it derives from base excision repair of monobasic photoproducts, including thymine glycols.

Materials and Methods

Materials. E. coli photolyase was generously provided by Dr. Aziz Sancar, Department of Biochemistry, University of North Carolina. *E. coli* endonuclease III was a gift from Dr. Richard Cunningham, Department of Biological Sciences, SUNY at Albany. Rad3 protein was provided by Dr. Hanspeter Naegeli in this laboratory and was purified as previously described (Naegeli et al., 1992). *Micrococcus luteus* pyrimidine dimer–DNA glycosylase (UV–DNA endonuclease) was purchased from Applied Genetics, Inc. Plasmid pGEM-3Zf(+) was purchased from Promega. Osmium tetroxide, *cis*-dichlorodiammineplatinum(II) (*cis*-platin), protease inhibitors, phosphocreatine (disodium salt), creatine phosphokinase, ATP, and deoxyribonucleoside triphosphates were purchased from Sigma Chemical Co. Sucrose (ultra pure) and acetylated bovine serum albumin were from BRL. Zymolyase 100-T was purchased from ICN Biomedicals, Inc.

Strains. E. coli strain DH5α was used to propagate plasmids, and was purchased from BRL. The *S. cerevisiae* strains used are listed in Table I. The human cell line HT1080 is a fibrosarcoma line (Rasheed et al., 1974) which is proficient in DNA repair.

Preparation of Yeast Nuclear Extracts. Yeast nuclear extracts were prepared essentially as described by Lue et al. (1991) and Verdier et al. (1991). Yeast cells were grown at 30 °C in YPD medium (1% yeast extract, 2% bactopeptone, and 2% glucose) to late-logarithmic phase. Cells were harvested by centrifugation, washed once in water, and resuspended to 0.1 g/mL in 0.1 M ethylenediaminetetraacetic acid (EDTA)[1]–KOH (pH 8.0)–10 mM dithiothreitol. Cell suspensions were incubated at 30 °C for 10 min with shaking (50 rpm) and centrifuged for 10 min at 6000 rpm in a JA-14 rotor (Beckman) at 4 °C. Cell pellets were resuspended to 1 g/mL in YPD medium containing 1 M sorbitol, and zymolyase 100-T was added to 1 mg/g of cells. Following incubation at 30 °C for ~1 h with shaking (50 rpm), ice-cold YPD medium containing 1 M sorbitol was added (10 mL/g of cells). Cells were collected by centrifugation as before, washed twice in the same medium, resuspended to 0.1 g/mL in the YPD–sorbitol medium, and incubated at 30 °C for 30 min with shaking (50 rpm), followed by the addition of PMSF to 0.5 mM. Cells were pelleted by centrifugation, washed once in 1 M sorbitol, and resuspended to 0.25 g/mL in 5 mM Tris-HCl (pH 7.4), 20 mM KCl, 2 mM EDTA–KOH (pH 7.4),

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; TE, 10 mM Tris-HCl (pH 7.5)–1 mM EDTA.

0.125 mM spermidine, 0.05 mM spermine, 18% ficoll, 1% thiodiglycol, and protease inhibitors (pepstatin, leupeptin, chymostatin, and antipain at 1 μg/mL each, 300 μg/mL benzamidine, and 1 mM PMSF). Spheroplasts were lysed in a motor-driven homogenizer with 10 strokes. Cell debris and unlysed spheroplasts were removed by centrifugation twice at 7500 rpm in a JA-20 rotor (Beckman) for 10 min at 4 °C, followed by repeated 5-min centrifugations at 6000 rpm to obtain a clear supernatant.

Nuclei were pelleted from the supernatant by centrifugation at 13 000 rpm in a JA-20 rotor for 30 min at 4 °C and resuspended to 0.6 mL/g of cells in 100 mM Tris–acetate (pH 7.9), 50 mM potassium acetate, 10 mM $MgSO_4$, 2 mM EDTA, 3 mM dithiothreitol, 20% glycerol, and protease inhibitors. A solution of 4 M ammonium sulfate (neutralized with NaOH) was then slowly added to 0.9 M, gently stirred, and centrifuged at 170000*g* for 1 h at 4 °C. The supernatant was adjusted to 75% saturation with solid ammonium sulfate and neutralized with NaOH. Precipitates were collected by centrifugation at 15000*g* for 15 min at 4 °C, resuspended in $^1/_{15}$th volume of the high-speed supernatant in 20 mM Hepes–KOH (pH 7.6), 10 mM $MgSO_4$, 10 mM EGTA, 5 mM dithiothreitol, 20% glycerol, and protease inhibitors, and dialyzed against the same buffer. Precipitates formed during dialysis were removed by centrifugation, and the resulting nuclear extracts were stored at −70 °C.

Preparation of Human Cell-Free Extracts. Whole cell extracts were prepared from the human cell line HT1080 as previously described (Wang et al., 1991).

DNA Damage. Plasmid DNA was isolated and purified by alkaline lysis and CsCl–ethidium bromide equilibrium centrifugation. UV radiation-damaged DNA was prepared by irradiating plasmid DNA (50 μg/mL) on ice in a thin layer under a germicidal lamp at a dose of 450 J/m^2, unless otherwise indicated. *cis*-Platin-damaged plasmid DNA was prepared as described (Hansson & Wood, 1989). Briefly, plasmid pUC18 (100 μg/mL in TE buffer) was incubated with *cis*-platin at a drug:nucleotide ratio of 0.005, at 37 °C for 20 h in the dark. The reactions were stopped by the addition of NaCl to 0.5 M, and the DNA was precipitated with ethanol. To obtain osmium tetroxide-damaged DNA, plasmid pUC18 (100 μg) was mixed with osmium tetroxide at concentrations of 150, 300, or 600 μg/mL in 300 μL of a buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 100 mM NaCl. After incubation at 70 °C for 90 min, the DNA was concentrated to a volume of ~200 μL in a SpeedVac (Savant) and loaded onto a linear 5–20% sucrose gradient in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.5 M NaCl, in an SW41Ti rotor (Beckman). Supercoiled plasmid DNA was separated from nicked DNA by centrifugation at 28 000 rpm for 17 h at 4 °C. Fractions (0.5 mL) were collected from the bottom of the tube, and 3 μL of each fraction was electrophoresed on a 1.2% agarose gel to identify DNA bands. Fractions containing form I plasmid DNA were pooled and precipitated with ethanol.

Photoreactivation of UV-Irradiated DNA. UV-irradiated plasmid pUC18 DNA (5 μg) was mixed with 3.35 μg of *E. coli* photolyase in 25 mM Tris-HCl (pH 7.5), 10 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, and 100 μg/mL acetylated bovine serum albumin. After incubation in the dark at room temperature for 30 min, the mixture was irradiated under two Black-Ray long-wavelength UV lights (Ultra-violet Products Co.) for 30 min at room temperature. The UV light was filtered through a 3-mm glass plate and a plastic 96-well culture plate cover to remove short-wave UV radiation. The DNA was then purified by phenol and chloroform extraction and precipitated with ethanol.

Preparation of UV-Irradiated DNA without Endonuclease III-Sensitive Lesions. UV-irradiated plasmid pUC18 DNA (400 μg) was digested with 40 μg of *E. coli* endonuclease III for 2 h at 37 °C in 16 mL of a buffer containing 40 mM Hepes–KOH (pH 8.1), 100 mM KCl, 1 mM EDTA, and 0.5 mM dithiothreitol. The DNA was concentrated in a SpeedVac to ~3.5 mL, and CsCl and ethidium bromide were added. After centrifugation, form I plasmid DNA band was collected. This treatment reduced the endonuclease III-sensitive sites in the UV-irradiated pUC18 DNA from an average of 0.7 site per molecule to 0.2 site per molecule. To completely remove DNA molecules with endonuclease III-sensitive lesions, 20 μg of the treated DNA was again incubated with 5 μg of endonuclease III at 37 °C for 2 h in a volume of 100 μL. Supercoiled plasmid DNA was again separated from nicked DNA by centrifugation through a linear 5–20% sucrose gradient as described above, and 10 μL from each fraction was electrophoresed on a 1.2% agarose gel to identify the DNA bands. Fractions containing form I plasmid DNA were pooled and precipitated with ethanol.

DNA Repair Synthesis. The DNA repair synthesis assay was adapted from that of Wood et al. (1988) and was described previously (Wang et al., 1991). Briefly, the standard reaction mixture (50 μL) contained 300 ng each of damaged plasmid pUC18 DNA and undamaged plasmid pBR322 or pGEM-3Zf(+) DNA, 45 mM Hepes–KOH (pH 7.8), 7.4 mM $MgCl_2$, 0.9 mM dithiothreitol, 0.4 mM EDTA, 2 mM ATP, 20 μM each of dATP, dGTP, and TTP, 8 μM dCTP, 1 μCi of [α-^{32}P]dCTP (3000 Ci/mmol), 40 mM phosphocreatine (disodium salt), 2.5 μg of creatine phosphokinase, 4% glycerol, 100 μg/mL bovine serum albumin, and yeast nuclear extracts. The assay measures incorporation of [^{32}P]dCMP into DNA during repair synthesis reactions, and the undamaged DNA was used as the internal control for nonspecific background DNA synthesis. After incubation for 2 h at 30 °C, plasmid DNA was purified, linearized with *Hin*dIII restriction endonuclease, and separated by 1% agarose gel electrophoresis. The gel was stained with ethidium bromide, dried, and autoradiographed. To quantitate [^{32}P]dCMP incorporation, DNA bands were sliced out of the dried gel, and the radioactivity was measured by scintillation counting.

Measurement of Base Excision. Plasmid pUC18 DNA was labeled with [^{3}H]thymine by nick translation in the presence of [*methyl*-^{3}H]TTP (80 Ci/mmol) and yielded radiolabeled DNA with a specific activity of 1.2×10^7 dpm/μg. The DNA was treated with UV irradiation (4 KJ/m^2) or osmium tetroxide (1200 μg/mL) as described above, except that osmium tetroxide was removed by ether extraction and the DNA was precipitated with ethanol and dissolved in TE buffer. Untreated, UV-irradiated, or osmium tetroxide-treated [^{3}H]DNA (100 ng) was mixed with 15 μg of yeast nuclear extract or 0.5 μg of *E. coli* endonuclease III in 40 mM Hepes–KOH (pH 8.1), 100 mM KCl, 1 mM EDTA, and 0.5 mM dithiothreitol. After incubation at 30 °C for 2 h, solutions were dried in a SpeedVac and dissolved in 30 μL of methanol. Following centrifugation in a microcentrifuge for 5 min, the supernatants were spotted onto CEL 300 PEI thin-layer chromatography plates (Macherey-Nagel). Chromatograms were developed with the lower layer of a mixture of chloroform–methanol–H_2O (4:2:1) to which 5 mL of methanol was added for each 100 mL of the organic phase. When the solvent had ascended to 19 cm, plates were dried, and each sample lane was sliced into 1-cm fractions. Radioactivity in each fraction was

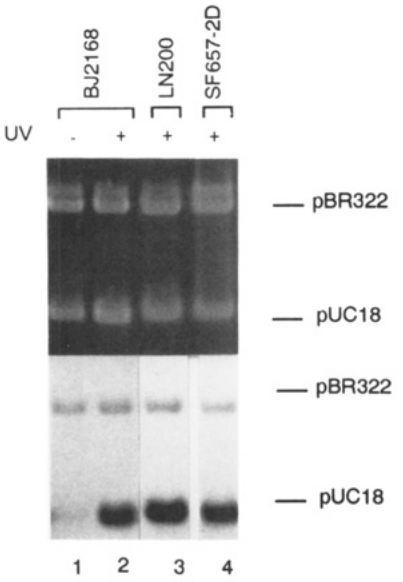

FIGURE 1: DNA repair synthesis in wild-type yeast nuclear extracts. Plasmid pUC18 DNA irradiated with UV (+) or unirradiated (−) was used for repair synthesis in the presence of 50 μg of yeast nuclear extract from the wild-type strain BJ2168 (lanes 1 and 2) or 80 μg of extract from the wild-type strains LN200 (lane 3) and SF657-2D (lane 4). Unirradiated plasmid pBR322 DNA was included in each reaction as an internal control. Top panel, ethidium bromide staining of the gel; bottom panel, autoradiograph of the gel.

measured by liquid scintillation counting.

Results

Repair Synthesis of UV-Irradiated DNA in Yeast Nuclear Extracts. A human cell-free extract originally described by Manley et al. (1983) supports both transcription and nucleotide excision repair in vitro (Wood et al., 1988). Recently, a nuclear extract has been developed from *S. cerevisiae* which supports in vitro transcription (Lue & Kornberg, 1987; Lue et al., 1991). We therefore examined the capacity of such yeast extracts to support excision repair of DNA treated with UV radiation.

Excision repair was measured by the incorporation of radiolabeled nucleotides into UV-irradiated plasmid DNA during repair synthesis catalyzed by nuclear extracts. Figure 1 demonstrates the incorporation of radioactivity into plasmid pUC18 DNA. This incorporation was strictly dependent on UV irradiation of plasmid DNA, since unirradiated plasmid pUC18 DNA (Figure 1, lane 1), or unirradiated plasmid pBR322 DNA included as an internal control, showed only background levels of nonspecific DNA synthesis.

The amount of DNA synthesis observed with yeast nuclear extracts increased linearly with increasing amounts of extract, reaching a plateau at ~80 μg of protein (Figure 2A). In addition, the activity increased with increasing dose of UV radiation, showing a linear response up to 500 J/m^2 (Figure 2B). On the basis of these results, we conclude that the DNA synthesis observed in plasmid DNA represents repair synthesis associated with excision repair of UV radiation-induced damage.

Repair Synthesis in Yeast Nuclear Extracts Does Not Reflect Nucleotide Excision Repair. The extent of the repair synthesis observed in plasmid DNA incubated with yeast nuclear extracts approximates that in human cell-free extracts tested under the same experimental conditions. In the presence of 80 μg of yeast protein, ~150 fmol of dCMP was incorporated into UV-irradiated plasmid pUC18 DNA (Figure 2A). This value is close to the level of incorporation (100–300 fmol

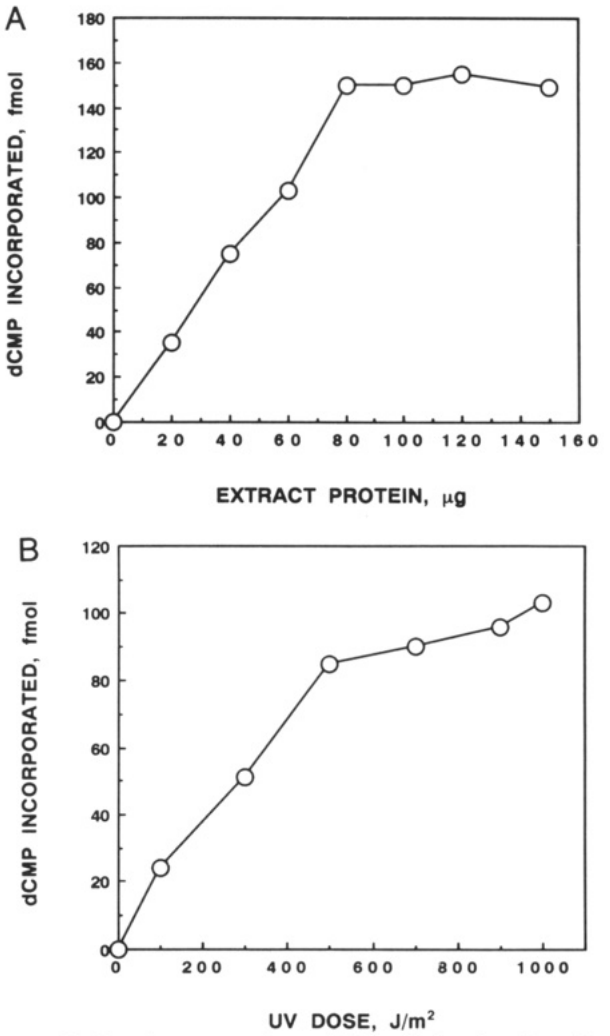

FIGURE 2: Protein concentration and UV radiation dose dependence of DNA repair synthesis in yeast nuclear extracts. (A) UV-irradiated pUC18 plasmid DNA was incubated with the indicated amounts of yeast BJ2168 nuclear extract for 2 h at 30 °C. Incorporation of dCMP into DNA was determined as described under Materials and Methods. Background dCMP incorporation was subtracted. (B) Plasmid pUC18 DNA was irradiated at the doses indicated and incubated with 80 μg of yeast BJ2168 nuclear extract for 2 h at 30 °C. Incorporation of dCMP into DNA was similarly determined.

of dCMP) observed with human cell-free extracts (Wood et al., 1988; Wang et al., 1991). In light of this observation and previous observations that extracts of human cells which are known to be defective in nucleotide excision repair are defective in repair synthesis in cell-free extracts (Wood et al., 1988), we carried out further experiments to determine whether the repair synthesis observed in yeast nuclear extracts reflects nucleotide excision repair.

We measured repair synthesis supported by nuclear extracts prepared from mutant strains defective in the *RAD1*, *RAD2*, *RAD3*, *RAD4*, or *RAD10* genes, which are known to be required for nucleotide excision repair in yeast (Friedberg et al., 1991). In each case, we observed that the levels of repair synthesis of UV-irradiated plasmid DNA were similar relative to that observed with wild-type extracts (Figure 3). These results suggest that the repair synthesis observed in yeast nuclear extracts is not associated with nucleotide excision repair.

UV-irradiated DNA contains multiple photoproducts which are recognized as substrates by both the nucleotide and base excision repair pathways. In order to discriminate between these pathways, we prepared plasmid DNA treated with *cis-*

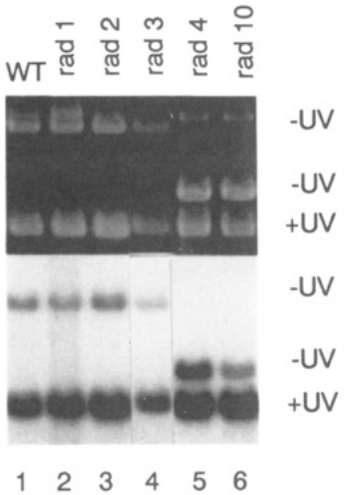

FIGURE 3: DNA repair synthesis in yeast *rad* mutant nuclear extracts. UV-irradiated plasmid pUC18 DNA (+UV) was incubated at 30 °C for 2 h in the presence of 50 μg of nuclear extract from the wild-type (WT) strain BJ2168 (lane 1), the *rad1* mutant strain SF657-2D *rad1*Δ (lane 2), or the *rad2* mutant strain BJ2168 *rad2*::*URA3* (lane 3) or in the presence of 80 μg of nuclear extract from the *rad3* mutant strain LN203-2 (lane 4), the *rad4* mutant strain SX46A *rad4*::*URA3* (lane 5), or the *rad10* mutant strain BJ2168 *rad10*Δ (lane 6). Unirradiated (−UV) plasmid pBR322 or pGEM-3Zf(+) was used as the internal control as indicated. The band at the top of the ethidium bromide-stained gel in lanes 5 and 6 originated from endogenous yeast DNA in the extracts. Top panel, ethidium bromide staining of the gel; bottom panel, autoradiograph of the gel.

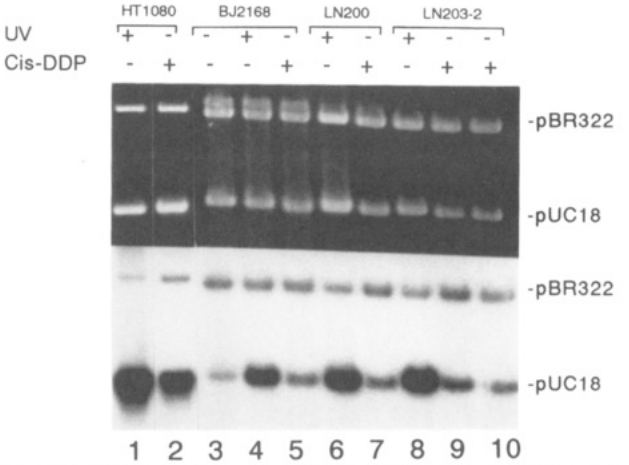

FIGURE 4: DNA repair synthesis in *cis*-platin-damaged DNA. *cis*-Platin-damaged plasmid pUC18 DNA (*cis*-DDP+) or UV-irradiated pUC18 (UV+) was incubated with 80 μg of various cell-free extracts as indicated, at 30 °C for 2 h. Extracts were from the human cell line HT1080 (lanes 1 and 2), the yeast wild-type strains BJ2168 (lanes 3–5) and LN200 (lanes 6 and 7), and the *rad3* mutant yeast strain LN203-2 (lanes 8–10). Purified Rad3 protein (100 ng) was added to the repair reaction in lane 10. Undamaged plasmid pBR322 DNA was used as the internal control, in addition to an undamaged pUC18 DNA control (lane 3). Top panel, ethidium bromide staining of the gel; bottom panel, autoradiograph of the gel.

platin. The available evidence suggests that both in *E. coli* and in mammalian cells *cis*-platin adducts are excised exclusively via a nucleotide excision repair pathway (Van Houten, 1990; Hansson & Wood, 1989; Sibghat-Ullah et al., 1989). Human cell-free extracts supported significant levels of repair synthesis in plasmid pUC18 DNA modified with *cis*-platin (Figure 4, lane 2). In contrast, only background levels of DNA synthesis were observed in nuclear extracts from both wild-type and *rad3* mutant yeast strains (Figure 4, lanes 5, 7, and 9). Repair synthesis was again readily detected in UV-irradiated pUC18 DNA incubated with these extracts in parallel experiments (Figure 4, lanes 4, 6, and 8).

Similar results were obtained when the incubation times with *cis*-platin-treated DNA were extended to 6 h (data not shown). The addition of up to 100 ng of purified Rad3 protein to *rad3* mutant extracts also had no effect on the levels of repair synthesis in *cis*-platin-modified DNA (Figure 4, lane 10), suggesting that the apparent lack of nucleotide excision repair activity is not due to limited amounts of Rad3 protein. Collectively, these results demonstrate that unlike human cell-free extracts, yeast nuclear extracts prepared for in vitro transcription do not support detectable levels of nucleotide excision repair under the experimental conditions used.

Yeast Nuclear Extracts Do Not Repair Pyrimidine Dimers in UV-Irradiated DNA. Exposure of DNA to UV radiation at wavelengths of ~254 nm leads principally to the formation of pyrimidine dimers and 6-4 photoproducts (Friedberg, 1985). In addition to nucleotide excision repair initiated by a direct-acting endonuclease such as the UvrABC enzyme of *E. coli* (Van Houten, 1990), the selective excision of pyrimidine dimers can be initiated by a specific pyrimidine dimer–DNA glycosylase. Such an enzyme has been demonstrated in *M. luteus* and in *E. coli* cells infected with bacteriophage T4 (Haseltine et al., 1980; Radany & Friedberg, 1980). We investigated whether a similar enzyme activity is associated with the repair synthesis of UV-irradiated DNA observed in yeast nuclear extracts.

Plasmid pUC18 DNA radiolabeled with [^{3}H]thymine by nick translation was exposed to UV radiation in the presence of Ag^+ as a sensitizer, in order to increase the yield of thymine dimers (Radany & Friedberg, 1980). Following incubation with yeast nuclear extracts and purification of the DNA, monomerization of pyrimidine dimers was effected by incubation with purified *E. coli* DNA photolyase (Sancar & Sancar, 1988) or by direct photoreversal (Radany & Friedberg, 1980). These experimental conditions result in the release of free radiolabeled thymine if either of the glycosylic bonds in pyrimidine dimers was cleaved by a pyrimidine dimer–DNA glycosylase during the initial incubation. We used purified *M. luteus* pyrimidine dimer–DNA glycosylase as a positive control for this assay. The lower limit of activity detection by this assay was 200 ng of the purified *M. luteus* pyrimidine dimer–DNA glycosylase. In contrast, we were unable to detect any pyrimidine dimer–DNA glycosylase activity in yeast nuclear extracts even when 1 mg of the extract protein was used in extended (2 h) incubations.

In order to provide further evidence that pyrimidine dimers are not a substrate for the repair synthesis observed in yeast nuclear extracts, we selectively removed these photoproducts from UV-irradiated plasmid DNA by photoreactivation with purified *E. coli* DNA photolyase. The monomerization of pyrimidine dimers was confirmed by subsequent incubation of the DNA with purified *M. luteus* pyrimidine dimer–DNA glycosylase. When plasmid pUC18 DNA was UV-irradiated at a dose of 450 J/m^2 and treated with DNA photolyase in the presence of photoreactivating light, most of the DNA was rendered insensitive to subsequent attack by the *M. luteus* enzyme (Figure 5A, compare lanes 3, 4, and 5). However, when we incubated this substrate with wild-type nuclear extracts or extracts prepared from the excision repair-defective mutant *rad3*, the levels of DNA repair synthesis were unaltered relative to that observed in DNA from which dimers were not removed (Figure 5B).

In these experiments, the repair synthesis was not the result of DNA damage sustained during the enzymatic photoreactivation, since repair synthesis was not detected in unirradiated plasmid pUC18 DNA treated identically (Figure 5B, lanes

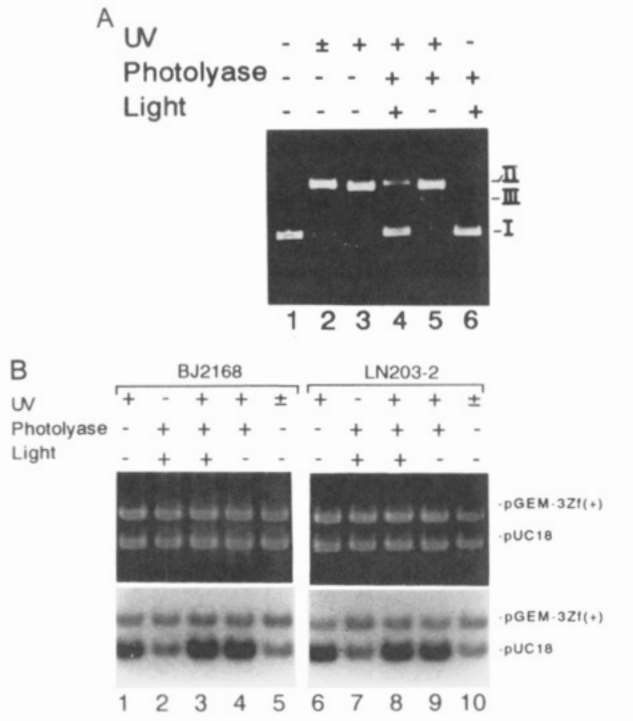

FIGURE 5: Effect of enzymatic photoreactivation on DNA repair synthesis in yeast nuclear extracts. (A) Plasmid pUC18 DNA was irradiated with UV light at a dose of 20 J/m^2 (±) or 450 J/m^2 (+). Photolyase was added (+), and photoreactivation was activated with long-wavelength UV light (+) as described under Materials and Methods. The DNA was then digested with 0.5 μg of *M. luteus* pyrimidine dimer–DNA glycosylase at 30 °C for 2 h in 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 40 mM NaCl and analyzed by 1.2% agarose gel electrophoresis. Unirradiated plasmid pUC18 without or with photoreactivation treatment is also shown in lanes 1 and 6, respectively. The position of supercoiled, relaxed, and linear plasmid DNA is indicated by I, II, and III, respectively. (B) Plasmid pUC18 DNA UV-irradiated at 20 J/m^2 (±) or 450 J/m^2 (+) was treated with (+) or without (−) photolyase and photoreactivating light as indicated. The DNA was incubated with yeast wild-type (BJ2168) or *rad3* mutant (LN203-2) nuclear extracts at 30 °C for 2 h. Undamaged plasmid pGEM-3Zf(+) without photoreactivation treatment was used as the internal control. Top panel, ethidium bromide staining of the gel; bottom panel, autoradiograph of the gel.

2 and 7). When plasmid pUC18 DNA was UV-irradiated at a dose of only 20 J/m^2, the extent of pyrimidine dimer formation was significantly greater than the residual level of pyrimidine dimers following photoreactivation of DNA irradiated at 450 J/m^2 (Figure 5A, compare lanes 2 and 4). Nonetheless, DNA repair synthesis was not detected in the former substrate (Figure 5B, lanes 5 and 10).

Collectively, these results suggest that the repair synthesis observed in UV-irradiated plasmid pUC18 DNA during incubation with yeast nuclear extracts is not initiated by a specific pyrimidine dimer–DNA glycosylase and is in fact independent of the presence of pyrimidine dimers in DNA.

Yeast Nuclear Extracts Support Base Excision Repair of UV-Irradiated Plasmid DNA. Since pyrimidine dimers were apparently not recognized by the yeast nuclear extracts, we investigated the possibility that other lesions produced by UV radiation might be responsible for the repair synthesis observed. UV radiation of DNA can lead to strand breakage (Friedberg, 1985). However, we did not detect a significant loss of form I plasmid pUC18 DNA at doses as high as 1000 J/m^2 (data not shown). To further confirm that the repair synthesis did not result from UV radiation-induced strand breakage, we repurified form I plasmid pUC18 DNA by CsCl–ethidium bromide gradient centrifugation after UV radiation. The extensively purified DNA continued to provide an effective

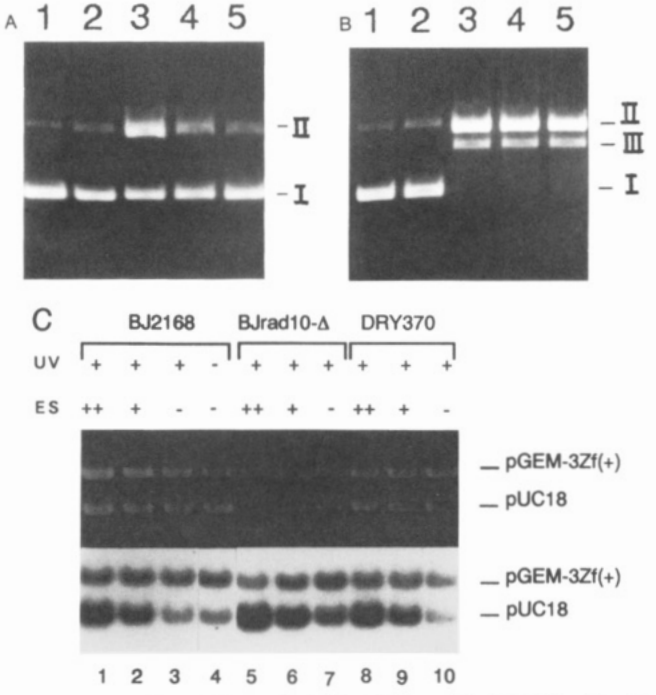

FIGURE 6: DNA repair synthesis in UV-irradiated DNA without endonuclease III-sensitive sites. (A) Plasmid pUC18 DNA (100 ng) was digested with 50 ng of *E. coli* endonuclease III at 37 °C for 2 h under standard conditions (lanes 2–5) and analyzed by 1.2% agarose gel electrophoresis. Lane 2, unirradiated pUC18; lane 3, UV-irradiated pUC18 without treatment with endonuclease III; lane 4, UV-irradiated pUC18 treated with endonuclease III and purified by CsCl–ethidium bromide centrifugation; lane 5, the DNA in lane 4 was further digested with endonuclease III and repurified in a sucrose gradient. Unirradiated pUC18 without endonuclease III digestion is shown in lane 1. (B) Plasmid pUC18 DNA (100 ng) was digested with 0.5 μg of *M. luteus* pyrimidine dimer–DNA glycosylase as described in Figure 5A and analyzed by agarose gel electrophoresis. Gel lanes are as in (A). Supercoiled, relaxed, and linear plasmid DNAs are indicated by I, II, and III, respectively. (C) UV-irradiated plasmid pUC18 DNA not treated with *E. coli* endonuclease III (lanes 1, 5, and 8), treated once with the enzyme (lanes 2, 6, and 9), or treated twice with the enzyme (lanes 3, 7, and 10) was used as substrate for repair synthesis with yeast nuclear extracts. The extracts (80 μg) were from wild-type (BJ2168) (lanes 1–4), *rad10* mutant (BJ*rad10*-Δ) (lanes 5–7), or *apn1* mutant (DRY370) (lanes 8–10) strains. Unirradiated, untreated plasmid pGEM3Zf(+) DNA was used as the internal control, in addition to an unirradiated untreated pUC18 control (lane 4). ES, endonuclease III-sensitive lesions. Top panel, ethidium bromide staining of the gel; bottom panel, autoradiograph of the gel.

substrate for repair synthesis (data not shown).

These observations led us to suspect that the repair synthesis observed in yeast nuclear extracts reflects base excision repair of DNA, presumably initiated by DNA glycosylase-catalyzed excision of monobasic photoproducts. To examine this possibility, we inactivated UV-irradiated plasmid pUC18 DNA molecules containing monobasic photoproducts by incubation with purified *E. coli* endonuclease III, which specifically attacks DNA containing such lesions (Breimer & Lindahl, 1984, 1985; Boorstein et al., 1989; Doetsch & Cunningham, 1990). Following CsCl–ethidium bromide gradient centrifugation, form I DNA was reisolated. Such treatment reduced the endonuclease III-sensitive sites of the plasmid DNA from an average of ~0.7 lesion/DNA molecule to a level of ~0.2 lesion/molecule (Figure 6A, compare lanes 3 and 4). Further incubation of the form I plasmid DNA with endonuclease III followed by sucrose gradient centrifugation resulted in essentially complete removal of DNA molecules containing endonuclease III-sensitive lesions (Figure 6A, lane 5).

The level of other UV radiation-induced lesions is expected to be unaffected by treatment of the DNA with endonuclease

Table II: Comparison of the Excision of Radiolabeled Damaged Bases of DNA by Yeast Nuclear Extracts and *E. coli* Endonuclease III[a]

	E. coli endonuclease III			yeast nuclear extracts		
fraction	no damage (dpm)	UV (dpm)	OsO_4 (dpm)	no damage (dpm)	UV (dpm)	OsO_4 (dpm)
1	1357	543	3324	143	103	1570
2	24	60	412	20	40	149
3	30	100	1000	21	75	247
4	30	60	122	30	44	42
5	26	68	40	25	28	27
6	40	72	46	57	50	32
7	27	89	39	24	55	32
8	40	88	46	29	96	43
9	59	192	91	32	207	38
10	45	318	40	43	176	30
11	30	59	39	33	47	36
12	42	59	55	20	47	27
13	58	118	99	26	52	38
14	171	418	396	38	117	55
15	1086	2035	1768	75	128	33
16	5071	6566	4594	211	67	25
17	1854	2622	3524	51	41	41
18	25	59	202	27	38	29
19	32	30	26	31	29	13

[a] The release of damaged bases was performed as described under Materials and Methods. The [^{3}H]DNA substrate was either untreated (no damage) or treated with UV irradiation (UV) or osmium tetroxide (OsO_4). The source of the radioactive peaks observed near the chromatographic front following incubation of all three DNA substrates with endonuclease III is unknown.

III. This conclusion was supported by the direct demonstration that the level of pyrimidine dimers was not altered (Figure 6B). In parallel with the reduction of the endonuclease III-sensitive sites, repair synthesis supported by both wild-type and *rad* mutant nuclear extracts was significantly reduced after the first treatment with endonuclease III and diminished to background levels after the second treatment (Figure 6C). In contrast, such DNA remained an effective substrate for repair synthesis in human cell-free extracts (data not shown).

Endonuclease III-sensitive sites constitute quantitatively minor lesions in DNA UV-irradiated at ~254 nm. We therefore prepared a substrate containing thymine glycol as the major source of DNA damage. This was achieved by treating plasmid pUC18 DNA with osmium tetroxide, an agent that promotes oxidative damage in DNA (Burton & Riley, 1966). Plasmids which sustained strand breaks during this treatment were removed by sucrose gradient centrifugation. As shown in Figure 7, incubation of this DNA with yeast nuclear extracts led to significant levels of repair synthesis, which increased as a function of increasing levels of thymine glycol in the DNA.

A quantitatively major AP endonuclease from *S. cerevisiae* has recently been characterized and is designated yeast AP endonuclease 1 (Apn1). This enzyme is the yeast homologue of an *E. coli* enzyme designated AP endonuclease IV (Popoff et al., 1990), which is involved in the repair of AP sites and some types of oxidative damage in DNA (Johnson & Demple, 1988a,b; Popoff et al., 1990). Yeast nuclear extracts prepared from an Apn1-defective mutant strain remained fully active in supporting repair synthesis in UV-irradiated DNA (Figure 6C, lane 8).

Release of Damaged Bases from DNA. In *E. coli*, base excision repair of single-base photoproducts such as thymine glycols is initiated by the DNA glycosylase activity of endonuclease III (Breimer & Lindahl, 1984, 1985; Boorstein et al., 1989; Doetsch & Cunningham, 1990). In order to provide direct confirmation of base excision repair in yeast nuclear extracts, we incubated [^{3}H]thymine-labeled UV-irradiated or osmium tetroxide-treated DNA with either yeast extract or purified endonuclease III, and compared the distribution of radiolabeled products in the methanol-soluble fraction by thin-layer chromatography. In the chromatographic system

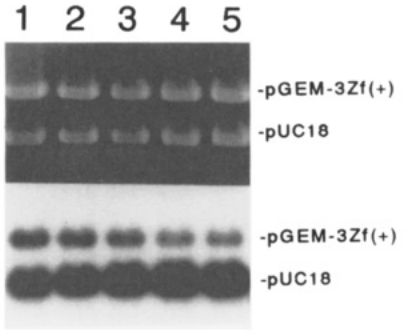

FIGURE 7: DNA repair synthesis in osmium tetroxide-treated DNA. Plasmid pUC18 DNA was treated with 150 (lane 2), 300 (lane 3), or 600 μg/mL (lane 4) osmium tetroxide as described under Materials and Methods. In lane 5, a portion (20 μg) of osmium tetroxide-treated (600 μg/mL) pUC18 DNA was incubated with 20 μg of *M. luteus* pyrimidine dimer–DNA glycosylase at 37 °C for 4 h and purified by sucrose gradient centrifugation. The osmium tetroxide-treated DNAs (lanes 2–5) as well as the UV-irradiated DNA (lane 1) were incubated with 80 μg of yeast wild-type (BJ2168) nuclear extract under standard conditions. Undamaged plasmid pGEM-3Zf(+) DNA was used as the internal control. Top panel, ethidium bromide staining of the gel; bottom panel, autoradiograph of the gel.

employed, DNA and nucleotides remain at the origin while different modified free bases migrate to different positions on the thin-layer plates. As shown in Table II, yeast nuclear extracts catalyzed the release of radiolabeled thymine derivatives from both UV-irradiated DNA (fraction 3, fractions 8–10, and fractions 14–15) and osmium tetroxide-treated DNA (fractions 2–3). Peaks of radioactivity with identical chromatographic mobility were observed following incubation of UV-irradiated or osmium tetroxide-treated DNA with purified *E. coli* endonuclease III (Table II), suggesting that they represent free modified bases released during base excision repair. These results support our conclusion that the repair synthesis in yeast nuclear extracts derives from base excision repair pathway, and suggest that the repair is initiated by DNA glycosylase activities.

Discussion

The results of these studies demonstrate that yeast nuclear extracts which can support accurate transcription in vitro are also able to support repair synthesis of UV-irradiated plasmid DNA. Our studies suggest that this repair synthesis reflects

a base excision repair mode which is associated with the repair of endonuclease III-sensitive lesions in DNA. In *E. coli*, such lesions are recognized by a specific DNA glycosylase with associated 3′ AP endonuclease/lyase activity designated endonuclease III (Breimer & Lindahl, 1984, 1985; Boorstein et al., 1989). This enzyme, which is a product of the *E. coli nth* gene, recognizes pyrimidines in DNA which have been damaged by ring saturation, ring fragmentation, or ring contraction (Cunningham & Weiss, 1985; Breimer & Lindahl, 1984). The structural feature(s) common to all of these alterations is (are) not known. However, it has been suggested that it might involve saturation of the 5,6-double bond and/or loss of the planar configuration in pyrimidine derivatives which lack this double bond (Breimer & Lindahl, 1984).

Following attack by the DNA glycosylase function of endonuclease III, cleavage of the sugar–phosphate backbone is believed to be universally mediated by an AP endonuclease activity. With respect to *E. coli*, there is considerable controversy as to whether such cleavage is mediated by a particular enzyme among the multiple proteins with either 5′ or 3′ AP endonuclease/lyase activity (Doetsch & Cunningham, 1990). In vivo, hydrolysis of phosphodiester bonds at sites of base loss may be primarily if not exclusively mediated by 5′ AP endonucleases, thereby generating 5′-terminal deoxyribophosphate residues which are excised by a distinct enzyme designated DNA deoxyribophosphodiesterase (Franklin & Lindahl, 1988). The major 5′ AP endonuclease in *E. coli* is exonuclease III. *E. coli* also contains a second AP endonuclease of this class designated endonuclease IV.

A DNA glycosylase that specifically recognizes DNA containing thymine glycols has not been characterized from yeast. However, an enzyme called yeast redoxyendonuclease (Gossett et al., 1988) has many properties reminiscent of *E. coli* endonuclease III. The yeast enzyme cleaves UV-irradiated and oxidized DNA substrates. Additionally, it cleaves DNA containing apurinic sites 3′ to such sites. In this regard, the observation that extracts from the *apnI* mutant (which is defective in a quantitatively major 5′ AP endonuclease activity) supported normal levels of repair synthesis is interesting. One interpretation of this finding is that in yeast multiple AP endonuclease are essentially redundant for base excision repair of monobasic photoproducts, as may be the case in *E. coli*. However, an alternative interpretation is that in yeast the redoxyendonuclease catalyzes both base excision by a DNA glycosylase and cleavage of the sugar–phosphate backbone by its 3′ AP endonuclease. Hence, *Apn*I endonuclease may not play a role in the repair of endonuclease III-sensitive lesions in yeast. This is consistent with the phenotype of *apn1* mutants, which are not abnormally sensitive to UV radiation, γ-rays, or some types of oxidative damage (Ramotar et al., 1991). The availability of a yeast cell-free system for base excision repair should facilitate the elucidation of these alternative possibilities using appropriate mutant strains.

In a standard repair synthesis reaction containing 80 μg of the yeast extract protein, we observed that the presence of $\sim$95 fmol of endonuclease III-sensitive sites led to $\sim$150 fmol of damage-specific incorporation of dCMP into repair synthesis patches. Assuming 100% efficiency of the excision repair of endonuclease III-sensitive sites, we estimate that the average repair synthesis patch cannot be smaller than seven nucleotides.

In contrast to the results obtained in extracts of human cells in which repair synthesis largely, if not exclusively, reflects nucleotide excision repair of DNA (Wood et al., 1988), the repair synthesis observed with yeast nuclear extracts apparently reflects exclusively base excision repair. This system therefore offers the potential for defining and characterizing discrete biochemical events associated with base excision repair in yeast. Our inability to detect repair synthesis mediated by nucleotide excision repair of DNA in yeast merits further comment. This result pertained under several other conditions for the preparation of cell-free extracts, including the use of extracts recently reported to support homologous recombination in yeast (Symington, 1991), and cell-free extracts prepared directly according to the Manley protocol (Manley et al., 1983) used in human cells. In both of these situations, we observed unacceptably high levels of background DNA synthesis on undamaged plasmid DNA. The addition of yeast nuclear extracts to human cell-free extracts did not inhibit repair synthesis mediated by the latter extracts, suggesting that the yeast extracts do not contain inhibitors of nucleotide excision repair.

Previous studies on the expression of the yeast *RAD* genes required for damage-specific recognition and incision of DNA have demonstrated that these genes are expressed extremely weakly (Friedberg, 1988). Hence, it is possible that the levels of one or more *RAD* gene products limit the in vitro detection of nucleotide excision repair in yeast. If so, it would appear that at least under constitutive conditions, the relative proportions of enzymes for nucleotide excision repair and base excision repair are different in yeast and mammalian cells.

Thymine glycols are important lesions produced by ionizing radiation and oxidative damage. Therefore, this in vitro excision repair should provide a useful system for the studies on DNA repair of ionizing radiation and oxidative damage in the yeast *Saccharomyces cerevisiae*. Since the yeast cell-free system specifically measures repair synthesis of DNA, this system should be informative for investigating which of the several known yeast DNA polymerases catalyzes this synthesis during base excision repair. Mutants defective in four genes known to encode distinct DNA polymerases in yeast have been isolated in several laboratories (Hartwell et al., 1973; Lemontt, 1971; Morrison et al., 1990), and studies on these mutants are currently in progress.

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