Nucleotide Excision Repair in Yeast Is Mediated by Sequential Assembly of Repair Factors and Not by a Pre-assembled Repairosome*

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In yeast and humans, nucleotide excision repair (NER) of ultraviolet (UV)-damaged DNA requires a large number of highly conserved protein factors, which include the multisubunit RNA polymerase II transcription factor TFIIH. Here, we examine whether NER occurs by sequential assembly of different repair factors at the site of DNA damage or by the placement there of a “pre-formed” reairosome containing TFIIH and all the other essential NER factors. Contrary to the recent report (Svejstrup, J. Q., Wang, Z., Feaver, W. J., Wu, X., Bushnell, D. A., Donahue, T. F., Friedberg, E. C., and Kornberg, R. D. (1995) Cell 80, 21–28), our results provide no evidence for a pre-assembled reairosome; instead, they support the sequential assembly model. By several independent criteria, including copurification, immunoprecipitation, and gel filtration of homogeneous proteins, we show that the damage recognition factor Rad14 exists in a ternary complex with the Rad1-Rad10 nuclease. We also find that Rad14 interacts directly with Rad1, but only slightly with Rad10, and that it interacts with the Rad1-Rad10 complex much more efficiently than with Rad1 alone. In the reconstituted NER system, a higher level of incision of UV-damaged DNA is achieved with the Rad1-Rad10-Rad14 complex, which we designate as nucleotide excision repair factor-1, NEF-1.

Nucleotide excision repair (NER)1 represents the most important pathway for the removal of DNA damage inflicted by ultraviolet (UV) light and by a variety of other DNA-damaging agents that distort the DNA helix. NER is accomplished by dual incision of the damage containing DNA strand, thus excising the damage in the form of a short DNA fragment (reviewed in Refs. 1 and 2). A defect in NER results in xeroderma pigmentosum (XP) in humans; XP patients are extremely sensitive to sunlight, and they suffer from a high incidence of skin cancers. Cells derived from XP individuals show elevated mutation rates because of misincorporation of bases opposite the unexcised UV lesions by DNA polymerases during replication of the damaged DNA template.

Studies in the yeast Saccharomyces cerevisiae have been instrumental in defining the functions of NER genes in different stages of the repair reaction and in revealing their roles in cellular processes other than NER. Seven genes, namely RAD1, RAD2, RAD3, RAD4, RAD10, RAD14, and RAD25, are essential for the incision step of NER, and four other genes, namely RAD7, RAD16, RAD23, and MMS19, affect the proficiency of the repair reaction (1). In addition to their NER function, RAD3 and RAD25 genes are essential for viability because of their requirement in RNA polymerase II transcription (3–5). Rad3 and Rad25 constitute two of the subunits of the transcription factor TFIIH (6). RAD1 and RAD10 also function in mitotic recombination in a pathway that is distinct from the RAD52 recombination pathway (7, 8).

The products encoded by the seven RAD genes indispensable for the incision of UV damaged DNA have been purified to near homogeneity and their biochemical activities have been determined. Rad14 is a zinc metalloprotein that binds preferentially to UV-damaged DNA (9). Rad3 protein is a DNA helicase (10) and it binds UV-damaged DNA preferentially in an ATP-dependent manner (11). The Rad25 protein also possesses a DNA helicase activity (5). Rad1 and Rad10 proteins form a complex that has single-stranded DNA endonuclease activity (12, 13). Rad2 protein is also a single-stranded DNA endonuclease (14). The Rad4 protein exists in a stoichiometric complex with Rad23, and available evidence suggests a role of this complex in the assembly of the repair factors at the damage site (15, 16).

Using purified protein factors, we have recently reconstituted the damage-specific incision reaction of NER (16). This work has indicated the requirement for the Rad1-Rad10 complex, Rad2 protein, the Rad4-Rad23 complex, Rad14 protein, replication protein A (RPA), and the RNA polymerase II transcription factor TFIIH in the incision reaction. ATP-dependent dual incision of the UV-damaged DNA by the combination of purified factors results in the release of 24–27-nucleotide-long DNA fragments. No incision of the damaged DNA occurs if any of the aforementioned protein factors is omitted (16). The requirement for such a large body of protein factors in the incision reaction indicates that this reaction likely involves a series of highly coordinated events dictated by stringent temporal and spatial assemblies of the protein factors at the damage site. One of our main goals now is to define the manner and sequence by which these proteins are assembled onto the DNA damage site. These studies, however, will be greatly facilitated by a prior understanding of how the NER proteins are organized into functional subassemblies, since such a determination not only would enable us to begin dissecting the biological functions and significance of each subassembly, but it would also be a prerequisite for logical experimental designs in working toward the goal of defining the order and manner of recruitment of the subassemblies at the DNA damage site.

From previous work, core TFIIH is known to contain the following six subunits, Rad3, Rad25, TFB1, SSL1, p55, and p38.
(6, 16, 17). RPA is a heterotrimer of 69-, 36-, and 13-kDa subunits (18). Rad1 and Rad10 form a complex (19, 20), as do Rad4 and Rad23 (16). It is not known whether or not these multimeric NER factors are physically associated with another and with the other NER proteins in one complex forming a repair holoenzyme. Should there be a higher order complex consisting of the aforementioned NER factors, however, co-purification of these factors will be observed upon chromatographic fractionation of cell extract; precisely the same approach has been used in identifying various functional subassemblies that help mediate transcription and DNA replication. Here, we find co-purification of the damage recognition protein Rad14 with the Rad1-Rad10 complex. Results from additional experiments indicate that Rad14 binds directly to the Rad1-Rad10 complex with a dissociation constant \( K_d \) of \( <5.3 \times 10^{-8} \) M, and that Rad1-Rad10-Rad14 function together as a subassembly in the NER reaction.

Recently, it has been reported that TFIIH exists in a physical complex with products of NER genes RAD1, RAD2, RAD4, RAD10, and RAD14, forming a nucleotide excision “repairosome” (21). Contrary to this report, we find no evidence for a pre-assembled complex of TFIIH with Rad1, Rad4, Rad10, and Rad14 proteins. Our data support the model wherein different repair factors assemble at the site of DNA damage in a sequential manner.

**MATERIALS AND METHODS**

**Antibodies**—The sources for the affinity purified rabbit polyclonal antibodies are: anti-Rad1 (19), anti-Rad3 (22), anti-Rad4 (16), anti-Rad10 (23), anti-Rad14 (9), anti-Rad25 (5), and anti-Rad51 (24). For raising anti-TFB1 antibodies, an Escherichia coli produced GST-TFB1 hybrid polypeptide (25) was purified and used as antigen (16), and for raising anti-SSL1 antibodies, an E. coli-produced 6-histidine tagged SSL1 (6) was purified and used as antigen (16). These antibodies were purified from rabbit antiserum by affinity chromatography on columns of cyanogen bromide activated Sepharose containing covalently cross-linked antigens, as described previously (19).

**Chromatographic Fractionation of Extract—** Yeast strain YPH/FTB1.HIS (21) was precultured in complete synthetic medium lacking uracil and diluted with 10 volumes of YPD, followed by incubation at 30°C in 10-liter batches in fermenters until the density had reached \( 1 \times 10^8 \) cells/ml. Cells were harvested by centrifugation and extracted as described in Ref. 22 and the buffer conditions described in Ref. 26. Buffers A, B, C, and D were as described in Ref. 26. All the column fractionation steps were carried out at 4°C, and all of the dialysis steps were made at 4°C.

**The extract** (Fraction I; 1,200 ml) was clarified by centrifugation (100,000 \( \times \) g for 60 min) and dialyzed against 10 liters of buffer A for 14 h. The ionic strength of the dialysate was adjusted to 120 mM KOAc with buffer A before it was applied onto a column of Bio-Rex 70 (5 \( \times \) 10.2 cm; 200 ml matrix total) equilibrated in buffer A + 120 mM KOAc. The column was washed with 600 ml each of buffer A + 120 mM KOAc and buffer A + 200 mM KOAc, before the bound proteins were eluted with buffer A + 600 mM KOAc, collecting 10-ml fractions. The Bio-Rex 70 protein pool (Fraction II; 120 ml) was dialyzed against 2 liters of buffer B + 100 mM KOAc for 14 h and then applied onto a DEAE-Sephacel column (2.5 \( \times \) 10.2 cm; 50 ml matrix total) equilibrated in buffer B + 100 mM KOAc. After loading, the DEAE column was washed with 100 ml of buffer B + 100 mM KOAc, 150 ml of buffer B + 200 mM KOAc, and then eluted with buffer B + 500 mM KOAc, collecting 4-ml fractions. The protein pool (Fraction III; 30 ml) from the 500 ml KOAc wash was dialyzed against 1 liter of buffer C without EDTA for 14 h. The dialysate was clarified by centrifugation (20,000 \( \times \) g, 15 min) and applied onto a column of hydroxyapatite (Bio-Gel HTP from Bio-Rad; 1.6 ml matrix total) equilibrated in buffer C + 200 mM KOAc, and which was developed with a 120-mM gradient from buffer C to buffer D, collecting 2.4-ml fractions. The hydroxyapatite fractions were screened by immunoblotting for the Rad1, Rad2, Rad4, Rad10, and Rad4 proteins.

**Affinity Chromatography on Nickel-Agarose—** The fractions (2 ml each) were screened by immunoblotting to locate Rad1, Rad4, Rad10, and Rad14 proteins. The column was developed with a 90-ml gradient of 50–500 mM KCl in buffer K, collecting 2.5-ml fractions; the Rad14 protein peak was located by Coomassie Blue staining after electrophoresis of the fractions in 10% SDS-polyacrylamide gels. Rad14 protein eluted from S-Sepharose at \(-270 \) mM KCl, the pool of which (Fraction III; 7.5 ml) was applied onto a Bio-Gel HT column (1 \( \times \) 2.5 cm; 2 ml matrix total; purchased from Bio-Rad) equilibrated in buffer K + 100 mM KC1 and developed with a 40-ml gradient of 0–280 mM KH$_2$PO$_4$ in the same buffer. The pool of Rad14 protein (Fraction IV; 2 ml), eluting at \(-100 \) mM KH$_2$PO$_4$, was diluted with 4 ml of 10% glycerol and then fractionated in a Mono S column (HR5/5) with a 40-ml, 100–400 mM KCl gradient in buffer K. The Rad14 protein eluted from Mono S at \(-200 \) mM KCl, the pool of which (Fraction V; 16 ml) was concentrated to 1 ml in a Centricon-30 microconcentrator. The final yield of nearly homogeneous Rad14 protein (16) was 1.2 mg.

**Binding of Purified Rad Proteins to Nickel-Agarose**—Purified Rad1, Rad10, and Rad14 proteins, 10 \( \mu \)g each in 0.6 ml of buffer E, were mixed individually with 0.3 \( \mu \)g of nickel-agarose in an Eppendorf tube for 3 h at 4°C, washed and then centrifuged in a microcentrifuge. The supernatant, the nickel matrix was washed in the Eppendorf tube with 600 \( \mu \)l each of 10, 20, 30, 40, and 100 mM imidazole in buffer E. The various fractions were subjected to immunoblotting to examine their content of the Rad proteins.

**Molecular Sizing Analyses—** In the experiment described in Fig. 2B, the 20 mR and 30 mR imidazole washes were combined and concentrated to 0.6 ml in a Centricon-30 concentrator (Amicon) and subjected to sizing analysis in a column of Sephacryl-300 HR (1 \( \times \) 42 cm; total 33 ml) in buffer F (20 \( \mu \)g Tris acetate, pH 7.7, 20% glycerol, 0.2 mM EDTA, and 0.01% Nonidet P-40) containing 100 mM KOAc, at a flow rate of 0.1 ml/min. The fractions (0.5 ml each) were subjected to immunoblot analyses to locate TFIIH and Rad1, Rad10, and Rad14 proteins.

In the experiment described in Fig. 4A, purified Rad14 protein, 6.9 \( \mu \)g or 160 pmol, in 400 \( \mu \)l of buffer F also containing 60 \( \mu \)g of BSA was applied onto a column of Sephacryl-S-200 HR (1 \( \times \) 42 cm; total 33 ml) in buffer F containing 100 mM KOAc, at a flow rate of 0.1 ml/min and collecting 0.5 ml fractions. In the experiment described in Fig. 4B, 20 \( \mu \)g of Rad1 and 160 pmol of Rad14 were subjected to sizing analysis in the S-200 HR using the same conditions described for Rad14 protein. The fractions were subjected to immunoblot analyses to locate Rad1, Rad10, and Rad14 proteins.

**Immunobeads—** Affinity-purified anti-Rad1, anti-Rad10, and anti-Rad51 antibodies in phosphate-buffered saline (15 mM NaH$_2$PO$_4$, pH 7.2, 150 mM NaCl), 2 mg each, were mixed with 0.3 ml of protein A-agarose (Bio-Rad) for 10 h to bind \( >95\% \) of the antibodies. After dialysis was mixed gently at 4°C on a rotating platform with 1.0 ml of nickel NTA-agarose (Qiagen) for 3 h, transferred into a glass column with an internal diameter of 0.6 cm, and washed sequentially with 4 ml of 10, 20, 30, 40, and 100 mM imidazole in buffer F. A portion of the various fractions was boiled in SDS sample buffer and run in 8% denaturing polyacrylamide gels, followed by either staining with Coo-
washed the beads with 10 ml of 0.1 M sodium borate, pH 9.0, the bound antibodies were cross-linked covalently to the beads using dimethylpimelimidate as described previously (19). The immunobeads were washed with phosphate-buffered saline and stored at 4°C.

In Vitro Transcription and Translation—A 1.77-kb Sp6-Xbal fragment containing the RAD14 gene was placed downstream of the T7 promoter in the vector pCRII to generate plasmid pFR14.12. To obtain 35S-labeled Rad14 protein, 10 μg of pFR14.12 DNA was incubated with 200 μl of TNT reticulocyte lysate (Promega) for 90 min at 30°C in the presence of 200 μCi of [35S]methionine (1,000 Ci/mmol; Amersham Corp.) and T7 RNA polymerase in a final volume of 400 μl, following the instructions of the vendor (Promega). To precipitate radiolabeled Rad14 protein, 400 μl of 4 M ammonium sulfate was added to the translation mix, followed by a 30-min incubation on ice. The protein precipitate was harvested by centrifugation (20,000 × g, 30 min), dissolved in 400 μl of buffer G (20 mM potassium HEPES, pH 7.5, 75 mM KCl, 20% glycerol, 5 mM sodium bisulfite, 4 mM MgCl2, 0.5 mM EDTA, 1 mM DTT, 0.1% Tween 20, 10% glycerol, and 100 μg/ml BSA), concentrated to 60 μl in a Centricon-30 microconcentrator (Amicon), diluted with 1 ml of buffer G, and reconstituted to 100 μl in the same concentrator. The final protein solution was used in immunoprecipitation experiments as described below.

Immunoprecipitation—In the experiment described in Fig. 2B, the Sephacryl S-300 fractions 36–40 were combined, and 0.3 ml of the pool was mixed gently on a rotary platform for 2 h at 4°C with 10 μl of protein A-agarose beads coated with antibodies specific for Rad1, Rad10, Rad14, or Rad51 or with protein A-agarose alone. The beads were washed as described above and then treated with 2% SDS and the eluates, 2 μl each, were analyzed by immunoblotting for their content of Rad1, Rad10, and Rad14 (Fig. 3). In the experiment described in Fig. 4B, the Sephacryl S-500 fractions 26–30 were combined and 0.3 ml of the pool was mixed with protein A-agarose beads bearing antibodies specific for Rad1, Rad10, Rad14, or Rad51 or with protein A-agarose alone. The beads were washed as described above and then treated with 2% SDS, and the eluates, 2 μl each, were analyzed by immunoblotting (Fig. 4C). In the experiment described in the legend to Fig. 5, Rad1 (1.25 μg or 10 pmol), Rad10 (0.25 μg or 10 pmol), and the Rad1-Rad10 complex (1.5 μg or 10 pmol) were incubated for 12 h on ice with 10 μl of the radiolabeled Rad14 protein solution in a final volume of 30 μl in buffer F containing 100 mM KOAc, 1 mM DTT, and 10 μg/ml BSA. After adjusting the volume of the reaction mixtures to 150 μl with buffer F, 10 μl of the appropriate Immunobeads were added, followed by gentle mixing for 2 h at 4°C. The beads were washed twice with buffer G, and the bound proteins were eluted with 15 μl of 2% SDS for 5 min at 37°C. The SDS eluates, 3 μl each, were run in 9% SDS-polyacrylamide gels, which were dried and subjected to fluorography to reveal the content of radiolabeled Rad14.

NER in Vivo—For use in the NER reaction, M13mp18 DNA (>90% supercoiled form) was irradiated with a UV dose of 400 J/m2. For the time course experiment described in the legend to Fig. 6, the in vitro repair reaction, 60 μl in final volume, was assembled in buffer R (45 mM potassium HEPES, pH 7.9, containing 6 mM MgCl2, 120 μM MgCl2, 1.5 mM DTT, 2 mM ATP, and an ATP-regenerating system consisting of 30 mM creatine phosphate and 250 ng of creatine kinase and also 60 mM potassium acetate and 25 mM KCl due to the addition of various purified protein factors (16). To carry out the repair reaction, 600 ng of TFIIH, 300 ng of RPA, 100 ng of Rad2 protein, 100 ng of the Rad4-Rad23 complex were preincubated at 25°C for 10 min, followed by the incorporation of 600 ng of UV-irradiated M13 DNA, and Rad1 (100 ng), Rad10 (3 ng), Rad14 (34 ng) proteins added either as the pre-assembled Rad1-Rad10-Rad14 complex in 1 μl or as the pre-assembled Rad1-Rad10 complex in 1 μl plus free Rad14 in 1 μl. The complete reaction mixtures were transferred to 30°C, and a 10-μl portion was withdrawn at various times and treated with 0.5% SDS and 200 μg/ml proteinase K at 37°C for 5 min to deproteinize the reaction mixtures. Deproteinized samples were run in 0.8% agarose gels in TAE buffer (20 mM Tris acetate, pH 7.4, 0.5 mM EDTA), which were stained with ethidium bromide (1 μg/ml) for 60 min and destained in H2O for 3 h before being photographed with Polaroid type 55 films through a red filter. The photographic negatives were analyzed in a Bio-Rad GS-670 imaging densitometer to obtain data points for a graphical representation of the results.

Fig. 1 Affinity binding of TFIIH to nickel NTA-agarose. A, fractionation scheme used. Details for the chromatographic steps are given under "Materials and Methods." B, the hydroxyapatite pool (ST) containing TFIIH, Rad1, Rad10, and Rad14 proteins was mixed with nickel-agarose at 4°C for 150 min and then centrifuged to collect the matrix. After decanting the supernatant (SP), the nickel matrix was transferred to a column and washed with 10, 20, 30, 40, and 100 mM imidazole to elute the bound proteins. The different fractions were run along with molecular size markers (M) in 9% denaturing polyacrylamide gels, which were either stained with Coomassie Blue in B or subjected to immunoblotting with the indicated anti-purified antibodies in C. The Rad1, Rad25, and TFB1 subunits of TFIIH in the 100 mM imidazole wash are marked in B.

RESULTS

Lack of Physical Association of TFIIH with Various NER Factors—To examine whether TFIIH is physically associated with the Rad1, Rad4, Rad10, and Rad14 proteins, we used cell extract prepared from yeast strain YPH/TB1.6HIS in which a 6-histidine tag has been added to the C terminus of the TFB1 subunit of TFIIH (17). After high speed centrifugation, the yeast extract was fractionated by stepwise elutions with potassium acetate in columns of Bio-Rex 70 and DEAE-Sepharose to enrich for TFIIH and the other NER factors (Fig. 1A). The 500 μl acetate wash from the DEAE-Sepharose step was dialyzed and fractionated in a hydroxyapatite (HAP) column using a potassium phosphate gradient from 20 to 300 mM, and the 50 fractions collected were subjected to immunoblotting with antibodies specific for various TFIIH subunits (Rad3, Rad25, TFB1, and SSL1) and for the Rad1, Rad4, Rad10, and Rad14...
proteins. The peak of TFIIH was found in fractions 21–27, while Rad1, Rad10, and Rad14 proteins co-eluted in fractions 15–23, and Rad4 protein was found later in the gradient in fractions 28–36. HAP fractions 21–27 containing the peak of TFIIH and ~30% of the total Rad1, Rad10, and Rad14 proteins, but a negligible amount of Rad4 protein, were pooled and dialyzed. The presence of the 6-histidine tag on TFB1 allowed the immobilization of TFIIH on nickel NTA-agarose, to which proteins containing a 6-histidine sequence bind avidly and from which they can be eluted by imidazole. The dialysate from the HAP TFIIH pool was mixed with nickel-agarose, and the bound proteins were eluted from the matrix in a stepwise fashion with 10, 20, 30, 40, and 100 mM imidazole (Fig. 1A). The starting material and various other fractions were run in denaturing polyacrylamide gels, which were then either stained with Coomassie Blue to examine the protein makeup (Fig. 1B) or probed for the content of TFIIH and of Rad1, Rad10, and Rad14 proteins by immunoblotting (Fig. 1C). Rather surprisingly, staining of the gel revealed that a great number of proteins present in the starting material in fact bound to the nickel-agarose and that the bulk of these proteins were eluted from 10 to 30 mM imidazole, with only a small fraction of the bound proteins eluting at 40 and 100 mM imidazole (Fig. 1B). It is clear that the great majority, or all, of the less tightly retained proteins eluted by 10–30 mM imidazole bound nonspecifically to the nickel matrix rather than bound through an interaction with TFIIH, because (i) the relatively minute amount of TFIIH present in the output (<0.4% of total protein) could not have accounted for the retention of such a large body of proteins, (ii) the bulk of TFIIH was eluted later in the 40 and 100 mM imidazole washes (see below), thus arguing strongly against association of the loosely retained proteins with the transcription factor, and (iii) importantly, purified Rad proteins that do not contain a 6-histidine tag bind nickel-agarose by themselves (see below).

Densitometric analysis of the immunoblot in Fig. 1C revealed that essentially all (>95%) of the TFIIH in the output was immobilized on the nickel-agarose, and that ~15% and ~75% of the bound TFIIH was eluted by 40 mM and 100 mM imidazole, respectively, consistent with specific binding of this fraction of the transcription factor to the nickel matrix through the 6-histidine tag on TFB1. Approximately 3 and 7% of the total TFIIH was found in the 20 and 30 mM imidazole washes, respectively, which might correspond to immobilization of TFIIH through nonspecific interaction of one or more of the subunits of TFIIH with the nickel matrix. Essentially all (>95%) of Rad1, Rad10, and Rad14 proteins in the starting material also bound to the nickel-agarose, and they were co-eluted from 10 to 30 mM imidazole, with 3, 48, and 47% of the total found in the three washes with increasing imidazole, respectively. Considering the large number of proteins in the output that bound nonspecifically to nickel-agarose (Fig. 1B), most plausibly, the Rad1-Rad10 complex and Rad14 protein were also retained via nonspecific interactions with the matrix. However, though highly unlikely, it could not be formally excluded that the bound Rad1, Rad10, Rad14 were associated with the small amount of TFIIH found in the 10–30 mM imidazole washes (Fig. 1B).

To establish whether or not there was a physical association between Rad1, Rad10, and Rad14 with TFIIH, we conducted the following two experiments. First, purified Rad1, Rad10, Rad14 proteins, all greater than 95% homogeneous (12, 16, 23), were mixed individually with nickel-agarose, which was then eluted with increasing concentrations of imidazole. The various fractions with increasing imidazole were subjected to immunoblotting, which indicated that, with the exception of Rad10 protein, Rad1 and Rad14 proteins by themselves bind quantitatively to nickel-agarose, with the bulk of the bound proteins eluting from 10 to 40 mM imidazole in both instances (Fig. 2A). In fact, we found that bovine serum albumin also binds nickel-agarose and is eluted from 10 to 20 mM imidazole (data not shown). Second, the 20 and 30 mM imidazole washes from the nickel-agarose step (Fig. 1, B and C) were combined, concentrated to a small volume, and subjected to molecular sizing in a column of Sephacryl S-300 HR as described under "Materials and Methods." The column output (OP) and fractions 24–48 from the column were subjected to immunoblotting to locate TFIIH and Rad1, Rad10, and Rad14 proteins.

A Complex of Rad1-Rad10-Rad14 Proteins—Since Rad1 and Rad10 are known to exist as a complex (1), it was not unexpected that the two proteins should co-elute from the nickel-agarose (Fig. 1C) and during the sizing analysis in the S-300 HR column (Fig. 2B). It was of particular interest, however, that Rad14 protein (M, 43,000) co-eluted with the Rad1-Rad10 complex (combined M, 150,700) from the S-300 molecular siz-
Rad14 protein (stably with the Rad1-Rad10 complex in the absence of any other NER component. Since the majority of the Rad14 protein remained associated with the Rad1-Rad10 protein in the S-200 column fractions over the course of >24 h (Fig. 4C), the kinetic constant for dissociation (K_d) of the Rad1-Rad10-Rad14 complex is at or below the concentration of 5.3 × 10^{-8} M of the proteins present in the S-200 column fractions.

Incorporation of Rad14 Requires the Rad1-Rad10 Complex—In principle, association of Rad14 protein with the Rad1-Rad10 complex can be realized by physical interaction of Rad14 with Rad1 or Rad10 alone, with both Rad1 and Rad10 separately, or with the Rad1-Rad10 complex. To distinguish among these possibilities, we made use of radiolabeled Rad14 protein obtained by in vitro translation of RAD14 mRNA in the presence of [35S]methionine. The translated Rad14 protein was enriched from the rabbit reticulocyte lysate by ammonium sulfate precipitation and subjected to dialysis in a Centricon-30 microconcentrator to remove residual ammonium sulfate. The 35S-labeled Rad14 protein was incubated with the same molar amount of Rad1, Rad10, or the Rad1-Rad10 complex on ice overnight to allow for protein-protein interactions to occur and then subjected to immunoprecipitation with protein A-agarose beads bearing anti-Rad1 or anti-Rad10 antibodies and, as control, also with protein A-agarose beads bearing anti-Rad51 antibodies. The various immunoprecipitates were washed and then treated with 2% SDS to elute the bound proteins. The various eluates were analyzed by immunoblotting for their content of the three Rad proteins.

As shown in Fig. 5, Rad1 protein alone interacts with Rad14 protein, as indicated by the fact that ~12% of the input Rad14

![Figure 3](image-url)  
**FIG. 3.** Rad14 is physically associated with the Rad1-Rad10 complex. Sephacryl S-300 HR fractions 36–40 containing the peak of the Rad1, Rad10, and Rad14 proteins were combined and a portion of the pool was mixed with protein A-agarose (lane 1) or with protein A-agarose bearing covalently conjugated anti-Rad1 (α1; lane 2), anti-Rad10 (α10; lane 3), anti-Rad14 (α14; lane 4), or anti-Rad51 (α51; lane 5) antibodies. After washing, the immunoprecipitates were treated with 2% SDS to elute the bound proteins, and the eluates were subjected to immunoblotting to examine their content of the Rad proteins.

![Figure 4](image-url)  
**FIG. 4.** Assembly of a Rad1-Rad10-Rad14 complex in vitro. A, purified Rad14 protein was subjected to sizing analysis in Sephacryl S-200 HR. The column output (OP) and fractions 20–48 from the column were analyzed by immunoblotting for their content of Rad14. B, purified Rad1, Rad10, and Rad14 proteins were incubated for 12 h on ice and then subjected to sizing analysis in the same S-200 HR column. The column output (OP) and fractions 20–48 from the column were analyzed by immunoblotting for their content of the three Rad proteins. C, the S-200 HR fractions 26–30 described in B that contained the peak of the Rad1-Rad10-Rad14 complex were combined and a portion of the pool was mixed with protein A-agarose (lane 1) or with protein A-agarose bearing anti-Rad1 (α1; lane 3), anti-Rad10 (α10; lane 4), anti-Rad14 (α14; lane 5), or anti-Rad51 (α51; lane 2) antibodies. The immunoprecipitates were washed and then treated with 2% SDS to elute the bound proteins. The various eluates were analyzed by immunoblotting for their content of the three Rad proteins.
co-precipitated with the added Rad1 protein by the anti-Rad1 immunobeads (lane 3), but not by the same immunobeads in the absence of Rad1 protein (lane 2), nor by beads bearing anti-Rad51 antibodies in the presence of Rad1 (lane 1). By contrast, less than 2% of the input Rad14 co-precipitated with Rad10 protein (Fig. 5, lanes 4 -6), thus indicating a much weaker interaction of Rad14 with Rad10 than with Rad1. Interestingly, we found that greater than 70% of the input Rad14 protein co-precipitated with the Rad1-Rad10 complex by either anti-Rad1 or anti-Rad10 immunobeads (Fig. 5, lanes 7-9). Thus, the amount of Rad14 that co-precipitated with the Rad1-Rad10 complex was much greater (~5-fold) than the sum of Rad14 protein that co-precipitated with Rad1 and Rad10 singly. The finding that Rad14 combines with the Rad1-Rad10 complex much more efficiently than with either Rad1 or Rad10 protein alone was verified in two other independent experiments (data not shown). Taken together, our results indicate that the Rad1-Rad10-Rad14 complex is the physiologically relevant entity for interaction with Rad14.

Rad1-Rad10-Rad14 Complex Is the Functional Entity in NER—In our in vitro reconstituted system for NER (16), incision of DNA damaged by ultraviolet light requires the Rad1-Rad10 nuclease, the Rad2 nuclease, the complex of Rad4-Rad23, Rad14, TFIIH, and RPA. In the standard reaction, these protein factors are preincubated together to allow for necessary protein-protein interactions to occur, before the addition of the damaged DNA substrate (16). ATP-dependent incision of the damaged DNA substrate is evidenced by conversion of the supercoiled form of the damaged DNA to the open circular form as assayed by agarose gel electrophoresis, followed by staining of the DNA species with ethidium bromide. Excision of the DNA damage is revealed by labeling the excision DNA fragments (24-27 nucleotides) with [α-32P]dATP and calf thymus terminal transferase, followed by electrophoresis in DNA sequencing gels and autoradiography to visualize the labeled fragments (16).

Since the agarose gel method (16; see “Materials and Methods”) represents a highly sensitive means for determining the initial rate of incision of the UV-damaged substrate, we used it to examine the relevance of the Rad1-Rad10-Rad14 complex to the damage specific incision reaction. To do this, the standard protocol was modified so that all the protein components except for Rad1, Rad10, and Rad14 were still subjected to preincubation, and then the latter three proteins were added, together with the DNA substrate, either as the Rad1-Rad10-Rad14 complex plus free Rad14 or as pre-assembled Rad1-Rad10-Rad14 complex. The incision reaction was then allowed to proceed at 30°C and terminated at 3, 6, 9, 12, and 15 min by adding SDS and proteinase K. The reaction samples were run in an agarose gel and then stained with ethidium bromide to reveal the proportions of open circular and supercoiled forms in the various samples. The gel in Fig. 6A was also subjected to image analysis to obtain data points for a graphical representation of the results (Fig. 6B). As reported in our recent work (16) and shown here in Fig. 6, ATP-dependent incision of the UV-damaged plasmid DNA occurred, as indicated by the accumulation of the open circular form with increasing reaction time. Importantly, a higher level of damage specific incision occurred when Rad1, Rad10, and Rad14 were added as a preformed complex than when they were added as the Rad1-Rad10 complex and free Rad14 (Fig. 6, A and B). This observation was confirmed in two other independent experiments. Thus, complex formation between Rad1-Rad10 nuclease and Rad14 represents an important step that has a direct influence on incision proficiency.

**FIG. 5.** The Rad1-Rad10 complex is the molecular entity for Rad14 recruitment. [35S]-Labeled Rad14 protein obtained by in vitro translation was incubated with Rad1 protein (lanes 1 and 3), with Rad10 protein (lanes 4 and 6), with the Rad1-Rad10 complex (lanes 7-9), or without any added protein (lanes 2 and 5) and then subjected to immunoprecipitation with protein A-agarose beads bearing anti-Rad1 (α1; lanes 2, 3, and 8), anti-Rad10 (α10; lanes 5, 6, and 9), or anti-Rad51 (α51; lanes 1, 4, and 7). Proteins were eluted from the immunoprecipitates with 2% SDS and then subjected to SDS-polyacrylamide gel electrophoresis and fluorography to reveal the content of radiolabeled Rad14 in each case.

**DISCUSSION**

TFIIH Is Not Physically Associated with Rad1, Rad4, Rad10, and Rad14—We initiated this work on the premise that if TFIIH and various Rad proteins were physically associated, we
would see co-purification of the transcription factor with the latter. To determine whether there was physical association of the NER factors, we subjected cell extract from a yeast strain in which the TFB1 subunit of TFIIH bears a 6-histidine tag at its C terminus (17) to initial fractionation on Bio-Rex 70 and DEAE-Sepharose. The smallest possible volume of column matrices for quantitative binding of TFIIH and various Rad proteins and step elutions of bound proteins were employed in these initial column steps in order to maintain a high protein concentration that may be necessary to preserve physical complexes of lesser stability. The great majority of TFIIH, Rad1, Rad4, Rad10, and Rad14 proteins present in the cell extract was recovered in the DEAE pool, representing ~100-fold enrichment of these protein factors. When the DEAE pool was subjected to gradient fractionation in hydroxyapatite, we found that (i) Rad1, Rad10, and Rad14 proteins eluted earliest from the column at the same position and the peak of these Rad proteins partially overlapped the peak of TFIIH, which eluted later, and that (ii) Rad4 protein eluted later than TFIIH and away from TFIIH. The second observation indicated that there was no physical association of Rad4 with TFIIH or with Rad1, Rad10, and Rad14. Because of the overlap noted in (i) above, the pool of TFIIH from hydroxyapatite contained ~30% of the total Rad1, Rad10, and Rad14 proteins.

The presence of the 6-histidine tag in the TFB1 protein allowed the immobilization of TFIIH and, theoretically, any protein that is physically associated with TFIIH on nickel-agarose. To test whether the presence of Rad1, Rad10, and Rad14 proteins in the hydroxyapatite TFIIH pool was due to a simple partial overlap of the peaks of the Rad proteins and TFIIH or actually reflected a physical association between the Rad proteins and TFIIH, we mixed the TFIIH pool with nickel agarose and then washed the nickel matrix with increasing concentrations of imidazole to elute the bound proteins. From examining the protein makeup and content of TFIIH and Rad proteins in the various fractions (Fig. 1, B and C), it was clear that (i) a large number of the protein species in the starting material bound nonspecifically to the nickel matrix and were eluted from 10 to 30 mM imidazole, (ii) the bulk of TFIIH bound specifically through the 6-histidine tag to the nickel matrix and was eluted by 100 mM imidazole; the 100 mM imidazole eluate was ~50-fold enriched in TFIIH, but contained no Rad1, Rad10, and Rad14 proteins, and (iii) a small fraction (~10%) of TFIIH and almost all (~95%) of the Rad1, Rad10, and Rad14 were found in the 20 and 30 mM imidazole eluates. The co-existence of the three Rad proteins and TFIIH in the 20 and 30 mM imidazole washes as noted in iii was due to fortuitous, nonspecific interaction of these protein factors with the nickel matrix as indicated in two additional experiments. First, the three Rad proteins were well separated from TFIIH when the 20 and 30 mM imidazole washes were combined and subjected to molecular sizing in Sephacryl S-300 HR (Fig. 2B). This sizing analysis demonstrated clearly that TFIIH was not physically associated with the Rad proteins in these imidazole fractions, because if they had been associated, precise co-elution of these factors would have been seen. Second, when purified Rad1 and Rad14 proteins that do not possess a 6-histidine tag were mixed with nickel agarose in the absence of any other protein component, quantitative nonspecific binding of these proteins to the matrix occurred (Fig. 2A). Because Rad10 protein by itself does not show any affinity for nickel-agarose (Fig. 2A), its retention on nickel-agarose (Fig. 1) was effected via its association with Rad1 and Rad14 proteins (see below), both of which bind the nickel matrix (Fig. 2A). Taken together, our results indicate that TFIIH is not physically associated with any of Rad4, Rad1, Rad10, and Rad14 proteins in a stable form that can be isolated by column chromatography.

Our results differ from the recent report (21) that TFIIH, Rad1, Rad4, Rad10, and Rad14 proteins existed in a physical complex, which the authors called "repairosome." In deriving their conclusion, these workers used the same yeast strain as was used in our work and observed that when a phosphocellulose column fraction enriched in TFIIH (TFB1-6His) and Rad proteins was mixed with nickel-agarose, retention of Rad1, Rad10, Rad14, and other Rad proteins occurred and that all of the bound Rad proteins were eluted by lower concentrations of imidazole than was needed to elute the bulk of TFIIH. Svejstrup et al. (21) also subjected the 20 mM imidazole eluate from the nickel-agarose step that contained Rad proteins and some TFIIH to molecular sizing in a column of Sepharose CL-2B and found co-elution of these factors. However, our studies indicate that Rad1 and Rad14 proteins bind nickel-agarose by themselves. Also, since the molecular mass of TFIIH and that of the other NER proteins, as well as the size differences between the former and latter, are rather insignificant relative to the large pore size of Sepharose CL-2B (exclusion size limit: 4 x 10^7 daltons), all of these protein factors will be retarded to very similar degrees in the Sepharose matrix, resulting in poor separation of factors. In fact, when we subjected the combined 20 and 30 mM imidazole eluates from nickel-agarose to sizing in Sepharose CL-4B, which has a smaller pore size (exclusion size limit: 2 x 10^7 daltons) than that of Sepharose CL-2B, even then the poor resolution of TFIIH from the Rad1-Rad10-Rad14 complex resulted in substantial overlap of the peaks of the two entities, creating the impression that they might be physically associated (data not shown). For this reason, we selected Sephacryl S-300 HR (exclusion size limit: 1.5 x 10^7 daltons) in our study (Fig. 2B) for effective separation of TFIIH from the Rad1-Rad10-Rad14 complex. In summary, our results do not support the premise that TFIIH is physically associated with all the essential NER proteins in a "repairosome."

Rad1-Rad10-Rad14 Proteins Exist as a Complex—In molecular sizing, while purified Rad14 protein behaved as a monomer, Rad14 protein in the imidazole eluates from nickel agarose co-migrated with the Rad1-Rad10 complex at a much earlier position than free Rad14. The Rad14 protein was co-immunoprecipitated with Rad1 and Rad10 proteins from fractions containing the peak of these proteins, indicating that it was physically associated with the Rad1-Rad10 complex (Figs. 2B and 3). We have shown that a complex of Rad1-Rad10-Rad14 that can be isolated in a sizing column and by immunoprecipitation is formed upon incubation of purified components (Fig. 4, B and C). And, in the in vitro NER system, a higher level of incision of UV damaged DNA is afforded by the pre-assembled Rad1-Rad10-Rad14 complex than by the mixture of Rad1-Rad10 complex and free Rad14 (Fig. 6).

To gain insight into how assembly of the Rad1-Rad10-Rad14 ternary complex occurs, 35S-labeled Rad14 protein obtained by in vitro coupled transcription-translation was incubated with Rad1, with Rad10, or with the Rad1-Rad10 complex. In each case, the amount of Rad14 that associated with the other component was determined by a combination of immunoprecipitation, gel electrophoresis, and fluorography. We found that Rad14 interacts directly with Rad1 protein but very weakly with Rad10 protein. Importantly, a much higher level of Rad14 was bound by the Rad1-Rad10 complex than could be accounted for by the sum of Rad14 amounts that were found associated with free Rad1 and Rad10 proteins (Fig. 5). These observations indicate that formation of the Rad1-Rad10-Rad14 ternary complex is mediated via interaction of Rad14 with the Rad1-Rad10 binary complex. It remains to be determined whether Rad14 protein makes substantial contacts with both
Rad1 and Rad10 in the ternary complex, or when present in the Rad1-Rad10 binary complex, Rad1 protein adopts a conformation that is conducive for binding Rad14.

The human Rad1 homolog XPF associates with the Rad10 homolog ERCC1, and like Rad1-Rad10, the XPF-ERCC1 complex is a DNA endonuclease (27). ERCC1, and the XPA protein, which is the counterpart of Rad14, were shown to interact in a yeast two-hybrid system (28), and ERCC1 protein was found to bind to amylose-agarose containing a hybrid polypeptide of maltose-binding protein-XPA (28, 29). The results with ERCC1 and XPA proteins are not necessarily incompatible with our finding that Rad10 interacts with Rad14 only very weakly, because the two-hybrid system and the affinity binding method used in detecting ERCC1-XPA interaction are designed to reveal even weak and transient protein-protein interactions. Our results would suggest that XPA exists as a complex with ERCC1 and XPF proteins in vivo and that XPF has a major role in the formation of this complex.

Functional Protein Assemblies in NER—Based on results from this work and from previous studies, we can begin to formulate a framework for understanding the roles of various NER protein complexes in the recognition and incision of damaged DNA. Here, we have shown that Rad1-Rad10-Rad14 exist in a physical complex that we designate as nucleotide excision repair factor-1 or NEF-1 (Table I). Rad1 and Rad10 together constitute an endonuclease (12, 13), and both of these proteins bind DNA but show no preference for UV damaged DNA (12, 23). The association of the damage recognition factor Rad14 (9) provides an efficient means for targeting the Rad1-Rad10 endonuclease to the site of DNA damage. The Rad4 and Rad23 proteins exist in a tight complex, which we have purified to near homogeneity and shown to be indispensable for NER (16). Rad4 protein interacts in vitro with TFIIH (30), and Rad23 protein interacts with Rad10 protein and TFIIH (15). These observations suggest that the Rad1-Rad23 complex, that we designate here as NEF-2 (Table I), may act primarily as a molecular tether that connects NEF-1 to TFIIH at the damage site. TFIIH contains two DNA helicases, Rad23 and Rad23, that are both indispensable for NER (5, 10, 31, 32). In addition, the Rad3 protein shows ATP-dependent binding to UV-damaged supercoiled DNA (11). Upon incorporation of TFIIH via its interaction with NEF-2 and the ability of Rad3 to bind the DNA damage, the combined helicase function of Rad3 and Rad23 could catalyze localized unwinding of the damaged duplex, creating a single-stranded region for the loading of RPA, which has high affinity for single-stranded DNA and that may help stabilize the unwound region. Human RPA interacts with XPA and XPG (33, 34), which represent the human counterparts of Rad14 and Rad2 proteins, respectively. An interaction of yeast RPA with Rad14 protein may facilitate the entry of RPA into the repair complex. Rad2 protein has DNA endonuclease activity, but has no affinity for damaged DNA (14). Rad2 interacts in vitro with TFIIH (30) and, as noted above, possibly with RPA. These interactions of Rad2 could effect its placement at the damage site to complete the assembly process, resulting in the dual incision of the damaged DNA strand.

While the order and the manner by which the NER machinery is assembled at the DNA damage site remains to be determined, our studies provide support to the notion that NER involves the sequential assembly of different protein factors at the damage site, rather than the placement of a "preformed" repairosome containing all the factors required for incision.

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TABLE I
Functional subassemblies in NER

| Repair factor | Components | Function or activity |
|---------------|------------|----------------------|
| NEF1          | Rad1 (XPF), Rad10 (ERCC1), Rad14 (XPA) | DNA damage recognition, DNA endonuclease |
| NEF2          | Rad1 (XPF), Rad23 (HR23) | Promote assembly of NEF1 with TFIIH |
| TFIIH         | Rad2 (XPD), Rad25 (XPB), TFB1, SSL1, p55, p38 | DNA helicase, DNA damage recognition |
| RPA           | p69, p36, p13 | DNA binding |
| Rad2          | Rad2 (XPG) | DNA endonuclease |

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