Saccharomyces cerevisiae RAD7 and RAD16 genes function together in the nucleotide excision repair of transcriptionally inactive DNA. The RAD7- and RAD16-encoded proteins exist as a tight complex named nucleotide excision repair factor 4 or NEF4. Previously, we showed that NEF4 binds UV-damaged DNA with high specificity and with a dependence upon ATP and that inclusion of NEF4 to the reconstituted nucleotide excision repair system consisting of purified NEF1, NEF2, NEF3, and replication protein A results in marked stimulation of damage-specific DNA incision. Here we show that NEF4 possesses an ATPase activity that is entirely dependent on a DNA cofactor and that double-stranded DNA is twice as effective as single-stranded DNA in activating ATP hydrolysis. Even though DNA binding is promoted by the nonhydrolyzable ATP analogue adenosine 5’-O-(thiotriphosphate) (ATPγS), damage binding is more proficient with ATP than with ATPγS. Interestingly, UV irradiation of double-stranded DNA results in a pronounced attenuation of the ATPase activity. Taken together, our results suggest a model in which ATP hydrolysis by NEF4 fuels the translocation of NEF4 on DNA in search of UV lesions and damage binding by NEF4 leads to a down-regulation of the ATPase activity. Damage-bound NEF4 could then serve as a nucleation point for the assembly of other repair components.

Nucleotide excision repair (NER) of ultraviolet-damaged DNA in eukaryotes is a complex process requiring the products of a large number of genes. The structure and function of the nucleotide excision repair genes are highly conserved among eukaryotes from yeast to humans (1, 2). Biochemical fractionation of yeast cell extracts have revealed the organization of the NER proteins into distinct functional subassemblies called nucleotide excision repair factors or NEFs. Specifically, we have shown that NEF1 contains the damage recognition protein Rad4 and the Rad1-Rad10 endonuclease (3), NEF2 comprises the Rad4 and Rad23 proteins (4), and that the Rad2 endonuclease and the six-subunit RNA polymerase II transcription factor TFIIH form NEF3 (5). Our biochemical studies have further demonstrated that the combination of NEF1, NEF2, NEF3, and the heterotrimeric single-stranded DNA (ssDNA) binding factor replication protein A (RPA) is sufficient for dual incision of UV-damaged DNA to occur (3–6). These studies have suggested that the basic yeast NER machinery consists of NEF1, NEF2, NEF3, and RPA. Human equivalents of these yeast NER proteins also carry out the dual incision of damaged DNA (7–9).

In addition to the aforementioned NER factors, genetic studies have indicated a role of four additional genes, RAD7, RAD16, RAD26, and MMS19, in NER (1, 2). The MMS19-encoded protein affects cell viability and functions in RNA polymerase II transcription, probably as a regulatory component by modulating the activity of TFIIH and of proteins that function in other cellular processes (10). The Rad26 protein is a member of the Swi2/Snf2 family of proteins, possesses a DNA-dependent ATPase activity (11), and is required for the preferential repair of the transcribed DNA strand (12). The RAD7 and RAD16 genes, on the other hand, are specifically required for the nucleotide excision repair of nontranscribed DNA (13–16). The RAD7 gene product does not possess significant homology to any other known protein, whereas the RAD16-encoded product is another member of the Swi2/Snf2 protein family (17). Recently, we showed that the Rad7 and Rad16 proteins are associated in a stoichiometric complex, which we have named NEF4. NEF4 has high affinity for UV-damaged DNA, and addition of NEF4 to the reconstituted NER system consisting of NEF1, NEF2, NEF3, and RPA results in marked stimulation of damage-specific incision (18). Here we describe our studies demonstrating a DNA-dependent ATPase activity in NEF4. Interestingly, UV irradiation of the DNA cofactor results in a marked down-regulation of the NEF4 ATPase activity. We suggest a model in which the free energy from ATP hydrolysis is utilized to fuel the translocation of NEF4 on DNA to search for DNA lesions. Binding of NEF4 to a DNA lesion results in suppression of ATP hydrolysis, and the stable NEF4-DNA damage complex serves as the nucleation site for the assembly of other NER factors.

MATERIALS AND METHODS

NEF4 Purification—Extract was prepared from 500 g of cells of strain LY2 co-harboring pR7.8 (2 μm, GAL-PGK-RAD7) and pH6.15 (2 μm, ADCl-RAD16) using a French press and clarified by centrifugation (100,000 × g for 120 min). The clarified extract was subjected to ammonium sulfate precipitation (35% saturation) to precipitate NEF4 and approximately 15% of the total protein. The precipitate was harvested by centrifugation (20,000 × g, 30 min) and dissolved in K buffer (20 mM KH₃PO₄, pH 7.4, 10% glycerol, 1 mM dithiothreitol, 0.5 mM EDTA) followed by sequential fractionation in columns of Q-Sepharose, SP-Sepharose, hydroxyapatite, and Mono S to yield fraction V1 NEF4 (18). The yield of NEF4 was 90 μg with a recovery of ~15%. When fraction V1 NEF4 was analyzed in an SDS-polyacrylamide gel and stained with Coomassie Blue, only the Rad7 and Rad16 proteins were seen (18), indicating that the preparation was nearly homogeneous. Frac-
NEF4 Is a DNA-dependent ATPase

**ATPase Assay**—Purified NEF4, 120 ng, was mixed with 250 ng of dX ssDNA or 250 ng of dX double-stranded DNA (dsDNA), 0.5 mM ATP and 1 μCi of [γ-32P]ATP (3,000 Ci/mmol; Amersham Life Science, Inc.) in buffer R (30 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM dithiothreitol, 100 μg/ml bovine serum albumin). The reaction mixtures were incubated at 30 °C for 30 min or as indicated. For quantifying ATP hydrolysis, a 0.5-μl portion of the reaction mixtures was spotted onto a polyethyleneimine cellulose sheet, which was developed with 1 M formic acid containing 0.3% LiCl. Quantification was done by phosphoimage analysis in a Molecular Dynamics PhosphorImager.

**DNA Helicase Assay**—The substrate for the DNA helicase assay was obtained as described previously (19). A 5'-32P-labeled 17-base DNA fragment was hybridized to the viral (+) strand of M13mp18 to generate a partial duplex. The nonhybridized labeled DNA fragment was separated from the partial duplex DNA by gel filtration in Sepharose G-50 matrix equilibrated in 50 mM Tris-HCl, pH 7.5, 0.1 M NaCl at 4 °C. Fractions containing the peak of partial duplex DNA were pooled and concentrated to a small volume. The helicase substrate, 5 ng, was mixed with the indicated amount of NEF4 protein in 10 μl of buffer R containing 2 mM ATP. After incubation for 45 min at 30 °C, the reaction was stopped by adding 5 μl of 1% SDS, 50 mM EDTA, 20% glycerol, 0.02% bromphenol blue. The reaction mixture was run in 12% polyacrylamide gels, which were then dried and subjected to autoradiography. A DNA helicase assay employing Rad3 protein was carried out as described (20).

DNA Mobility Shift Assay—A 0.9-kilobase AT-rich DNA fragment from plasmid pS288 (21) was cloned into pUC19 to generate the plasmid pTB402. A 130-base pair HindIII-SalI fragment that contained eight consecutive thymines was isolated from plasmid pTB402 and labeled with [γ-32P]ATP at the 3' end by treatment with Klenow polymerase (22). UV irradiation of the fragment was carried out using a gernicidal UV lamp emitting at 254 nm, as described previously (22). Purified NEF4, 60 ng, was incubated with 2 ng of the 32P-labeled DNA fragment and 20 ng of linear dX dsDNA as cold competitor in 10 μl of reaction buffer R that contained 2 mM ATP or ATP/S. After the addition of 2 μl of loading buffer (40% glycerol, 0.1 mM Tris acetate, pH 7.0, 10 mM EDTA, 0.02% acrylamide-bis), the reaction mixtures were run in 4% polyacrylamide gels at 30 mA and 4 °C for 1 h. The gels were dried onto Whatman 3MM paper and then exposed to Kodak MR film or subjected to phosphoimage analysis to quantify the binding reaction.

**RESULTS**

By immunoprecipitation and other criteria, we have shown recently that Rad7 and Rad16 are associated in a stable complex (K₆ = 4 × 10⁻¹⁰ M) termed NEF4, which has been purified to near homogeneity (18). The Rad16 protein contains Walker types A and B nucleotide binding motifs (23) suggestive of an ability to bind and hydrolyze ATP. To examine whether purified NEF4 has ATPase activity, it was incubated with [γ-32P]ATP in the presence of Mg²⁺, and the reaction mixtures were analyzed by thin layer chromatography in polyethyleneimine cellulose sheets using lithium chloride and formic acid as the developing solvent (11, 24). However, without DNA, we did not detect significant hydrolysis of ATP (≤2%) over the pH range of 6.0–9.0 (data not shown). Since NEF4 functions in DNA repair and binds DNA, we tested whether in the presence of DNA NEF4 would hydrolyze ATP. Interestingly, the addition of either ssDNA or dsDNA resulted in substantial hydrolysis of ATP by NEF4 (see below). When the column fractions from the last step of NEF4 purification in Mono S were subjected to immunoblotting with anti-Rad7 and anti-Rad16 antibodies to determine their NEF4 content and also assayed for ssDNA- and dsDNA-dependent ATP hydrolysis, we found that both the ssDNA-dependent and dsDNA-dependent ATP hydrolysis activities co-eluted with NEF4 from Mono S fractions 9–21 from the last step of NEF4 purification were subjected to immunoblot analyses with anti-Rad7 and anti-Rad16 antibodies to determine their NEF4 content and also assayed for ssDNA- and dsDNA-dependent ATPase activities. For the immunoblot analyses, 1.0 μl of the fractions was used, and for the ATPase assays, 4.0 μl of the fractions were used.

![Fig. 1. DNA-dependent ATPase activity co-elutes with NEF4.](image)

 Mono S fractions 9–21 from the last step of NEF4 purification were subjected to immunoblot analyses with anti-Rad7 and anti-Rad16 antibodies (●) and ssDNA-dependent (▲) and dsDNA-dependent (●) ATPase activities. For the immunoblot analyses, 1.0 μl of the fractions was used, and for the ATPase assays, 4.0 μl of the fractions were used.

Consistently, we found that single-stranded dX DNA was about 50% as effective as double-stranded dX DNA in activating ATP hydrolysis by NEF4 (Fig. 2 and Table I). To investigate the possibility that ATP hydrolysis seen with dX ssDNA might have been due to the presence of secondary structure in the DNA, we examined whether the single-stranded homopolymer poly(dA), which is relatively free of secondary structure, could also activate ATP hydrolysis by NEF4. As shown in Table I, poly(dA) was also effective in activating ATP hydrolysis. Taken together, these results indicate that NEF4 hydrolyzes ATP upon binding either ssDNA or dsDNA, with the latter being the preferred DNA cofactor. No ATP hydrolysis was detected with the polynucleotides poly(A) and RNA.

Although the Rad7 protein does not show any discernible conserved sequence motifs, the Rad16 protein belongs to the Swi2/Snf2 family of proteins whose members function in diverse chromosomal processes including transcription, transcription-coupled repair, post-replication repair, and recombinational repair (see Ref. 17 for discussion). All of the Swi2/Snf2 family of proteins possess the seven consensus helicase-like motifs that may be primarily involved in coordinating DNA binding, ATP binding, and the hydrolysis of ATP, as none of them has yet been shown to have DNA helicase activity (11, 25–27). To determine whether Rad16 protein was an exception, we hybridized a 17-base 32P-labeled DNA fragment to circular M13 ssDNA and used the resulting partial duplex as substrate to test whether NEF4 had helicase activity. Incubation of the helicase substrate with as much as 300 ng of NEF4 for 45 min did not result in displacement of the hybridized fragment from the partial duplex (Fig. 2C), whereas 100 ng of Rad3 protein, a known helicase (20), resulted in >80% displacement of the hybridized fragment (data not shown). NEF4 also did not unwind a forked DNA substrate that contained 20-nucleotide-long
and 5'-overhanging tails adjoining a 30-base pair duplex region (data not shown). Thus, it appears that, like the other Swi2/Snf2 family proteins that have been examined to date (11, 25–27), ATP hydrolysis by Rad16 is utilized for purposes other than extensive disruption of base pairing in dsDNA.

Rad16 protein contains two potential zinc binding, DNA binding motifs, a C4 motif and a C3HC4 motif (23), which could confer damage-specific DNA binding activity to NEF4. C4 motifs are present in other known damage recognition factors including the *Escherichia coli* UvrA protein and the yeast Rad14 and human XPA proteins (22, 28–31). The DNA binding activity of NEF4 was examined by a DNA mobility shift assay in which purified NEF4 was incubated with a 130-base pair DNA fragment that was end-labeled with $^{32}$P in the presence of cold $\phi$X 174 dsDNA as competitor to titrate out the nonspecific binding and then analyzing the reaction mixtures on nondenaturing polyacrylamide gels followed by autoradiography of the dried gels to detect nucleoprotein complexes. As reported in our recent work (18) and reiterated here in Fig. 3, in the presence of ATP and ATPγS, NEF4 exhibits DNA binding activity.

### TABLE I

| DNA ATPase activity | DNA  |
|---------------------|------|
| No DNA             | 0%   |
| Single-stranded DNA| 55%  |
| $\phi$X 174, $-\text{Mg}^{2+}$| 0% |
| Poly(dA)           | 36%  |
| Duplex DNA         |      |
| $\phi$X 174        | 100% |
| $\phi$X 174, $-\text{Mg}^{2+}$| 0% |
| Poly(dA) · Poly(dT) | 55% |
| Poly(dA) · (dT)$_{12-18}$ | 57% |

**FIG. 2.** Characterization of the NEF4 ATPase activity. A, dsDNA-dependent (●) and ssDNA-dependent (▲) ATPase activities of NEF4 (120 ng) as a function of the reaction time. B, dsDNA-dependent (●) and ss DNA dependent (▲) ATPase activities as a function of the NEF4 amounts. The incubation time was 30 min. C, NEF4 does not exhibit DNA helicase activity. NEF4, at 25, 50, 100, and 300 ng was incubated with 5 ng of the 17-base pair partial duplex substrate for 45 min at 30 °C (lanes 3–6). Lane 1, DNA substrate. In lane 2, the DNA substrate was boiled for 2 min to release the annealed $^{32}$P-labeled DNA fragment. nt, nucleotides.

**FIG. 3.** Effects of ATP and ATPγS on damage recognition by NEF4. A, NEF4, 60 ng, was incubated with nondamaged DNA and with DNA irradiated with 2 and 10 kJ/m² of UV light in the absence or presence of 2 mM ATP or ATPγS, as indicated. N/P, no protein; F, free DNA probe; C, nucleoprotein complex.

**NEF4 Is a DNA-dependent ATPase**

6294
of ATP, NEF4 binds specifically to UV-irradiated DNA. For instance, whereas less than 2% of the nondamaged DNA fragment was bound by NEF4 (Fig. 3A, lane 4; Fig. 3B), UV irradiation of the DNA fragment with doses of 2 and 10 kJ/m² resulted in binding of about 40 and 90% of the DNA fragment (Fig. 3A, lanes 8 and 12; Fig. 3B), respectively. The omission of ATP from the binding reaction diminished damage-specific DNA binding markedly, such that only 5 and 24% of the fragments irradiated with 2 and 10 kJ/m² were bound by NEF4, respectively (Fig. 3).

Previously, we showed that the nonhydrolyzable ATP analogue ATPγS promotes damage binding by NEF4 (18), indicating that damage recognition can occur in the absence of ATP hydrolysis. To determine if ATP hydrolysis modulates the level of damage recognition, here we examine the effect of ATP and ATPγS on the ability of NEF4 to bind DNA that had been irradiated with 2 or 10 kJ/m² of UV light. As shown in Fig. 3, even though ATPγS promotes damage binding, the level of damage binding achieved with ATPγS (26 and 67% for the 2- and 10 kJ/m²-irradiated fragments) was lower than that seen with ATP (40 and 90% binding of the 2- and the 10-kJ/m² irradiated fragments). These results suggest that ATP hydrolysis increases the efficiency of damage recognition by NEF4.

To further analyze the relationship between damage recognition and ATP hydrolysis by NEF4, we incubated NEF4 with δX dsDNA that has been exposed to increasing doses of UV light (0.1–10 kJ/m²) and 32P-labeled ATP. Unexpectedly, we found that UV irradiation of the dsDNA cofactor in fact results in a marked inhibition of the NEF4 ATPase activity (Fig. 4). For instance, irradiating the dsDNA with a dose of 500 J/m², which introduced approximately five photoproducts per kilobase pair, resulted in 30% inhibition of the ATPase activity, with more inhibition occurring at progressively higher UV doses (Fig. 4). Thus, the results indicate that the binding of NEF4 to UV lesions is accompanied by an attenuation of ATP hydrolysis.

**DISCUSSION**

Consistent with the presence of nucleotide binding motifs in the Rad16 protein, purified NEF4 has an ATPase activity that requires either ssDNA or dsDNA for its activation. In this regard, dsDNA is approximately twice as effective as ssDNA in activating ATP hydrolysis. NEF4 binds preferentially to UV damaged DNA (Ref. 18 and this work), and ATP is required for maximal damage-specific binding. Even though ATPγS promotes damage-specific DNA binding, this reaction occurs more efficiently with ATP than with ATPγS. Furthermore, we find that UV irradiation of the dsDNA cofactor results in an attenuation of the ATPase activity. Taken together, our results strongly suggest that ATP hydrolysis by NEF4 is primarily utilized in steps before the binding of NEF4 to the UV damage in the target DNA.

The strong dependence of the NEF4 damage recognition ability on ATP distinguishes it from two other known damage recognition factors, Rad14 and RPA, which are integral components of the NER machinery (4). The yeast Rad14 protein and its human counterpart XPA both contain a zinc finger motif and have affinity for UV damage, but the damage binding activity of these yeast and human NER factors is not influenced by ATP (22, 30). RPA has also been shown to have an affinity for UV damage, but it does so in the absence of ATP (32). From the results of genetic and biochemical studies, we surmise that NEF4, by utilizing the free energy from ATP hydrolysis, translocates on chromosomal DNA to survey the DNA for the presence of bulky damages such as UV photoproducts (Fig. 5). Stable binding of NEF4 to the DNA lesion results in an attenuation of the ATPase activity, and the now damage-associated NEF4 serves as the nucleation site for the recruitment of other NER factors including the aforementioned damage recognition proteins to initiate the assembly of the NER machinery. This working model for NEF4 action predicts that Rad7 and Rad16 interact with one or more protein components in NEF1, NEF2, NEF3, and RPA to effect the coupling of the damage recognition process to the assembly of the basic NER machinery comprising the latter protein complexes.

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