The yeast Rad4 and Rad23 proteins form a complex that is involved in nucleotide excision repair (NER). Their function in this process is not known yet, but genetic data suggest that they act in an early step in NER. We have purified an epitope-tagged Rad4Rad23 (tRad4Rad23) complex from yeast cells, using a clone overproducing Rad4 with a hemagglutinin-tag at its C terminus. tRad4Rad23 complex purified by both conventional and immuno-affinity chromatography complements the in vitro repair defect of rad4 and rad23 mutant extracts, demonstrating that these proteins are functional in NER. Using electrophoretic mobility shift assays, we show preferential binding of the tRad4Rad23 complex to damaged DNA in vitro. UV-irradiated, as well as N-acetoxy-2-(acetylamino)fluorene-treated DNA, is efficiently bound by the protein complex. These data suggest that Rad4Rad23 interacts with DNA damage during NER and may play a role in recognition of the damage.

Nucleotide excision repair (NER) is the main mechanism responsible for the error-free removal of many distinct types of DNA damage. This process is strongly conserved in eukaryotes ranging from yeast to man and involves several proteins. NER consists of the following basic steps: (i) damage recognition, (ii) DNA unwinding around the lesion, (iii) dual incision on either side of the lesion, and (iv) template-dependent DNA synthesis followed by ligation of the remaining nick (1). The factors involved in the NER reaction have been identified, and biochemical activities have been assigned to most of them.

A detailed model for NER has been proposed, but the exact mechanism of DNA damage recognition is still poorly understood. Identification of the factors implicated in damage recognition is a prerequisite to gain insight in this first step of NER. Although some yeast proteins that bind damaged DNA have been identified, notably the Rad14 and Rad7-Rad16 proteins (2, 3), other proteins may also be involved in DNA damage recognition.

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HTP hydroxylapatite (in this case, a linear gradient of KPO$_4$, pH7.4 in buffer A containing 50 mM NaCl was used; tRad4 peak around 240 mM KPO$_4$), single-stranded DNA-cellulose (Sigma) (410 mM NaCl), Hi-Trap SP-Sepharose (1 ml) [500 mM NaCl], and finally either Resource Q (1 ml) or Mono Q (HR5/5) [350 mM NaCl], which both gave comparable results. The DNA was incubated overnight with 10 mM (7 mg/ml) DTT, 100 mM DTT, 100 mM DTT, and the sample was heated to 95 °C for 5 min and allowed to cool to 37 °C. Unincorporated label was removed by a Sephadex G-50 spin column. DNA was ethanol precipitated and redissolved in water.

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200–400 pmol of each oligonucleotide was 5'-phosphorylated in 200 μl reaction containing 200 μl of kinase buffer (70 mM Tris-HCl, pH 7.6, 10 mM MgCl$_2$, 5 mM DTT) for 1.5 h at 37 °C. DNA was irradiated with UV in 15-μl droplets of water at 0–40 kJ/m$^2$ at 254 nm. NA-AAF treatment of DNA (data not shown). Samples were loaded on 3.5% polyacrylamide (acrylamide:N,N$^\prime$-methylenebisacrylamide, 37.5:1) gel and run in 25 μm Tris-Glycine, pH 8.5, 1 mM DTT, and 1 μl of tRad4 from a 1 ml reaction containing 30 μM Tris-HCl, pH 8.0, 50 mM NaCl, 1.5% glycerol, 0.5 mM MgCl$_2$, 1 mM DTT, 100 μg/ml bovine serum albumin, 1 mM ATP, and 10 units T4-ligase (Fermentas) (DNA fragment containing a single guanine residue. After purification via phenol-chloroform extraction, precipitated twice with ethanol, and redissolved in water.

Linear DNA fragments containing a single AAF adduct were constructed using a protocol based on Refs. 15 and 20. The DNA substrate consists of six oligonucleotides comprising a 146-bp fragment. –92 to +54 bp relative to the transcription start site from the Lytechinus variegatus 5S rDNA gene (21). Each strand is derived from three oligonucleotides (from 5' to 3': top strand, 71, 19, and 56 bp; bottom strand, 48, 39, and 59 bp). The central 19-mer oligonucleotide (5'-CTTACATTCGCAACACACCA) in the top strand was treated with NA-AAF to yield near-complete adduct formation at the single guanine residue. After purification via phenol-chloroform extraction and ethanol precipitation of the damaged or mock treated 19-mer, 200–400 pmol of each oligonucleotide was 5'-phosphorylated in 200 μl of kinase buffer (see above) by using 40 units of T4 polynucleotide kinase (Amersham Pharmacia Biotech) and $^{32}$P-ATP (ICN; 7000 Ci/mmol) for 1 h at 37 °C. The six phosphorylated oligonucleotides were mixed together, NaCl was added to 100 mM, the sample was heated to 95 °C for 5 min and allowed to cool slowly to room temperature to anneal the oligonucleotides. The DNA was ethanol precipitated, redissolved, and ligated in 50 μl containing 50 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10 mM MgCl$_2$, 5 mM DTT, 50 μg/ml bovine serum albumin, 1 mM ATP, and 10 units T4-ligase (Fermentas) for 16 h at 14 °C. DNA was extensively purified by elution of full-length fragments from two subsequent 10% acrylamide-urea gels to remove unligated oligonucleotides. Purified fragments consist of two 146-mer strands, one of which contains an AAF adduct at position 81 from the 5' terminus. These strands were rehydrbized, and double-stranded fragments were purified on a native 6% polyacrylamide gel. After elution, the DNA was precipitated twice with ethanol and redissolved in water. Typical yield of full-length double-stranded substrate containing a single AAF lesion was about 1 pmol. The presence and position of the AAF lesion was verified by incubation with UvrABC endonuclease (20) and by restriction analysis (a I recognition site is lost because of the appearance of slower migrating forms of DNA. When the DNA is irradiated with UV, tRad4 is co-purified via direct binding to tRad4, once more confirming a direct interaction between these proteins. To determine whether our purified tRad4/tRad23 preparations are still functional in NER, we attempted to rescue the defective repair activity of cell-free extracts of rad4, rad23, and rad4 rad23 deletion strains. In vitro NER was assayed by means of measuring DNA synthesis in AAF-damaged plasmids incubated with cell-free extracts (23, 24). The in vitro NER deficiency of cell free extracts from rad4, rad23, and rad4 rad23 disruption mutants (see also Refs. 5 and 18) is rescued when they are supplemented with tRad4/tRad23 (Fig. 1B), purified by either of the two approaches described above. This indicates that the complex is functional in NER.

RESULTS

Purification of the tRad4-Rad23 Complex—To characterize the Rad4-Rad23 complex, we purified these proteins from yeast cell lysates containing Rad4 containing a HA epitope at its C terminus, here referred to as t(aged)Rad4. Full-length RAD4 sequences cannot be propagated in Escherichia coli (22). Therefore, two partially overlapping clones bearing either the N-terminal or the C-terminal part of the RAD4 gene (kind gifts from Dr. K. Madura) were co-transformed to yeast to obtain a full-length clone via homologous recombination. This clone complements the UV sensitivity of a rad4Δ strain, indicating that a functional protein is produced (data not shown). The gene is under the control of a CUP1 promoter, which is inducible by Cu$^{2+}$ ions.

We have purified tRad4 in two distinct ways: first by sequential chromatography on phosphocellulose, hydroxylapatite, single-stranded DNA-cellulose, SP-Sepharose, and Resource Q columns; second, we used a two-step immuno-affinity purification protocol in which phosphocellulose fractions were immunopurified using 12CA5 monoclonal antibodies (anti-HA) and elution with synthetic HA-peptide.

Both procedures yield a complex of two proteins, with some minor contaminants as shown by silver staining of SDS-polyacrylamide electrophoresed gels (Fig. 1A). The band corresponding to a protein of apparent molecular mass of around 120 kDa (4) was shown to be tRad4 by Western blotting using anti-HA monoclonal antibodies (data not shown). A protein of 57 kDa co-purifies with tRad4, and in agreement with previous reports (4, 6), this protein was shown to be Rad23 anti-Rad23 antiserum and immunoblotting (not shown). Rad23 is not overproduced in these cells, but the endogenous level of Rad23 exceeds natural Rad4 levels (4). As immuno-affinity purification of tRad4/Rad23 proceeds via the HA-epitope of tRad4, Rad23 is co-purified via direct binding to tRad4, once more confirming a direct interaction between these proteins. To determine whether our purified tRad4/Rad23 preparations are still functional in NER, we attempted to rescue the defective repair activity of cell-free extracts of rad4, rad23, and rad4 rad23 deletion strains. In vitro NER was assayed by means of measuring DNA synthesis in AAF-damaged plasmids incubated with cell-free extracts (23, 24). The in vitro NER deficiency of cell free extracts from rad4, rad23, and rad4 rad23 disruption mutants (see also Refs. 5 and 18) is rescued when they are supplemented with tRad4/tRad23 (Fig. 1B), purified by either of the two approaches described above. This indicates that the complex is functional in NER.

NER, DNA Damage Binding by tRad4 and Rad23—Using the purified functional tRad4/Rad23 complex, we have characterized its DNA damage binding properties. We conducted electrophoretic mobility shift assays using a $^{32}$P-labeled 200-bp linear DNA fragment that runs as a single band when no protein was added (not shown). DNA binding by tRad4/Rad23 protein is observed as the appearance of slower migrating forms of DNA. When the DNA is irradiated with UV, tRad4/Rad23-DNA complex formation is markedly increased (Fig. 2). Binding of tRad4/Rad23
complex is specific for damaged DNA because complexes persist in the presence of a large excess of competitor DNA, whereas binding to undamaged DNA is strongly decreased (Fig. 2 A). Complex formation increases with increasing UV dose (Fig. 2 B) and was observed using independent protein preparations. In addition, we conducted a similar experiment using a DNA-probe containing adducts induced by NA-AAF. Also using this substrate, we found preferential binding by tRad4-Rad23 to damaged DNA in a dose-dependent manner (Fig. 3A), showing that damage-induced binding of Rad4-Rad23 is not confined to DNA containing UV-induced lesions. To further validate the damaged DNA binding by tRad4-Rad23, we assayed complex formation on a more defined DNA substrate. To this purpose, we constructed a linear 146-bp double-stranded DNA fragment containing a single positioned AAF adduct. Electrophoretic mobility shift analysis using this single AAF-adduct substrate also showed a clear enhancement of complex formation compared with undamaged DNA (Fig. 3 B), demonstrating that even a single DNA damage induces DNA binding by tRad4-Rad23. Therefore, preferential binding is not confined to DNA fragments containing multiple damaged sites.

In all experiments performed, at least two protein-DNA complexes were observed. Because these complexes are also observed using undamaged DNA or DNA containing a single lesion, the existence of more than one complex cannot be explained solely by the presence of multiple lesions per DNA fragment. To determine whether the protein-DNA complexes...
Rad4-Rad23 Binds Damaged DNA

...tRad4, we used 12CA5 monoclonal antibodies directed against the HA-epitope. Addition of this antibody to the binding reactions resulted in a supershift of the labeled fragment (Fig. 4), indicating that tRad4 is present in the observed protein-DNA complexes.

**DISCUSSION**

In this study, we report the preferential binding of the *Saccharomyces cerevisiae* Rad4-Rad23 complex to damaged DNA. We have purified the complex and assayed its binding characteristics to damaged DNA by mobility shift analysis.

Previous reports suggest a role for Rad4 in damage recognition. Rad4-independent repair *in vivo* exists in yeast (11). Likewise, repair in human cells can take place in the absence of XPC (12), the human homolog of *Rad4*. In addition, *in vitro* NER is observed in the absence of XPC for some DNA lesions (13–15). Apparently the lesion structure may circumvent the need for XPC in humans or Rad4 in yeast, suggesting a role for these proteins in recognition of DNA lesions. Our data provide direct evidence for this hypothesis, as we observe preferential binding of tRad4-Rad23 to damaged DNA. Two structurally distinct types of NER substrates (i.e. UV-induced photoproducients and AAF adducts) induce binding of tRad4-Rad23 to DNA.

Recently Sugasawa *et al.* (25) have shown by competition assays that human NER is initiated by XPC-hHR23B. Furthermore, they provide direct evidence that purified human XPC-hHR23B preferentially binds to DNA damage by means of an immuno-pull down assay and by DNase I footprinting. Our results of damaged DNA binding by yeast Rad4-Rad23 are consistent with and extend these data, using different methodology and proteins from a different eukaryotic origin. These observations again underscore the homology between yeast and human NER.

Rad23 is in complex with Rad4 but might also have functions independent of Rad4. Repair of rDNA that is independent from Rad4 (11), does depend on functional Rad23 (26). Also, biochemical experiments have shown that purified Rad23 interacts with Rad14 and TFIIH and promotes complex formation between these proteins (8). Furthermore, it has recently been shown that the NER complex can be linked to the 26 S proteasome via Rad23 (6). These observations point to a possible role for Rad23 in NER complex assembly and disassembly. Rad4-Rad23 may therefore act as an intermediate in damage binding and NER complex formation.

We observe a moderate enhancement of DNA binding by tRad4-Rad23 induced by DNA damage. Because a number of factors have now been implicated in damage recognition in yeast, it is possible that a coordinate action of different proteins, such as Rad14 (2), Rad7-Rad16 complex (3), and Rad4-Rad23 (this study) is needed to gain the specificity required at the genomic level. Interactions between Rad4 and Rad7 (5), as well as between Rad23 and Rad14 (8), have been reported, pointing to a link between these damage-recognizing proteins. Along this line, it has recently been suggested that human NER complex formation at the site of the damage only occurs when XPC-hHR23B, XPA, replication protein A (RPA), TFIIH, and XPG are present together, indicating a cooperative mode of binding to the damage (27).

On the basis of the evidence we present in this report, we suggest that Rad4-Rad23 acts early in the formation of the NER complex by directly binding to DNA damage, possibly in concert with other NER factors.

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**REFERENCES**