Affinity Purification and Partial Characterization of a Yeast Multiprotein Complex for Nucleotide Excision Repair Using Histidine-tagged Rad14 Protein*

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The nucleotide excision repair (NER) pathway of eukaryotes involves ~30 polypeptides. Reconstitution of this pathway with purified components is consistent with the sequential assembly of NER proteins at the DNA lesion. However, recent studies have suggested that NER proteins may be pre-assembled in a high molecular weight complex in the absence of DNA damage. To examine this model further, we have constructed a histidine-tagged version of the yeast DNA damage recognition protein Rad14. Affinity purification of this protein from yeast nuclear extracts resulted in the co-purification of Rad1, Rad7, Rad10, Rad16, Rad23, RPA, RPB1, and TFIH proteins, whereas none of these proteins bound to the affinity resin in the absence of recombinant Rad14. Furthermore, many of the co-purifying proteins were present in approximately equimolar amounts. Co-elution of these proteins was also observed when the nuclear extract was fractionated by gel filtration, indicating that the NER proteins were associated in a complex with a molecular mass of >1000 kDa prior to affinity chromatography. The affinity purified NER complex catalyzed the incision of UV-irradiated DNA in an ATP-dependent reaction. We conclude that active high molecular weight complexes of NER proteins exist in undamaged yeast cells.

The repair of base damage by the eukaryotic nucleotide excision repair (NER) pathway involves ~30 polypeptides. In the yeast Saccharomyces cerevisiae genes that encode polypeptides required for the events of base damage recognition, damage-specific excision, and oligonucleotide excision during NER can be divided into two broad groups. Genes such as RAD1, RAD2, RAD4, RAD10, and RAD14 are indispensable for NER but not for cell viability, whereas genes such as RFA1, RFA2, RFA3, RAD3, SSSL1, SSSL2, TFB1, TFB2, TFB3, and TFB4 are essential for both NER and cell viability (2–4). In addition, mutations in the RAD7, RAD16, or RAD23 genes confer modulate sensitivity to killing by ultraviolet (UV) radiation (5, 6), suggesting that their polypeptide products may either increase or decrease the efficiency of NER or participate in a subpathway(s) of this process.

The characterization of cell-free extracts from mammalian (7) and yeast cells (8) capable of catalyzing repair synthesis of DNA associated with NER in vitro has led to important insights into the molecular mechanism of this process. By using this assay it was demonstrated that Rad3 protein functions in NER as a component of TFIH, a multiprotein complex that is required for transcription initiation by RNA polymerase II (9). The identification of Rad3 as a subunit of this transcription factor provided an explanation for the essential function of the RAD3 gene (10). Additionally, the requirement for core TFIH in NER predicted that genes encoding the other six subunits of this complex are also indispensable for this process. This has now been directly demonstrated for all seven proteins using a yeast cell-free system (2, 9–13).2 Interestingly, extracts prepared from rad7, rad16, or rad23 strains, which exhibit moderate sensitivity to cell killing by UV radiation (5, 6), are also defective in repair synthesis in vitro (14). Recent studies have shown that rad7 and rad16 mutant extracts are proficient in DNA damage-dependent incision both in vitro and in vivo but fail to excise the oligonucleotide fragment containing the DNA lesion (15). Hence, the Rad7 and Rad16 proteins, which interact with each other (16), appear to be required for post-incision events during NER.

Distinct biochemical functions have been assigned to a number of the NER gene products. Rad14 protein recognizes and binds to UV radiation-induced DNA lesions, in particular (6–4) photoproducts, suggesting a role in the recognition of base damage (17). It appears that the heterotrimeric protein complex Rpa, which is required for DNA replication, is also involved in DNA lesion recognition (18–20). Rad3 and Ssl2 proteins, both of which are components of core TFIH, are DNA helicases that appear to be involved in unwinding the DNA duplex in the vicinity of DNA lesions, thereby generating NER “bubbles” that include sites of base damage (12, 21, 22).

Finally, Rad2 and a heterodimeric complex of Rad1 and Rad10 proteins are DNA structure-specific endonucleases that are similar in that they cleave DNA at duplex/single-strand junctions but differ in terms of the polarity of the single strand that they cleave (23–25). These junctions are likely generated by TFIH-mediated “bubble” formation. Rad2 protein cleaves the 5′ single-strand at the junction and hence operates as a 3′ endonuclease with respect to sites of base damage. Reciprocally, Rad1 and Rad10 proteins are DNA structure-specific endonucleases that are similar in that they cleave DNA at duplex/single-strand junctions but differ in terms of the polarity of the single strand that they cleave (23–25). These junctions are likely generated by TFIH-mediated “bubble” formation. Rad2 protein cleaves the 5′ single-strand at the junction and hence operates as a 3′ endonuclease with respect to sites of base damage. Reciprocally, Rad1 and Rad10 proteins are DNA structure-specific endonucleases that are similar in that they cleave DNA at duplex/single-strand junctions but differ in terms of the polarity of the single strand that they cleave (23–25). These junctions are likely generated by TFIH-mediated “bubble” formation. Rad2 protein cleaves the 5′ single-strand at the junction and hence operates as a 3′ endonuclease with respect to sites of base damage. Reciprocally, Rad1 and Rad10 proteins are DNA structure-specific endonucleases that are similar in that they cleave DNA at duplex/single-strand junctions but differ in terms of the polarity of the single strand that they cleave (23–25). These junctions are likely generated by TFIH-mediated “bubble” formation. Rad2 protein cleaves the 5′ single-strand at the junction and hence operates as a 3′ endonuclease with respect to sites of base damage. Reciprocally, Rad1 and Rad10 proteins are DNA structure-specific endonucleases that are similar in that they cleave DNA at duplex/single-strand junctions but differ in terms of the polarity of the single strand that they cleave (23–25). These junctions are likely generated by TFIH-mediated “bubble” formation. Rad2 protein cleaves the 5′ single-strand at the junction and hence operates as a 3′ endonuclease with respect to sites of base damage.

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The abbreviations used are: NER, nucleotide excision repair; DTT, dithiothreitol; FPLC, fast protein liquid chromatography; his, histidine; TFIH, transcription factor IH; NTA, nitrilotriacetic acid; PCNA, proliferating cell nuclear antigen.

2 W. J. Feaver, W. Huang, and E. C. Friedberg, unpublished results.
cally, Rad1/Rad10 protein cleaves the 3’ single-strand at the junction and hence operates as a 5’ endonuclease with respect to sites of base damage (23–25). The combined action of these two endonucleases is responsible for the dual incision events that are characteristic of NER in all eukaryotes studied and that result in the removal of DNA lesion-containing oligonucleotides 27–30 nucleotides in length (20, 26). In addition to the proteins described above, a complex of Rad4 and Rad23 proteins is required for early events during NER (20); however, the precise roles of these proteins is not yet understood.

Recently, a relatively stable high molecular weight complex comprising core TFIIH and multiple NER subunits was identified and extensively purified from cell extracts prepared from yeast cells not exposed to exogenous DNA-damaging agents (11). These observations led to the suggestion that, in the yeast S. cerevisiae and possibly other eukaryotes, a NER complex is assembled in the absence of substrate base damage. In this initial study, the NER complex was called a repairosome although it was not shown to perform any of the reactions involved in NER. Here we define a repairosome as a stable complex of proteins that carry out the DNA damage recognition and incision reactions that are characteristic of NER. Recent studies have also provided evidence suggesting the existence of a high molecular weight complex of NER proteins in extracts from undamaged human cells that catalyze DNA damage-dependent DNA synthesis (27). Although it is plausible that such preformed multiprotein complexes may function to constantly monitor the genome for sites of base damage and catalyze NER in the presence of such damage, there is as yet no direct biochemical evidence for such functions by the repairosome. It has also not been established that yeast cells contain only a single multiprotein complex that comprises both TFIIH and NER polypeptides. Indeed, the primary question as to whether or not any NER complex is pre-assembled in yeast (and other eukaryotes) or is assembled in a step-wise process exclusively when cells are required to carry out NER is controversial (11, 28) (see “Discussion.”)

In initial studies that led to the elaboration of the repairosome hypothesis, extracts containing histidine (his)-tagged Tbf1 protein (one of the subunits of TFIIH) were fractionated by assaying for complementation of defective RNAP II transcription in vitro (11). To further test the hypothesis that a high molecular weight complex of proteins comprising both TFIIH and NER subunits exists in undamaged yeast cells, we have constructed a his-tagged derivative of the DNA damage recognition protein Rad4 (which is not required for RNAP II transcription) and expressed this recombinant protein in a yeast strain deleted of the chromosomal RAD4 gene. Here we present evidence that single step affinity chromatography of recombinant Rad4 protein from nuclear extracts results in the copurification of multiple other NER proteins, including TFIIH subunits, in a complex with a molecular mass >10,000 kDa. Additionally, we show that fractions containing this complex incise UV-irradiated DNA in an ATP-dependent reaction.

**EXPERIMENTAL PROCEDURES**

**Materials**—Purified mouse IgG was purchased from Pierce. The Rad1 IgG mononal antibody, 3E3 (29), and the rabbit polyclonal antibodies specific for Rad3 (30), Rad10 (31), Rad7, Rad16, and Rad23 (15), have been described previously. Antibodies for Tbf1 (32) and RPB1 were provided by Roger Kornberg, Stanford University. Antibodies for the 34-kDa subunit of Rpa (4), PCNA, and Rad4 (41) were supplied by Steve Brill, State University of New Jersey, Peter Burgers, Washington University, and Tom Koddek, University of Texas Southwestern Medical Center, respectively.

Rad1 and Rad10 proteins were purified as described previously (33). RPA was purified from the yeast strain RKT2275 that overexpresses each of the Rpa subunits, as described (34). Purified TFIIH containing his-tagged Tbf1 (35) and RNA polymerase II holoenzyme (36) were provided by Roger Kornberg. Recombinant XPG protein purified from baculovirus-infected insect cells was obtained from Rick Wood, ICRF, United Kingdom (37). Protein concentrations were measured by the Bradford assay (38) using bovine serum albumin as the standard.

**Plasmid Constructs**—The HindIII fragment from the plasmid pGEM4Z-SpRAD14, which contains the RAD14 open reading frame (39), was subcloned into pQE-30 (Qiagen) to produce the plasmid pQE-hisRad14, in which a truncated version of Rad14 deleted of the amino-terminal 14 amino acids was expressed as a fusion protein with a poly(hist) sequence at the amino terminus. An EcoRI fragment encoding his-tagged Rad14 was subcloned in the yeast plasmid pYES2 (Invitrogen) to produce plasmid pYES2-hisRad14, in which recombinant Rad14 was expressed from a GAL1-inducible promoter.

**Preparation of Recombinant Rad14 Protein**—The Escherichia coli strain M15 pREP4 (Qiagen) harboring pQE-hisRad14 was grown at either 30 or 37 °C for preparation of soluble and insoluble recombinant Rad14, respectively. Expression of recombinant Rad14 in exponentially growing cultures was induced by the addition of isopropyl thiogalactoside to a final concentration of 2 mM. After further incubation for 3 h, cell pellets were harvested by centrifugation, resuspended in 50 mM sodium phosphate (pH 7.8), 300 mM NaCl containing a mixture of protease inhibitors (33) and lysed by sonication. Recombinant Rad14 was purified from the clarified lysate by metal chelating affinity chromatography using Ni-NTA beads (Qiagen) and then by ion exchange chromatography using FPLC Mono Q and Resource S columns (Amersham Pharmacia Biotech). Approximately 0.4 mg of Rad14 that was >95% homogenous was obtained from 1 liter of culture.

The majority of recombinant Rad14, which was present in the insoluble fraction, was purified by immobilized metal affinity chromatography according to the manufacturer’s protocol (Qiagen). Recombinant Rad14 was eluted from the Ni-NTA beads with 8 M urea, 100 mM Na2HPO4, 10 mM Tris-HCl (pH 4.5) and was purified to near homogeneity by preparative native polyacrylamide gel (40). The position of Rad14 in the gel was identified by reverse staining with copper chloride using the Pro-Green Staining System according to the manufacturer’s protocol (Integrated Separation Systems). Rad 14 was electroeluted from these gel slices using an Electro-Eluter 422 (Bio-Rad) as suggested by the manufacturer. Approximately 1.5 mg of Rad14 was obtained from a 1-liter culture.

**In Vitro NER**—Yeast whole cell extract (40) containing overexpressed Rad2 protein (8), nuclear extract (200 μg) from SX46A ADRAD14::HIS3 cells, and recombinant Rad14 were incubated for 2 h at 26 °C with 300 ng each of N-acetoxy-2-acetylaminofluorene-treated pUC18 DNA and undamaged pGEM3zf(+) DNA, 45 mM Hepes-KOH (pH 7.8), 7.4 mM MgCl2, 0.9 mM DTT, 0.4 mM EDTA, 2 mM ATP, 20 μM each of dATP, dCTP, dGTP, and dTTP, 1 μCi of [α-32P]dATP (3,000 Ci/mmol), 40 mM phosphocreatine, 2.5 μg of bovine serum albumin, and 5% polyethylene glycol 8000 in a final volume of 50 μl. Plasmid DNA was purified and processed as described previously (8).

**Rad14 Antiserum**—Mice were initially immunized with recombinant Rad14 purified from the insoluble fraction as described above, plus Freund’s complete adjuvant. Subsequently the animals were immunized twice at 2-week intervals with the same antigen plus Freund’s incomplete adjuvant and bled 10 days after each injection.

**Preparation of Nuclei**—Stationary phase cultures of yeast strains grown in uracil dropout media were diluted 1:20 in 8 liters of 2% Bacto-peptone, 1% yeast extract containing 1% galactose, 1% raffinose as the carbon source. When the cultures reached late logarithmic phase cells were harvested, washed once with 0.1 x EDTA (pH 8.0), 1 ml proteinase K (1 mg/ml), and resuspended in the same buffer containing 10 μg/ml ethidium bromide (all 5% wet weight of cells). Cells were resuspended in 1% yeast extract, 2% Bacto-peptone, 1% sorbitol (1 mg/ml wet weight of cells) containing yeast extract yeast enzyme (ICN, 1.4 mg/g wet weight of cells) and incubated with shaking at 30 °C. When >80% of the cells had been converted to spheroplasts digestion was stopped, and spheroplasts were collected as described (8). Spheroplasts were resuspended in 50 mM Tris-HCl (pH 8.0), 20 mM KCl, 2 mM EDTA, 0.125
m spermidine, 0.05 mM spermine, 1% thioglycolic acid, 18% Ficoll plus a mixture of protease inhibitors (33) (5 mg/ml wet weight of cells), and after Dounce homogenization the lysate was cleared by centrifugation at 7000 rpm for 10 min in a Sorval SS34 rotor. The supernatant was centrifuged twice more at 7000 rpm for 5 min before a final spin at 15,000 rpm in a SS34 rotor which pelleted the nuclei. After removal of the supernatant the pellet of nuclei was flash-frozen in liquid nitrogen and stored at -80°C.

**Affinity Purification of Rad14 and Associated Proteins**—Nuclei were resuspended in 20 mM Tris acetate (pH 8.0), 300 mM potassium acetate, 20% glycerol, 5 mM p-mercaptoethanol and a mixture of protease inhibitors (34), lysed by Dounce homogenization, and centrifuged at 15,000 rpm for 30 min. In some cases, MgCl2 and DNase I were added to the cleared lysate to final concentrations of 10 mM and 20 μg/ml, respectively, prior to incubation at 25°C for 30 min. The cleared lysate (25 mg) was incubated with rotation at 4°C with 2 ml of a 50% slurry of Ni2+-NTA agarose beads (Qiagen) for 1 h. This mixture was poured into a column, and the beads were washed extensively with buffer A containing 5 mM imidazole. Bound proteins were eluted in a stepwise manner with 20, 50, 100, and 200 mM imidazole in buffer A. Protein-containing fractions from each eluate were detected using the Bradford assay (38). In some cases, the protein-containing fractions were pooled and concentrated by ultrafiltration using Centricron-10 (Amicon). The 20 mM eluates from the extracts with and without recombinant Rad14 contained 4 and 0.4 mg, respectively. The 50 mM eluates were added to the extracts with and without recombinant Rad14 contained 0.4 and 0.1 mg, respectively. Fractions were flash-frozen in liquid nitrogen and stored at -80°C.

**Immunoblotting**—Polypeptides were separated by denaturing gel electrophoresis and transferred electrophoretically to a nitrocellulose membrane. Following incubation with TBST (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20) containing 5% dried milk for 30 min at room temperature, the membrane was incubated overnight at 4°C with the primary antibody in TBST containing 2% dried milk overnight. Antigen-antibody complexes were detected by enhanced chemiluminescence (Pierce) after incubation for 60 min at room temperature with the appropriate secondary antibody linked to horseradish peroxidase. After a 15-min incubation, the membrane was washed three times in TBST containing 2% dried milk and incubated for 20 min in the dark. The membrane was then washed again (three times) in TBST containing 2% dried milk and immersed in an enhanced chemiluminescence detection solution for 5 min before being washed three times (30 min each) in TBST containing 2% dried milk. The membrane was then placed between two sheets of Whatman 3MM filter paper and exposed to X-ray film for up to 7 days.

**RESULTS**

**His-tagged Rad14 Protein Complements Both the UV Radiation Sensitivity and Defective NER of a rad14 Deletion Mutant**—We constructed a version of the DNA damage recognition protein Rad14 (39) in which the amino-terminal 14 amino acids were replaced by a polythiol (30) sequence. A plasmid that expresses this version of Rad14 protein from a GAL1 promoter restored the UV radiation sensitivity of a RAD14 deletion mutant to wild type levels under both inducing conditions (galactose medium, Fig. 1A) and non-inducing conditions (glucose medium, Fig. 1B). To monitor the expression of Rad14 protein, we generated polyclonal antibodies to recombinant Rad14 purified from *E. coli*. In glucose medium, the cellular level of recombinant Rad14 was similar to that of native Rad14 in the wild type strain, whereas in galactose medium the cellular level of recombinant Rad14 was about 100-fold higher (Fig. 2). As expected, no immunoreactivity was detected with similarly prepared nuclear extracts from the ΔRAD14 strain transfected with the plasmid vector (Fig. 2). Taken together these results demonstrate that the his-tagged version of Rad14 functions in NER in vivo.

Although the majority of recombinant his-tagged Rad14 protein expressed in *E. coli* is insoluble, we purified recombinant Rad14 from the soluble fraction to >95% homogeneity by affinity and ion exchange chromatography. Addition of purified recombinant Rad14 protein to cell-free extracts prepared from a ΔRAD14 mutant restored NER activity as measured by DNA damage-dependent DNA synthesis (data not shown). Thus, his-tagged recombinant Rad14 is active both in vivo and in vitro.

**Affinity Purification of Rad14 and Associated Proteins from Yeast Nuclear Extracts**—When a nuclear extract from the ΔRAD14 pYES2-hisRad14 strain was fractionated by immobilized metal affinity column chromatography, recombinant Rad14 was not detected in the flow-through fraction (Fig. 3A). However, substantial amounts of the protein were eluted in the 20 and 50 mM imidazole eluates (Fig. 3A). Smaller quantities were also detected in 100 and 200 mM imidazole eluates (data not shown). No immunoreactivity was detected when the nuclear extract from the ΔRAD14 pYES2 strain was fractionated by the same procedure (Fig. 3A).

The reaperosome complex described previously was isolated by affinity chromatography using his-tagged Tbh1 protein (11). We therefore asked whether Tbh1 co-purified with recombinant his-tagged Rad14. Nuclear extracts prepared from the ΔRAD14 pYES2 and the ΔRAD14 pYES2-hisRad14 strains contain equivalent amounts of Tbh1 protein (Fig. 3B). In the absence of recombinant Rad14 protein Tbh1 was detected in the column flow-through fraction but not in the 20 or 50 mM imidazole fractions. In contrast, Tbh1 protein was quantitatively bound by the affinity beads in the presence of recombinant Rad14 and
Purified recombinant Rad14 (0.4 g/ml) extract from cells grown in glucose and galactose medium, respectively. The products of the RAD1 and RAD10 genes are known to form a highly stable heterodimeric complex that is responsible for incisions 5′ to sites of base damage during NER (24, 25). Both Rad1 and Rad10 proteins co-eluted with recombinant his-tagged Rad14 during affinity chromatography (Fig. 3D).

The Rad7 and Rad16 proteins also form a stable heterodimeric complex (16). Recent studies have demonstrated that these proteins are required for post-incision events during NER (15). Both proteins co-fractionate with recombinant Rad14 (Fig. 3D), suggesting that they are also components of the RPA complex. Rad23 has been purified as a component of a stable Rad23-Rad4 complex which is required for the reconstitution of NER with purified yeast proteins (20). Hence, the co-fractionation of Rad23 and Rad4 with recombinant Rad14 (Fig. 3D) suggests that they are also components of the repairosome. Rad23 has been purified as a component of a stable Rad23-Rad4 complex which is required for the reconstitution of NER with purified yeast proteins (20). Hence, the co-fractionation of Rad23 and Rad4 with recombinant Rad14 (Fig. 3D) suggests that they are also components of the repairosome.

Co-elution of the proteins discussed above was not observed using extracts lacking Rad14 protein. Thus we consider it unlikely that the co-fractionation of multiple NER proteins is purely fortuitous. It is conceivable, however, that the association of multiple NER proteins is mediated by their independent binding to DNA in nuclear extracts. To address this issue, we performed the same fractionation procedure with nuclear extracts prepared from cells grown in media with glucose as the carbon source. Under these growth conditions, the cellular level of recombinant Rad14 is similar to that in the wild-type.
FIG. 3. Affinity purification of recombinant Rad14 and associated proteins from yeast nuclear extracts. The yeast strains SX46A ΔRAD14::HIS3 pYES2 and SX46A ΔRAD14::HIS3 pYES2-hisRad14 were grown and nuclei prepared as described under “Experimental Procedures.” The nuclei were lysed by Dounce homogenization, and the cleared lysates were fractionated by affinity chromatography as described under “Experimental Procedures.” Equal amounts of protein from the two extracts in the cleared lysate (20 μg, Load), pass-through fractions (15 μg, Pass), 20 mM imidazole eluates (23 μg, 20 mM), and 50 mM imidazole eluates (7 μg, 50 mM) were separated by denaturing gel electrophoresis and transferred to nitrocellulose membranes. Antigen-antibody complexes were detected by enhanced chemiluminescence. Extracts were prepared from SX46A ΔRAD14::HIS3 pYES2 (−) and SX46A ΔRAD14::HIS3 pYES2-hisRad14, (+) cells. A, immunoblot with Rad14 antiserum. B, immunoblot with Tfb1 antibody. C, immunoblot with RPB1 antibody. D, immunoblots with antibodies specific for the indicated NER proteins. E, immunoblot with PCNA antibody.
that of native Rad14 in wild type cells (Fig. 2). In these experiments Rad23, Rad7, Tfb1, RPB1, and Rad10 were retained by the affinity beads in a Rad14-dependent manner (data not shown), indicating that the association of these proteins is not a consequence of the abnormally high levels of recombinant Rad14.

It has been demonstrated that PCNA is required for repair synthesis, a late step in the NER process in eukaryotic cells (43). Remarkably, we did not observe co-fractionation of PCNA protein, either in the presence or absence of recombinant Rad14 (Fig. 3E). This result suggests that the yeast repairosome we have identified does not include components required for repair synthesis of DNA, an event that is in fact not specific for NER. Gratifyingly, this result also indicates that the binding of Rad14 to the affinity beads does not facilitate nonspecific binding of other proteins in nuclear extracts. To support this notion further, we examined the binding of Rad51, a relatively abundant nuclear protein that is not involved in NER, to the affinity beads. As expected, we did not detect binding of this protein either in the presence or absence of recombinant Rad14 protein (data not shown).

**Stoichiometry of the Putative Repairosome Components and Molecular Mass of the Repairosome Complex**—Since NER proteins were consistently detected in both the 20 and 50 mM imidazole eluates, we omitted the 20 mM imidazole elution step during subsequent experiments. When the affinity column was eluted with a single step of 50 mM imidazole, the same NER proteins co-purified (data not shown). In quantitative immunoblotting experiments (Fig. 4), we estimate that 5 μg of the 50 mM eluate contains 0.2 pmol of Tfb1, 0.1 pmol of RPB1, 0.1 pmol of Rpa2, 0.1 pmol of Rad10, and 0.1 pmol of Rad23. Thus, several of the putative repairosome subunits are present in approximately equimolar quantities.

It is prudent to consider the possibility that the association of proteins with recombinant Rad14 is a biochemically specific but biologically irrelevant consequence of the binding of Rad14 to the affinity beads. To determine whether NER proteins are associated in the absence of affinity chromatography, we applied nuclear extracts directly to an FPLC Superose 6 gel filtration column. In Fig. 5 we show the elution profiles of Rad14 and several NER proteins that co-purified with recombinant Rad14 on the affinity column. In these experiments extracts were treated with DNase I prior to filtration. Similar results were obtained with extracts that were not treated with DNase I (data not shown). Recombinant Rad14 and other NER proteins co-eluted in fractions corresponding to a molecular mass of 1000–1500 kDa, indicating that they are associated in a high molecular weight complex in the nuclear extract prior to the binding of recombinant Rad14 to the affinity beads.

**DNA Structure-specific Endonuclease Activity Associated with the Repairosome**—The results presented thus far support the notion that NER proteins exist in a high molecular weight complex in yeast cells not exposed to exogenous DNA damaging agents. For this complex to be designated as a functional repairosome, it should support some, if not all, of the reactions that transpire during the DNA damage recognition and incision steps of NER in vivo. We examined the ability of affinity purified Rad14 and associated proteins to specifically cleave Y-shaped DNA substrates that contain duplex/single-strand junctions known to be cleaved by the junction-specific Rad1/ Rad10 and Rad2 endonucleases (23–25). Fractions containing
Rad14 and associated NER proteins cleaved a Y-shaped substrate radiolabeled at the duplex end to produce a partial linear duplex product (Fig. 6A). In contrast, equal amounts of protein from a comparable fraction purified from a ΔRAD14 pYES2 nuclear extract did not contain junction-specific cleavage activity, although low levels of nonspecific nuclease activity were observed (Fig. 6A). Preincubation of the reparisome fraction with 5 μg of Rad1 monoclonal antibody 3E3, which inhibits the Rad1/Rad10 endonuclease (29), reduced cleavage of the Y structure by ~80%, whereas purified mouse IgG had no inhibitory effect (data not shown). These results indicate that the Rad1 and Rad10 proteins that co-purify with recombinant Rad14 are catalytically active.

Cleavage of the radiolabeled Y-shaped substrate described above by either Rad1/Rad10 or Rad2 endonuclease generates products that are indistinguishable by gel electrophoresis. However, if the non-complementary 5′ single-strand of the Y structure is end-labeled to produce the substrate shown in Fig. 6B, cleavage by the Rad2 endonuclease will produce a unique end-labeled single-strand oligonucleotide ~20 residues in length (23). In assays with such a substrate, fractions containing Rad14 and associated NER proteins generated both the partial linear duplex expected from cleavage by the Rad1/Rad10 endonuclease and a novel product with increased electrophoretic mobility (Fig. 6B). This product was not observed in assays with a comparable fraction purified from a ΔRAD14 pYES2 nuclear extract. However, it was detected in reactions with XPG protein, the human homolog of Rad2 protein (23, 25, 37, 44) (Fig. 6B). Thus, we are led to the conclusion that the affinity purified fractions containing recombinant Rad14 possess Rad2 activity. Antibodies to Rad2 protein were not available to demonstrate specific inhibition of this activity. However, preincubation with 5 μg of the Rad1 monoclonal antibody 3E3, resulted in significant inhibition (~80%) of the putative Rad2 activity (data not shown). Since the Rad1 antibody is known to inhibit the NER reaction catalyzed by yeast extracts (29), it is possible that binding of the antibody to the Rad1 component of the reparisome precludes the activity of the Rad2 endonuclease.

**Nicking of UV-irradiated Supercoiled DNA by the Reparisome**—In reconstitution experiments with purified NER proteins, specific nicking of UV-irradiated DNA is ATP-dependent (20). We asked whether the fraction from the affinity column that contains recombinant Rad14 and other NER proteins could perform the same reaction. ATP-dependent nicking of UV-irradiated plasmid DNA was indeed observed in assays with this fraction, whereas no nicking of undamaged or damaged plasmid DNA occurred in the absence of ATP (Fig. 7A, lane 6). Similarly, no nicking was observed with an equivalent amount of protein from a comparable fraction purified from the nuclear extract lacking recombinant Rad14. Fractions containing the reparisome supported some nicking of undamaged plasmid DNA in the presence of ATP (Fig. 7A, lane 5) but to a significantly lower extent than that observed with UV-irradiated DNA.

Since the Rad1 monoclonal antibody 3E3 inhibits both the cleavage of Y structures by the Rad1/Rad10 endonuclease and in vitro NER by yeast cell-free extracts (29), we examined the effect of this antibody on the ATP-dependent nicking of UV-irradiated plasmid DNA. Preincubation of an affinity-purified fraction containing Rad14 and associated NER proteins with antibody 3E3 resulted in >50% inhibition of endonuclease activity (Fig. 7B, lane 5). In contrast, preincubation of the same fraction with mouse IgG had no such effect (Fig. 7B, lane 6).

In the experiment shown in Fig. 8, we employed reaction conditions essentially identical to those described for the reconstituted NER reaction that includes the Rad7-Rad16 complex (16). UV-irradiated plasmid DNA containing approximately one UV photoproduct/molecule was rapidly cleaved such that ~50% of the substrate was converted to the nicked circular form in 2 min in the presence of 100 fmol of reparisome (assuming a molecular weight of 1.6 × 10^6 from all the identified subunits) (Fig. 8, A and B). The rate of endonucleolytic cleavage was estimated to be 0.2 nicks/min/reparisome. After 30 min essentially all the supercoiled plasmid DNA was nicked (Fig. 8, A and B). Undamaged plasmid molecules were nicked at a similar initial rate (Fig. 8B). However, the reaction reached a plateau when ~30% of the substrate molecules were nicked (Fig. 8B). This reaction profile could be explained if a proportion of the putatively undamaged plasmid molecules contain some sort of DNA lesion that is recognized by the reparisome. In the NER reaction reconstituted from purified subcomplexes (16), the rate of cleavage of the UV-irradiated substrate was about 0.006 nicks/min/reparisome subcomplex. After subtracting the nicking of undamaged DNA, it appears that the affinity-purified reparisome is at least 10-fold more efficient at cleaving the UV-irradiated DNA substrate than the combination of purified NER subcomplexes (16). This may re-
Reactions (15 m) supercoiled plasmid DNA and aliquots (1 μg) of affinity purified fractions from the SX46A reaction. More of the subcomplexes may not have been purified in a dependent incision. Alternatively, it is possible that one or affinity-purified fraction that enhance the rate of DNA damage-complex and/or the presence of additional factors in the affinity purification regardless of which NER protein is tagged with poly-histidine residues, with the obvious caveat that the affinity purification method (nucleotide excision repairosome) exists in yeast cells, the as-suggested that a large multiprotein complex partially purified by affinity chromatography. We also consider it unlikely that the binding of overexpressed recombinant Rad14 to the affinity column specifically promotes the association of NER (and possibly other) proteins in the nuclear extract for the following reasons: (i) when nuclear extracts were applied directly to a gel filtration column many of the NER proteins, which co-purified with recombinant Rad14 by affinity chromatography, co-eluted with Rad14 in fractions that correspond to a molecular mass of >1000 kDa; (ii) although the NER proteins that co-purify with recombinant Rad14 were present in approximately equimolar amounts, the relatively abundant nuclear proteins PCNA and Rad51, which are not involved in the DNA damage recognition and incision steps of NER (events that are specific to NER), were not retained by the affinity column in either the presence or absence of recombinant Rad14; (iii) a similar spectrum of NER proteins was retained by the affinity column in a Rad14-dependent manner when recombinant Rad14 was expressed at the same level as the native protein.

A high molecular weight complex of NER proteins was initially detected after affinity purification of a his-tagged version of Tfb1 expressed at wild type levels (11). The putative repairosome containing his-tagged Tfb1 complemented the repair defect in extracts from several yeast strains mutated in NER genes (11). However, this complex was not investigated for its ability to directly support reactions involved in NER. Here we have demonstrated that the multiprotein complex partially purified by affinity chromatography of his-tagged Rad14 protein incises UV-irradiated DNA in an ATP-dependent reaction. Hence, we conclude that the high molecular complex of NER proteins constitutes a functional repairosome.

Unlike the complex of human proteins purified by XPA affinity chromatography (27), we failed to detect co-purification of PCNA with NER proteins. Thus, it is unlikely that the affinity purified complex of yeast NER proteins can support DNA damage-dependent DNA synthesis. In fact the yeast NER complex is similar to another high molecular weight transcription-repair complex isolated from human cells in that it contains the catalytic subunit of RNA polymerase II (45).

Although our results provide support for the notion that repairosomes exist in undamaged yeast cells, it is not known what proportion of NER proteins are present in such complexes. Nor can we eliminate the possibility that affinity chromatography identifies several independent complexes containing NER proteins, some of which contain additional polypeptides dedicated to other cellular responses to DNA damage. However, the relative ease of genetic manipulation in
yeast make this an attractive organism to identify proteins that play important roles in the assembly and/or stability of multiprotein complexes. Finally, these results do not exclude the possibility that some, if not most, DNA lesions are in fact removed by the sequential assembly of NER subcomplexes at or near the site of DNA damage in vivo.

It seems reasonable to speculate that a preformed repairosome is dedicated to continual surveillance of the genome in eukaryotic cells for sites of base damage normally processed by NER. We suggest that when (and only when) such sites are encountered, the multiprotein complex undergoes specific conformational changes that strengthen the protein-DNA interactions. Additionally, these and/or further conformational changes may both facilitate localized unwinding of the DNA that flanks sites of base damage by the Rad3 and Ssl2 DNA helicases and position the Rad2 and Rad1/Rad10 endonucleases at the duplex/single-strand junctions generated by this unwinding, specifically on the damaged DNA strand.

Several observations suggest that the amount and/or composition of the nucleotide excision repairosome may change when yeast cells are exposed to UV radiation or other DNA-damaging agents that generate substrates for NER. For example, genes that encode several subunits of the repairosome are up-regulated under these conditions. Notable examples include RAD2 (46), RAD7 (47), RAD16 (48), and RAD23 (49). Additionally, recent studies in one of our laboratories (E. C. Friedberg) have demonstrated significant inhibition of RNAP II transcription in vitro in the presence of active NER (50). This inhibition can be relieved by supplementing in vitro reactions with purified holo-TFIH, a form of TFIH specifically required for transcription initiation. Hence, when cells are required to carry out NER, subunits of the repairosome such as TFIH and Rpa which are indispensable for transcription and replication, respectively, may be preferentially sequestered by the repairosome. This would not only enhance the cellular repair capacity but would also inhibit transcription and replication until the genome is repaired.

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