Affinity of Yeast Nucleotide Excision Repair Factor 2, Consisting of the Rad4 and Rad23 Proteins, for Ultraviolet Damaged DNA*  

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Saccharomyces cerevisiae Rad4 and Rad23 proteins are required for the nucleotide excision repair of UV light-damaged DNA. Previous studies have indicated that these two DNA repair proteins are associated in a tight complex, which we refer to as nucleotide excision repair factor 2 (NEF2). In a reconstituted nucleotide excision repair reaction, incision of UV-damaged DNA is dependent on NEF2, indicating a role of NEF2 in an early step of the repair process. NEF2 does not, however, possess an enzymatic activity, and its function in the damage-specific incision reaction has not yet been defined. Here we use a DNA mobility shift assay to demonstrate that NEF2 binds specifically to UV-damaged DNA. Elimination of cyclobutane pyrimidine dimers from the UV-damaged DNA by enzymatic photoreactivation has little effect on the affinity of NEF2 for the DNA, suggesting that NEF2 recognizes the 6-(1,2)-dihydro-2-oxo-4-pyrimidinedione-5-methyl-2,4-(1H,3H)-pyrimidinedione photoproducts in the damaged DNA. These results highlight the intricacy of the DNA damage-demarcation reaction during nucleotide excision repair in eukaryotes.

Nucleotide excision repair (NER) in yeast and other eukaryotic organisms is a highly intricate process that requires a large number of protein factors. The NER reaction entails damage recognition in the target DNA, sequential assembly of a number of repair protein complexes at the damage site, and dual incision of the damage-containing DNA strand, resulting in the excision of the damage in the form of a short DNA fragment ~27–30 nucleotides in length (1–6).

Important insights concerning the action mechanism of NER in eukaryotes have been garnered from genetic and biochemical studies in yeast, and the incision phase of NER has been reconstituted using highly purified repair factors. Using UV-damaged DNA as a substrate, we showed previously that the combination of the Rad14 protein, the Rad4-Rad23 complex, the six-subunit RNA polymerase II transcription factor TFIH, the heterotrimeric replication protein A (RPA), the Rad10 complex, and the Rad2 nucleosome mediates dual incision of the damaged DNA (1). Subsequent studies showed that Rad14 in fact exists as a complex with the Rad1-Rad10 endonuclease in the cell, and the complex consisting of Rad1-Rad10-Rad14 has been named nucleotide excision repair factor 1 (NEF1) (2). Likewise, Rad2 associates stably with the six-subunit TFIH to form NEF3 (3), whereas the complex of the Rad4 and Rad23 proteins is referred to as NEF2 (1). Recently, we showed that the Rad7 and Rad16 proteins, which genetic studies have implicated in the nucleotide excision repair reaction, also exist in a stoichiometric complex termed NEF4. Consistent with the genetic data, the addition of NEF4 to the reconstituted repair reaction stimulates the incision efficiency markedly (7). These studies have allowed us to deduce that the yeast nucleotide excision repair machinery comprises NEF1, NEF2, NEF3, NEF4, and RPA (7, 8).

Substantial information concerning the biochemical functions of NEF1, NEF3, and NEF4 has been gleaned from biochemical studies with purified components that constitute these NEFs, with the notable exception of NEF2. For understanding the function of NEF2 in the UV damage-specific incision reaction, we have overproduced NEF2 in yeast and purified it to near homogeneity. Here we report our biochemical studies, which directly link NEF2 to the damage recognition reaction.

MATERIALS AND METHODS

**Oxexpression and Purification of Rad4-Rad23 Complex (NEF2)—**

The Rad4 protein is highly toxic to *Escherichia coli*, and for this reason, a stuffer DNA fragment has to be inserted into the open reading frame of *Rad4* for stable maintenance of *Rad4*-containing plasmids in *E. coli* (9, 10). The Rad4 protein coding frame from the ATG initiation codon to 434 bp downstream of the TAG stop codon, and containing a 2.2-kb *Bgl*II DNA stuffer fragment, was placed under the control of the constitutively expressed *ADC1* promoter to yield plasmid pR4.1 (*p*Adh-Rad4). After removal of the stuffer fragment by *Bgl*II digestion, the plasmid was recircularized by ligation and then transformed into the protease-deficient yeast strain YRP11. YRP11 harboring pR4.1 was grown in synthetic medium lacking tryptophan to a density of 5 × 10⁷ cells/ml and then harvested by centrifugation. Yeast paste (500 g) was resuspended in cell breakage buffer and lysed using a French press. The crude lysate was clarified and treated with ammonium sulfate at 0.21 g/ml to precipitate NEF2. The ammonium sulfate pellet was dissolved in 300 ml of K buffer (20 mM KH₂PO₄, pH 7.4, 10% glycerol, 0.5 mM EDTA, 1 mM dithiothreitol) to give conductivity equivalent to 100 mM KCl and then loaded directly on a Q-Sepharose column (1.5 × 14 cm, 25 ml) equilibrated in K buffer with 100 mM KCl. The column was developed with a 250-ml gradient of 100–500 mM KCl in K buffer, and 50 fractions were collected. Fractions containing the peak of NEF2, eluting at 300 mM KCl, were identified by immunoblotting and pooled (Fraction III, 45 ml). The Q-Sepharose pool was dialyzed against 1× buffer before fractionation on an SP-Sepharose column (1 × 5 cm, 4 ml) equilibrated in K buffer with 100 mM KCl and 0.01% Nonidet P-40. The column was developed with a 50-ml gradient from 100 to 450 mM KCl in K buffer, collecting 50 fractions. The pool of NEF2 (Fraction IV, 8 ml), eluting at ~250 mM KCl,
was applied onto a 1-ml hydroxyapatite column, which was developed with a 30-ml gradient of 20–300 mM KH$_2$PO$_4$ in K buffer and 0.1% Nonidet P-40, collecting 30 fractions. The NEF2 peak (fraction V, 5 ml), eluting at ~150 mM KH$_2$PO$_4$, was concentrated to 0.6 ml and then fractionated in a 30-ml Sephacryl S300 column in K buffer with 100 mM KCl and 0.01% Nonidet P-40. The S300 NEF2 pool (fraction VI) was fractionated in a Mono Q column with a 30-ml 100–500 mM KCl gradient in buffer K and 0.01% Nonidet P-40, collecting 30 fractions. NEF2 elutes from Mono Q at ~300 mM KCl, the pool of which (fraction VII, 2 ml) was diluted with 4 ml of 10% glycerol that also contained 0.01% Nonidet P-40 and then further fractionated in a 2500-μl Mini S column, with a 5-ml gradient of 100–500 mM KCl, collecting 25 fractions. NEF2 elutes from Mini S at ~250 mM KCl in fractions 10–14, which were individually concentrated to 30 μl in Minicon-30 concentrators. NEF2 from the Mini S column (fraction VIII) was nearly homogeneous (Fig. 1B) and was used in all of the DNA binding experiments described herein.

**Production of Antibodies**—The entire open reading frame of RAD4 from the initiating ATG to 434 bp downstream of the stop codon and containing a 2.2-kb BglII DNA stuffer fragment was isolated at the BamHI site of the baculovirus vector pVL1393 (Invitrogen) to yield plasmid pR4.4, thus placing RAD4 under the control of the viral polyhedrin promoter. *Spodoptera frugiperda* (Sf9) cells were co-transfected with linear wild-type AcMNPV DNA (Invitrogen) and pR4.4 (minus the stuffer fragment) and recombinants were detected by visual screening for the exclusion-negative (polyhedrin-negative) phenotype and then examined for expression of Rad4 protein by comparing the lysate of insect cells infected with the RAD4 recombinant baculovirus with lysate from cells infected with the wild-type virus by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining. Rad4 protein was easily identified in extract of insect cells harvested from 36 to 72 h after infection with the Rad4 protein expressing recombinant baculovirus, but essentially all of the Rad4 protein is insoluble. The Rad4 protein in the insoluble fraction was solubilized by boiling in SDS buffer, purified by preparative SDS-polyacrylamide gel electrophoresis, and then injected into rabbits for generating polyclonal antiserum. Rad4 antigen (2 μg) was coupled to CNBr-Sepharose to generate an affinity matrix to predict antibody-purified antibodies were obtained as described previously (11).

**DNA Mobility Shift Assay**—A 130-bp HindIII-SalI fragment harboring a stretch of thymines in the middle was isolated from plasmid pTB402, labeled with $^{32}$P, and irradiated with UV light (254 nm) as described previously (7, 12). Purified NEF2 was incubated with the $^{32}$P-labeled, UV-irradiated DNA (2 ng) and 100 ng of unlabeled, unirradiated $\phi$X174 double-stranded DNA (linearized with HaeIII) in 10 μl of reaction buffer (40 mM Tris-HCl, pH 7.5, 5 μM MgCl$_2$, 0.1 μg/ml BSA, 1 mM dithiothreitol) at 30 °C for 30 min. The reaction mixture was then run on 3.6% polyacrylamide gels in Tris-acetate-EDTA buffer (40 mM Tris acetate, pH 7.0, 0.1 mM EDTA) at 30 mA at 4 °C for 1 h. The gels were dried and exposed to Eastman Kodak Co. MR films, and the autoradiograms were subjected to image analysis in a Bio-Rad GS670 densitometer to obtain data points for graphical representation of the results.

**Antibody Inhibition**—Purified NEF2 in 10 μl of DNA mobility shift reaction buffer was preincubated at 25 °C for 5 min with the indicated amounts of affinity-purified anti-Rad4 or anti-Rad6 antibodies added in 1 μl of storage buffer (10 μM KH$_2$PO$_4$, pH 7.2, 100 mM KCl). After the addition of the radiolabeled, UV-irradiated DNA substrate (10 kJ/m²) and the cold, unirradiated competitor DNA, the reaction mixtures were incubated at 30 °C for 30 min and then processed for polyacrylamide gel electrophoresis, as described above under “DNA Mobility Shift Assay.”

**Enzymatic Photoreactivation**—UV-irradiated, $^{32}$P-labeled DNA fragment (500 ng) was mixed with 3 μg of Escherichia coli photolyase (a gift from Aziz Sancar) in 100 μl of reaction buffer (20 mM Tris-Cl, pH 7.4, 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 100 μg/ml BSA) and placed under photoreactivating light emitting at ~360 nm for 20 min at 25 °C. An identical reaction mixture incubated with photolyase in the dark served as control. The reaction mixtures were extracted twice with phenol, and the DNA was precipitated with ethanol and redissolved in 20 μl of Tris-EDTA buffer. The complete removal of cyclobutane pyrimidine dimers from the UV-irradiated DNA was verified by incubating the photoreactivated DNA with T4 pyrimidine-dimer endonuclease, as described previously (12, 13).

**RESULTS**

To facilitate the purification of NEF2, we overexpressed the Rad4 protein in yeast. We have found that Rad23 protein is present in considerable molar excess over the Rad4 protein in wild-type yeast cells. Thus, overexpression of Rad4 protein alone was sufficient to obtain NEF2, and there was no advantage of simultaneously overexpressing Rad23 as well (data not shown). A purification procedure, which entails ammonium sulfate precipitation of cell extract, followed by chromatographic fractionation in columns of Q-Sepharose, SP-Sepharose, hydroxyapatite, Sephacyr S300, Mono Q, and Mini S, was devised to purify NEF2 ~10,000-fold to near homogeneity (Fig. 1A and B). During purification, the elution of NEF2 from various columns was monitored by immunoblot analysis for the Rad4 and Rad23 proteins. The Rad4 and Rad23 proteins remain quantitatively associated throughout all of the purification steps. When analyzed by SDS-polyacrylamide gel electrophoresis and staining with Coomassie Blue, the Rad4-Rad23 protein complex (NEF2) from the final Mini S column (fraction VIII) showed only the Rad4 and Rad23 protein bands, indicating that the NEF2 preparation was nearly homogeneous. Fraction VIII NEF2 was used in all of the biochemical studies described below. The yield of NEF2 from 500 g of starting yeast paste was ~50 μg.

Genetic studies have implicated the two constituents of NEF2, Rad4 and Rad23 proteins, in the incision step of nucleotide excision repair (14). Our *in vitro* studies with the recon-
stituted nucleotide excision repair system have indicated an essential function of NEF2 in the incision of UV-damaged DNA (1). Because NEF2 does not appear to possess an enzymatic activity, we examined whether NEF2 was involved in the recognition of UV-induced DNA damage. For examining the DNA binding properties of NEF2, a 130-bp DNA fragment containing a run of thymine residues in the middle was irradiated with a range of UV dose from 1 to 12 kJ/m² to introduce different levels of UV photoproducts into the DNA. The unirradiated DNA and UV-damaged DNAs were incubated with purified NEF2 at 30 °C in the presence of an excess of cold, unirradiated DNA to titrate out the nonspecific background binding. The reaction mixtures were run out on a native polyacrylamide gel at 4 °C, after which the gel was dried and exposed to x-ray films to detect nucleoprotein species. As shown in Fig. 2A, incubation of the UV-damaged DNA with NEF2 resulted in the formation of nucleoprotein complexes with a retarded mobility compared with the free probe, whereas little binding (<2%) to the unirradiated DNA fragment occurred. The amount of nucleoprotein complexes increased with the UV dose, and at 10 kJ/m², >80% of the DNA fragment was converted into nucleoprotein complexes (Fig. 2B). The amount of UV damage specific nucleoprotein complex formation was proportional to the amount of NEF2 in the reaction, and even at the highest concentration of NEF2, the level of background binding to the unirradiated fragment did not increase significantly (Fig. 3). These experiments (Figs. 2 and 3) showed that purified NEF2 had an associated UV damage-specific DNA binding activity. In other experiments, we found that the damage binding activity of NEF2 was not influenced by 1 mM ATP (data not shown).

In both Figs. 2 and 3, three distinct nucleoprotein complexes were detected. The levels of the slower-migrating species were much more prevalent at higher UV doses and NEF2 concentrations. Because the DNA fragment used contained multiple target sites for UV photoproduct formation, it seems quite possible that different nucleoprotein complexes correspond to increasing number of NEF2 molecules bound to separate UV lesions in the same DNA fragment.

The high degree of purity of our NEF2 protein preparations and the known involvement of NEF2 in the incision of UV-damaged DNA both in vivo (14) and in vitro (1) strongly suggested that the damage-specific binding activity observed was in fact an intrinsic property of NEF2. However, to obtain further corroborating evidence that NEF2 does indeed possess UV damage recognition activity, we examined the co-elution of this activity with NEF2 and also the specific inhibition of this activity by anti-Rad4 antibody. For the co-elution experiment, extract from 500 g of wild-type yeast cells without the Rad4-overexpressing plasmid was subjected to exactly the same chromatographic procedure as used for the purification of NEF2 from Rad4-overproducing cells (Fig. 1A). Fractions 6–20 from the Mini S step obtained from wild-type cells lacking the Rad4-overproducing plasmid as well as the equivalent fractions from the Mini S column obtained during the purification of NEF2 from 500 g of the Rad4-overproducing yeast strain were subjected to immunoblot analysis to determine their content of NEF2, and these fractions were also used in DNA mobility shift assays with the unirradiated DNA fragment and a DNA fragment irradiated with 10 kJ/m² of UV light. As shown in Fig. 4A, because of the low level of NEF2 in wild-type cells, it was barely detectable in the Mini S fractions derived from the extract of
wild-type cells, and accordingly, no binding to UV-damaged DNA was detected in these fractions. As expected, the DNA damage binding activity in the Mini S fractions derived from Rad4-overproducing cells precisely paralleled the NEF2 amounts in these fractions (Fig. 4A). For the antibody inhibition experiment, NEF2 was preincubated with increasing amounts of affinity-purified antibody against the Rad4 protein and also with control antibody specific for the Rad6 protein. As shown in Fig. 4B, whereas damage binding was not affected by even the highest amount (1 μg) of the control anti-Rad6 antibody, the addition of anti-Rad4 antibody resulted in strong inhibition of the reaction. Specifically, the level of binding of the UV-damaged DNA (10 kJ/m²) in the presence of increasing amounts of affinity-purified anti-Rad4 (●) or anti-Rad6 (●) antibody. The uninhibited level (100%) of damage binding corresponds to shifting of ~95% of the UV damage-containing DNA substrate. For experimental details, see "Materials and Methods."

We have previously described the purification of Rad23 protein from a yeast strain genetically tailored to overproduce this protein (15). In view of the UV damage binding activity present in NEF2, we examined whether Rad23 protein alone has DNA damage-specific binding activity. As shown in Fig. 5, Rad23 does not bind to the same DNA fragment used in the experiments with NEF2, regardless of whether the DNA was UV-irradiated. The result with Rad23 protein alone (Fig. 5) suggests the possibility that the damage recognition activity may reside in the Rad4 protein. However, we have been unable to purify Rad4 protein to test this hypothesis, because Rad4 protein expressed in yeast cells deleted for the RAD23 gene does not behave well chromatographically, and because Rad4 protein expressed in insect cells is insoluble (data not shown). UV light elicits the formation of two types of photoproducts in DNA, the cyclobutane pyrimidine dimers and the pyrimidine-pyrimidone (6-4) photoproducts. The kinetics of formation of these two classes of photoproducts differ in that the level of cyclobutane pyrimidine dimers reaches photostationary equilibrium at ~4 kJ/m², whereas the yield of the pyrimidine-pyrimidone (6-4) photoproducts continues to increase with the UV dose (16, 17). As shown in Fig. 2, the level of nucleoprotein complexes continues to rise beyond 4 kJ/m², where the content of pyrimidine dimers had already become saturated, indicating that NEF2 can recognize the pyrimidine-pyrimidone (6-4) photoproducts. We made use of enzymatic photoreactivation to determine whether NEF2 also has an affinity for pyrimidine...
DNA Damage Binding Activity of NEF2

To do this, DNA irradiated with a UV dose of 2 or 10 kJ/m² was treated with E. coli photolyase, which uses light energy to eliminate the pyrimidine dimers by converting the two cross-linked pyrimidine residues back to the monomeric form (18). The removal of pyrimidine dimers from the UV-irradiated fragments was verified using the T4 UV endonuclease, which nicks DNA at sites of pyrimidine dimers, as we have done before (12, 13). As shown in Fig. 6, the levels of NEF2-DNA nucleoprotein complexes obtained with either UV-irradiated DNA fragment did not diminish after photoreactivation, indicating that NEF2 has little affinity for the cyclobutane pyrimidine dimers.

**DISCUSSION**

Genetic studies have implicated the Rad4 and Rad23 proteins in the incision step of NER (14). Biochemical studies have indicated that these two proteins exist as a tight stoichiometric complex called NEF2, which is essential for the incision of UV-damaged DNA in our reconstituted nucleotide excision repair system (1). Here, we have shown that NEF2 binds UV-damaged DNA specifically, and photoreactivation of UV-damaged DNA did not diminish the interaction of NEF2 with the target DNA, strongly suggesting that NEF2 specifically recognizes the pyrimidine-pyrimidone (6-4) photoproducts in the damaged DNA. At 10 kJ/m², at which we observed a >80-fold preference for binding UV-damaged DNA, there is an average of ~1.5 pyrimidine-pyrimidone (6-4) photoproducts/molecule of the 130-bp DNA fragment. From this, it can be estimated that NEF2 shows greater than (80 × 130/1.5) 6000-fold preferential binding to pyrimidine-pyrimidone (6-4) photoproduct sites than to undamaged nucleotides. The damage recognition ability of NEF2 shows no dependence on ATP. We also find that Rad23 protein alone does not bind DNA, suggesting the possibilities that either the damage binding activity resides in Rad4 or that activation of the damage recognition ability is contingent on complex formation between the Rad4 and Rad23 proteins. We reported previously that Rad23 protein interacts with TFIIH and Rad14 (15), consistent with the premise that Rad23 functions to link NEF2 to other components of the incision machinery during UV damage removal.

The identification of NEF2 as a damage recognition factor underscores the complexity of the damage recognition reaction during NER in eukaryotes. To date, three other NER protein factors have been shown to have the ability to bind UV-damaged DNA, namely, NEF4, consisting of the Rad7 and Rad16 proteins (7, 8), the zinc-metalloprotein Rad14 (12), and the heterotrimeric RPA (13). Although recognition of DNA damage by Rad14 (12), RPA (13), and NEF2 (this work) does not show a dependence on a nucleoside triphosphate, damage binding by NEF4 is markedly stimulated by ATP (7, 8), which is consistent with the presence of a DNA-dependent ATPase activity in NEF4 (8). Our recent results have suggested that although ATP binding alone is sufficient to induce a conformation in NEF4 necessary for damage binding, ATP hydrolysis may in fact fuel the translocation of NEF4 on DNA in search of DNA lesions (8). These properties of NEF4 suggest that it may be the primary damage sensor that monitors chromatin for the presence of DNA damage in vivo. On encountering a DNA lesion, NEF4 becomes stably bound to the lesion and then recruits the other nucleotide excision repair factors, including NEF2, leading to the assembly of the incision repair complex and the DNA damage-specific incision.

XPC, the human homolog of RAD4, is required for the repair of nontranscribed regions of the genome but not for the repair of the transcribed strand of actively transcribed genes (19). Also, mouse embryo fibroblasts of XPC−/− mice are completely defective in the removal of pyrimidine-pyrimidone (6-4) photoproducts from the nontranscribed strand, but they repair significant levels of UV damage from the transcribed strand (20). In contrast to XPC, the RAD4 gene of yeast is essential for the repair of both transcribed and nontranscribed regions of the genome. The rad4Δ mutants are as highly sensitive to UV damage as deletions of other incision genes (10), and no incision of UV-damaged DNA occurs in the rad4Δ mutant in vivo (21). Furthermore, in the reconstituted system, no incision of UV-damaged DNA occurs in the absence of NEF2 (1). Even though the absence of RAD23 does not completely inactivate NER (22), the repair of both the transcribed and nontranscribed DNA is decreased in the rad23Δ mutant (23). Thus, all the evidence suggests that the yeast Rad4-Rad23 complex functions in the repair of both the transcribed and nontranscribed DNAs. In contrast to yeast, in which RAD23 is a single-copy gene, humans contain two copies of this gene, hHR23A and hHR23B, and XPC exists in vivo in a complex with hHR23B (24). It is possible that like hHR23, XPC also has become duplicated in humans and other mammals, and the other copy of XPC functions with hHR23A in the repair of the transcribed strand. Thus, like the Rad4-Rad23 complex, the XPC-hHR23 complexes may also be critical for the repair of both transcribed and nontranscribed DNAs.
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