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Communication

Reconstitution of Yeast Nucleotide Excision Repair with Purified Rad Proteins, Replication Protein A, and Transcription Factor TFIIH*

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Nucleotide excision repair (NER) functions to remove DNA damage caused by ultraviolet light and by other agents that distort the DNA helix. The NER machinery has been conserved in structure and function from yeast to humans, and in humans, defective NER is the underlying cause of the cancer-prone disease xeroderma pigmentosum. Here, we reconstitute the incision reaction of NER in *Saccharomyces cerevisiae* using purified protein factors. The Rad14 protein, the Rad4-Rad23 complex, the Rad2 nuclease, the Rad1-Rad10 nuclease, replication protein A, and the RNA polymerase II transcription factor TFIIH were purified to near homogeneity from yeast. We show that these protein factors are both necessary and sufficient for dual incision of DNA damaged by either ultraviolet light or *N*-acetoxy-2-aminoacetylfluorene. Incision in the reconstituted system requires ATP, which cannot be substituted by adenosine 5′-*O*-(3-thiotriphosphate), suggesting that the hydrolysis of ATP is indispensable for the incision reaction. The excision DNA fragments formed as a result of dual incision are in the 24–27-nucleotide range.

Nucleotide excision repair (NER)[1] represents the most important cellular mechanism for the removal of DNA damage induced by ultraviolet light (UV). Genetic studies in the yeast *Saccharomyces cerevisiae* have identified seven genes, *RAD1, RAD2, RAD3, RAD4, RAD10, RAD14,* and *RAD25*, that are essential for NER (1). To begin to define the biological roles of these genes, we have purified their encoded proteins from yeast and characterized their biochemical activities. This undertaking has allowed us to infer the involvement of these proteins in different steps of the incision process, *viz.* in damage recognition, in DNA unwinding, and in dual incision of the damaged DNA strand. Rad14 is a zinc metalloprotein with an affinity for UV-damaged DNA (2). Rad3 is a single-stranded DNA-dependent ATPase, and it also has DNA helicase and DNA·RNA helicase activities (3–5). Rad3 exhibits a preference for binding UV-damaged DNA that is dependent upon ATP and the degree of negative superhelicity in DNA (6). Rad25 also possesses a single-stranded DNA-dependent ATPase and DNA helicase activities (7). Rad1 and Rad10 exist as a complex *in vivo*, and complex formation between these proteins is essential for their biological action (8). Rad1 and Rad10 together comprise a DNA endonuclease (9, 10), and Rad2 also is a DNA endonuclease (11).

Another level of complexity in understanding the biological roles of NER genes was introduced from the observation that in addition to their requirement in NER, *RAD3* and *RAD25* are also essential for cell viability (1). Studies of temperature-sensitive conditional lethal mutations of these genes have indicated that both are essential for RNA polymerase II transcription (7, 12, 13). Studies with the *rad3* and *rad25* mutants defective in ATPase/DNA helicase activities have indicated that whereas Rad3 ATPase/helicase activity is required for NER, the Rad25 ATPase/helicase is essential for both NER and polymerase II transcription (7, 13, 14). Rad3 and Rad25 proteins are two of the six subunits of polymerase II transcription factor TFIIH, and it has been proposed that the entire TFIIH functions in NER (15–18).

In addition to the above mentioned protein factors, the single-stranded DNA binding protein RPA has been suggested to have a role in an early step of NER (19, 20). In a reconstituted system reported recently, Mu *et al.* (21) have shown that human RPA is essential for incision of a DNA substrate containing a cholesterol adduct.

Our goal has been to reconstitute nucleotide excision repair in yeast with purified proteins. Because of the amenability of *S. cerevisiae* to genetic and biochemical analyses, development of such a system is essential for a detailed understanding of processes that effect different steps of NER, including transcription-coupled DNA repair. Various yeast protein factors that have been implicated in NER in genetic and biochemical studies have now been purified to near homogeneity in our laboratory. Here, we present our results, which indicate that the incision step of NER can be accomplished by combining the following highly purified protein components: Rad14, Rad4-Rad23 complex, Rad1-Rad10 complex, Rad2, RPA, and TFIIH. The damage-specific incision reaction has a strict dependence on ATP, and our results imply that the two incision nicks are made in a highly coordinated fashion.

MATERIALS AND METHODS

UV Irradiation and AAF Treatment of Plasmid DNA—Replicative form M13 mp18 DNA (~90% supercoiled form) was purified from infected *Escherichia coli* strain JM101 by two rounds of cesium chloride banding. The DNA, at a concentration of 100 μg/ml in TE (10 mM Tris-HCl, pH 7.0, 0.2 mM EDTA), was irradiated in 15-μl droplets for 60 s with a germicidal lamp emitting at 254 nm and a fluence rate of 5 J/m^2/s to introduce ~2.5 photoproducts/1000 base pairs of DNA. For treatment with *N*-acetoxy-2-aminoacetylfluorene (AAF), the plasmid DNA, 10 μg or 30 nmol of nucleotides, was incubated at 37 °C for 12 h in the dark with 0.5 nmol of AAF in 200 μl of 50 mM sodium acetate, pH 5.8. After extraction with diethyl ether, the DNA was purified by ethanol precipitation and redissolved in TE to 100 μg/ml.

Reconstitution of NER in Vitro—(i) In the incision assay, reaction mixtures (10 μl, final volume) were assembled in buffer R (45 mM K-HEPES, pH 7.9, containing 8 mM $MgCl_2$, bovine serum albumin at

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[1] The abbreviations used are: NER, nucleotide excision repair; PAGE, polyacrylamide gel electrophoresis; RPA, replication protein A; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); AAF, *N*-acetoxy-2-aminoacetylfluorene.

120 μg/ml, 1.5 mM dithiothreitol, 2 mM ATP, an ATP-regenerating system consisting of 30 mM creatine phosphate and 200 ng of creatine kinase, and 60 mM potassium acetate and 20 mM KCl that were due to addition of the various NER protein factors) and contained 100 ng of TFIIH, 50 ng of RPA, 8 ng of Rad1-Rad10 complex, 10 ng of Rad2 protein, 20 ng of Rad4-Rad23 complex, and 10 ng of Rad14 protein. The Rad1-Rad10 complex for use in these studies was formed by incubating equimolar amounts of purified Rad1 and Rad10 proteins for 24 h on ice in 20 mM Tris-HCl, pH 7.5, containing 1 mM dithiothreitol and 500 μg/ml bovine serum albumin to give a final concentration of 1 μM of the protein complex. The combination of NER factors were incubated at 25 °C for 5 min before the undamaged DNA or damaged DNA, 100 ng each, was added in 1 μl. After incubation at 30 °C for 10 min, SDS and proteinase K were added to 0.5% and 200 μg/ml, respectively, followed by a 5-min incubation at 37 °C to deproteinize the reaction mixtures. Samples were run in 0.8% agarose gels in TAE buffer (20 mM Tris acetate, pH 7.4, 0.5 mM EDTA). The gels were treated with ethidium bromide (1 μg/ml in H_2O) to stain DNA, soaked in a large volume of water to reduce background staining, and then photographed through a red filter. (ii) For the excision assay, reaction mixtures (50 μl, final volume) were assembled in buffer R and contained 500 ng of TFIIH, 250 ng of RPA, 40 ng of Rad1-Rad10 complex, 50 ng of Rad2, 100 ng of Rad4-Rad23 complex, and 50 ng of Rad14. The combination of NER proteins was incubated at 25 °C for 5 min before 1 μg of undamaged or UV-damaged DNA was added in 10 μl of TE. The complete reaction mixtures were incubated at 30 °C for 60 min and then deproteinized by extraction with an equal volume of buffered phenol. The DNA was precipitated by ethanol and redissolved in 10 μl of TE, and 5 μl of which was treated with 7.5 units of calf thymus terminal transferase (Boehringer Mannheim) with 5 μCi of [α-^{32}P]dideoxy ATP (Amersham Corp.; 5000 Ci/mmol) for 60 min at 37 °C in a final volume of 20 μl of the buffer supplied by the vendor. The labeling mixtures were deproteinized by treatment with 0.5% SDS and 300 μg/ml proteinase K for 10 min at 37 °C, followed by the precipitation of the DNA. The pellets were dissolved in 4 μl of buffer and analyzed on 15% sequencing gels. The gels were dried and exposed to x-ray films (Kodak Bio Max MR) to reveal the excision DNA fragments.

RESULTS

Purification of NER Factors—Rad10 protein was purified ~1,000-fold to near homogeneity (Fig. 1*B*, *lane 1*) from yeast strain CMY135 harboring the overproducing plasmid pSUC8 as described (22). The *RAD14* gene contains an intron and encodes a protein with a predicted size of 43 kDa, and it exhibits a size of 48 kDa in SDS-PAGE (23). Rad14 protein has been purified ~1,000-fold to near homogeneity (Fig. 1*B*, *lane 2*) from yeast strain YRP11 harboring the overproducing plasmid pR14.15 containing *RAD14* under the control of the yeast alcohol dehydrogenase I (*ADC1*) promoter, with the purification scheme outlined in Fig. 1*A*. Rad1 protein was purified ~2,000-fold to near homogeneity (Fig. 1*C*, *lane 1*) from yeast strain CMY135 harboring the overproducing plasmid pRR168 as described previously (9). Rad2 protein was purified ~3,000-fold to near homogeneity (Fig. 1*C*, *lane 2*) from yeast strain LY2 harboring the overproducing plasmid pR2.26 as described (11). To overproduce the *RAD4*-encoded protein in yeast for purification, the protein coding frame of *RAD4* was fused to the yeast *ADC1* promoter to yield plasmid pR4.1, which was introduced into yeast strain YRP11. The scheme presented in Fig. 1*A* was used to purify Rad4 protein ~4,000-fold to near homogeneity (Fig. 1*C*, *lane 3*) from YRP11(pR4.1). The elution of Rad4 protein from various chromatographic matrices was monitored by immunoblotting using affinity-purified polyclonal antibodies raised against an insoluble form of Rad4 protein expressed in insect cells with the use of baculovirus (data not shown). Rad4 protein has a predicted molecular mass of 87 kDa (24), but it migrates in SDS-polyacrylamide gels with a relative molecular mass of 116 kDa. Throughout the purification of Rad4, we observed a precise co-elution of a protein with a relative molecular mass of 57 kDa, which is the same as that described for the *RAD23* gene product (25) that plays an important role in the proficiency of NER (1, 25). By immunoblotting using affinity-purified anti-Rad23 antibodies (25), we have verified that this 57-kDa species was indeed Rad23 protein. Because in wild type yeast Rad23 is more abundant than Rad4 (25), only overproduction of Rad4 was necessary to purify the Rad4-Rad23 complex (Fig. 1*C*, *lane 3*). TFIIH was purified >10,000-fold to near homogeneity (Fig. 1*D*) from yeast strain YPH/TFB1.6HIS that contains a 6-histidine tag in the TFB1 subunit of TFIIH (26) as outlined in Fig. 1*A*. During purification, the elution of TFIIH from various chromatographic matrices was monitored by immunoblotting using affinity-purified antibodies specific for the Rad3, Rad25, TFB1, and SSL1 subunits (23). The TFIIH preparation used in this study contained six subunits, Rad25, Rad3, TFB1, SSL1, p55, and p38 (Fig. 1*D*). RPA, consisting of three subunits of 69, 36, and 13 kDa, was purified to near homogeneity (Fig. 1*B*, *lane 3*) from yeast strain LP2749-9B using the procedure of Brill and Stillman (27).

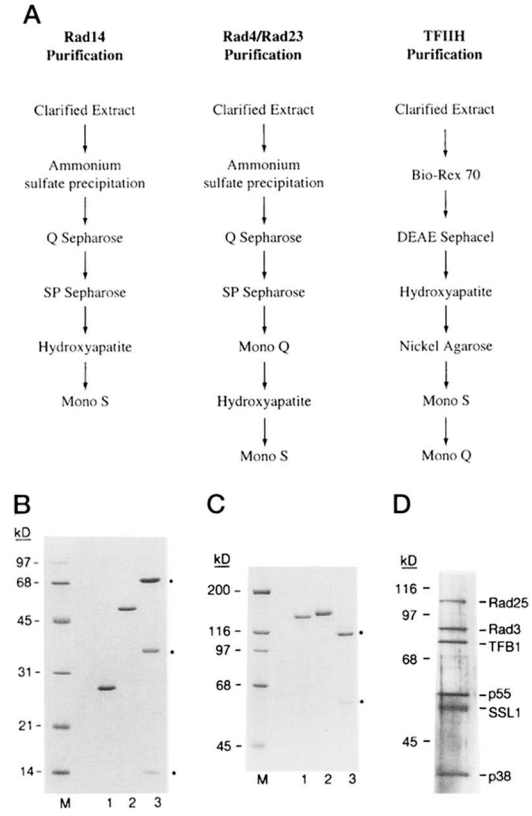

FIG. 1. **Protein factors for *in vitro* reconstitution of NER.** *A*, purification schemes for Rad14, Rad4-Rad23 complex, and TFIIH. *B–D*, SDS-PAGE of purified NER factors. *B*, Rad10 (1 μg in *lane 1*), Rad14 (1 μg in *lane 2*), and RPA (2 μg in *lane 3*) consisting of the 69-, 36-, and 13-kDa subunits each marked by an *asterisk* were run in a 12% denaturing polyacrylamide gel and stained with Coomassie Blue. *M*, molecular size standards. *C*, 1 μg each of Rad1 (*lane 1*), Rad2 (*lane 2*), and the complex of Rad4 (*upper band* marked by *asterisk* in lane 3) and Rad23 (*lower band* marked by *asterisk* in *lane 3*) were run in a 7% denaturing polyacrylamide gel and stained with Coomassie Blue. *M*, molecular size standards. *D*, TFIIH (500 ng total protein) was run in an 8% denaturing polyacrylamide gel and silver-stained with a kit purchased from Bio-Rad. The various subunits of TFIIH are indicated. The identity of subunits was verified by immunoblotting using affinity-purified anti-Rad25, anti-Rad3, anti-TFB1, and anti-SSL1 antibodies.

ATP-dependent Incision of UV-damaged DNA—To examine whether the purified yeast factors Rad14, Rad4-Rad23 complex, Rad1-Rad10 complex, Rad2, RPA, and TFIIH are sufficient for reconstituting NER *in vitro*, they were incubated at 30 °C in buffer containing ATP with plasmid DNA previously irradiated with UV light to introduce photoproducts into the DNA. Reaction mixtures were deproteinized and analyzed by agarose gel electrophoresis, followed by staining with ethidium

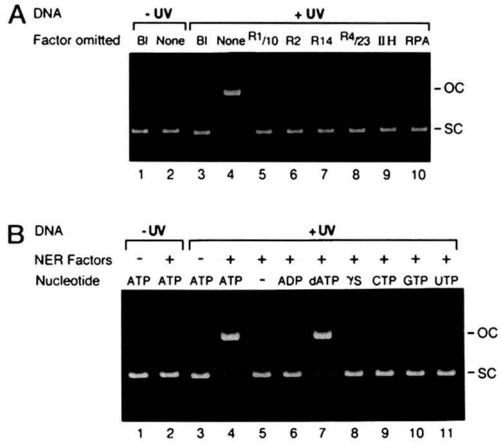

FIG. 2. **ATP-dependent incision of UV-damaged DNA.** *A*, reconstitution of the incision reaction using purified protein factors. The DNA used was either not treated (*−UV*, *lanes 1* and *2*) or treated with UV (*+UV*, *lanes 3–10*). The full complement of NER factors (Rad1-Rad10 complex, Rad2, Rad4-Rad23 complex, Rad14, TFIIH, and RPA) was incubated with undamaged DNA (*lane 2*) and UV-damaged DNA (*lane 4*) in buffer containing ATP at 30 °C for 10 min. One of the aforementioned NER factors was absent in the reaction mixtures in *lanes 5–10* that contained UV-damaged DNA and ATP; Rad1-Rad10 complex was omitted in *lane 5*, Rad2 was omitted in *lane 6*, Rad14 was omitted in *lane 7*, Rad4-Rad23 complex was omitted in *lane 8*, TFIIH was omitted in *lane 9*, and RPA was omitted in *lane 10*, as indicated at the *top* of the figure. *Bl*, DNA incubated in buffer without any of the NER factors (*lanes 1* and *3*). *B*, incision of UV-damaged DNA requires ATP. The full complement of NER factors (*lane 2* and *lanes 4-11*) was incubated with either undamaged DNA (*−UV*, *lane 2*) or with UV-damaged DNA (*+UV*, *lanes 4-11*) in the presence of ATP (*lanes 2* and *4*), ADP (*lane 6*), dATP (*lane 7*), ATPγS (*lane 8*), CTP (*lane 9*), GTP (*lane 10*), UTP (*lane 11*), or without any nucleotide (*lane 5*) at 30 °C for 10 min. In *lanes 6* and *8*, creatine kinase was omitted to inactivate the ATP-regenerating system. No NER factors were added in *lanes 1* and *3*. *SC*, supercoiled form; *OC*, open circular form.

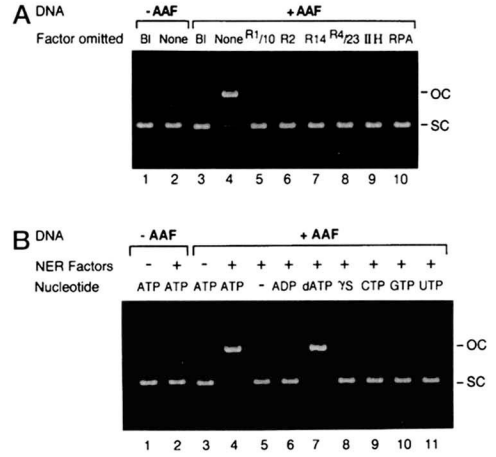

FIG. 3. **ATP-dependent incision of DNA containing AAF adducts.** *A*, the NER factors incise AAF-containing DNA. The undamaged DNA (*lane 2*) or damaged DNA (*lane 4*) was incubated with the Rad1-Rad10 complex, Rad2, Rad4-Rad23 complex, Rad14, TFIIH, and RPA at 30 °C for 10 min in buffer containing ATP. The reaction mixtures in *lanes 5–10* contained AAF-damaged DNA and ATP, but one of the NER factors was omitted; Rad1-Rad10 complex was absent in *lane 5*, Rad2 was absent in *lane 6*, Rad14 was absent in *lane 7*, Rad4-Rad23 complex was absent in *lane 8*, TFIIH was absent in *lane 9*, and RPA was absent in *lane 10*. *Bl*, DNA incubated in buffer without any of the NER factors (*lanes 1* and *3*). *B*, incision of AAF-containing DNA requires ATP. The full complement of NER factors (*lane 2* and *lanes 4–11*) was incubated with either undamaged DNA (*−AAF*, *lane 2*) or with AAF-containing DNA (*+AAF*, *lanes 4–11*) in the presence of ATP (*lanes 2* and *4*), ADP (*lane 6*), dATP (*lane 7*), ATPγS (*lane 8*), CTP (*lane 9*), GTP (*lane 10*), UTP (*lane 11*), or without any nucleotide (*lane 5*) at 30 °C for 10 min. In *lanes 6* and *8*, creatine kinase was omitted to inactivate the ATP-regenerating system. No NER factors were added in *lanes 1* and *3*. *SC*, supercoiled form; *OC*, open circular form.

bromide to examine the fate of the DNA substrate. Fig. 2*A* shows that when all the purified NER factors were present, greater than 80% of the UV-damaged supercoiled plasmid DNA was converted to the open circular form (*lane 4*), indicating that incision of the damaged DNA had occurred. By contrast, plasmid DNA that had not been subjected to UV treatment was not acted on by the same protein factors (Fig. 2*A*, *lane 2*). Thus, the combination of NER proteins carries out an incision reaction that is highly specific for UV damage. Importantly, omission of any of the components Rad1-Rad10 complex, Rad2, Rad14, Rad4-Rad23 complex, TFIIH, or RPA from the reaction mixture abolished the formation of the open circular form (Fig. 2*A*, *lanes 5–10*), indicating that incision of UV-damaged DNA requires all of these purified protein factors. It is of particular interest that nicking of the UV-damaged DNA did not occur when either the Rad1-Rad10 endonuclease or the Rad2 endonuclease was omitted from the reaction mixture (Fig. 2*A*, *lanes 5* and *6*). This observation strongly suggests that both the Rad1-Rad10 complex and the Rad2 protein, in addition to being the endonucleolytic components, have a pivotal role in the proper assembly of the incision enzyme complex at the damage site, thus ensuring that the two incision nicks are made in a coordinated fashion.

Because Rad3 and Rad25 proteins both possess an ATP (dATP)-dependent helicase activity that is required for NER (7, 13, 14), it was of considerable importance to determine whether the incision of damaged DNA in our reconstituted system requires ATP. We found that in the absence of ATP, the incision of UV-damaged DNA does not occur (Fig. 2*B*, compare *lanes 4* and *5*). Whereas dATP is as effective as ATP in promoting the incision reaction, ADP, CTP, GTP, and UTP are inactive (Fig. 2*B*). Interestingly, the non-hydrolyzable ATP analog ATPγS is also inactive (Fig. 2*B*, *lane 8*), suggesting that ATP hydrolysis is in fact required for the incision reaction. Furthermore, Mg^{2+} is also required for incision (data not shown).

Incision of DNA Damaged by AAF—The NER machinery has specificity for a variety of DNA lesions. We examined whether our purified NER proteins would also incise DNA damaged by *N*-acetoxy-2-aminoacetylfluorene. As shown in Fig. 3*A*, plasmid DNA containing the AAF adduct was incised when all the purified protein factors were present (*lane 4*) but not when any of the factors was omitted from the reaction mixture (*lanes 5–10*). The incision of AAF-modified DNA also has a specific requirement for ATP or dATP (Fig. 3*B*). ATPγS did not promote incision (Fig. 3*B*), again suggesting a requirement for ATP hydrolysis in the repair of AAF-damaged DNA.

Size of Excision DNA Fragments—NER in humans has been shown to occur by way of dual incision of the DNA strand that contains the lesion, resulting in the release of a DNA fragment 27–29 nucleotides in length (28). To detect the excision DNA fragment, we incubated plasmid DNA damaged by UV light in the reconstituted repair system. Following phenol extraction to remove repair proteins, the DNA was purified and treated with calf thymus terminal transferase in the presence of [α-^{32}P]dideoxy ATP to label any excision DNA fragments that might have been generated. As shown in Fig. 4, a series of DNA fragments ranging in size from 25 to 28 nucleotides was detected in the reaction mixture that contained UV-damaged DNA as substrate (*lane 5*) but not in the control that contained undamaged DNA (*lane 2*). Importantly, the DNA fragments were not produced if ATP was absent (Fig. 4, *lane 6*) or when TFIIH was omitted from the repair reaction (Fig. 4, *lane 4*). Because the ^{32}P-labeling protocol added one nucleotide to the excision DNA fragments, the actual size range of these fragments is 24–27 nucleotides.

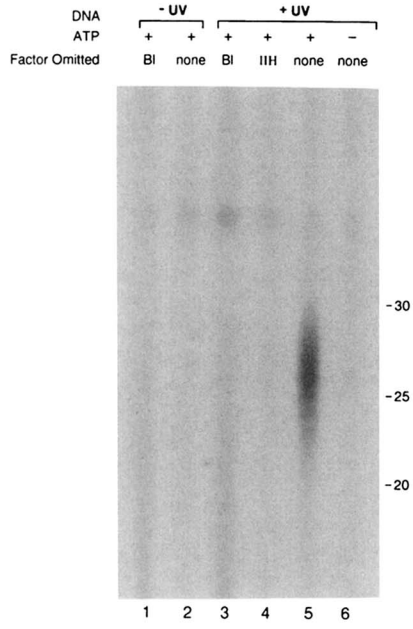

FIG. 4. **Size of the excision DNA fragments.** UV-irradiated (*lanes 3-6*) and unirradiated (*lanes 1* and *2*) plasmid DNAs were incubated with the full complement of NER factors (*lanes 2*, *5*, and *6*) in the presence of ATP (*lanes 2* and *5*) or in its absence (*lane 6*). The reaction mixture in *lane 4* contained ATP but lacked TFIIH. The numbers to the *right* of the autoradiogram indicate the positions in nucleotides of the DNA markers used. *Bl*, DNA incubated in buffer without any of the NER factors (*lanes 1* and *3*).

DISCUSSION

In this work we have achieved the reconstitution of a system that mediates dual incision of DNA damaged either by UV or AAF, by combining the following essentially homogeneous *S. cerevisiae* factors: Rad1-Rad10 complex, Rad2, Rad4-Rad23 complex, Rad14, RPA, and TFIIH consisting of Rad3, Rad25, TFB1, SSL1, p55, and p38 subunits. Since all these purified protein factors are indispensable for damage-specific incision, they represent the minimum set of factors for accomplishing this reaction.

In our reconstituted system, incision of damaged DNA shows a strict dependence on ATP or dATP, whose hydrolysis is required for the repair reaction because the non-hydrolyzable analog ATPγS does not promote the incision of damaged DNA. The requirement for ATP and its hydrolysis in the incision step of NER is consistent with the results from genetic studies conducted with mutant variants of Rad3 and Rad25 proteins that are defective in ATP hydrolysis (7, 13, 14). We suggest that at the expense of ATP hydrolysis, the combined helicase function of Rad3 and Rad25 creates a single-stranded region at the damage site for dual incision by the Rad1-Rad10 and Rad2 endonucleases.

It is intriguing that nicking of the damaged DNA does not occur if either the Rad1-Rad10 endonuclease or the Rad2 endonuclease is present alone with the remainder of the incision NER components. This finding strongly suggests that in addition to providing the endonucleolytic activities for dual incision, the Rad1-Rad10 protein complex and the Rad2 protein are also required for the proper assembly of the NER ensemble at the damage site. The involvement of the two endonucleolytic components in assembling the NER complex may serve to ensure that the endonucleolytic scissions at the damage site occur in a coordinated manner, as well as to minimize gratuitous nicking of DNA.

In summary, the following major conclusions emerge from this work. First, the damage recognition factor Rad14, the Rad4-Rad23 complex, the Rad1-Rad10 endonuclease, the Rad2 endonuclease, RPA, and TFIIH are all essential for the incision step of NER. Second, because of the high degree of purity of the protein factors used, we infer that the combination of these proteins is sufficient for the incision reaction. Third, the incision reaction occurs only in the presence of ATP, and our results strongly suggest a requirement of ATP hydrolysis in this reaction. Finally, our observation that the size of the excision fragment produced by the yeast incision enzyme complex resembles that in humans indicates that the yeast and human NER machineries act in a highly similar manner.

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