BIOCHEMICAL AND GENETIC ANALYSIS OF EXCISION DNA REPAIR

by

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A thesis submitted in conformity with the requirements for
the Degree of Doctor of Philosophy
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in the University of Toronto

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A thesis submitted by Johnson M. S. Wong
for the Degree of Doctor of Philosophy (1999)
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ABSTRACT

The integrity of the genetic material is constantly threatened by various DNA damaging agents. Hence, multiple DNA repair pathways have evolved to eliminate damage of different origins. Nucleotide excision repair (NER) is the primary mechanism by which both *S. cerevisiae* and human cells remove lesions, including those induced by ultraviolet light, that usually cause severe distortions of the DNA helix. I have described a simple protocol for preparing yeast whole cell extracts that can support efficient NER to facilitate biochemical studies of this repair pathway in yeast. This assay reflects *bona fide* NER as it depends on *RAD* genes such as *RAD14* and *RAD2*, which are known to be essential for excision repair. Interestingly, the assay was also dependent on the *RAD7* and *RAD16* genes, whose precise roles in NER are uncertain. Using this system, I also demonstrated that yeast replication protein A (Rpa), encoded by the *rfa2* gene, is required for NER, since *rfa2* mutations which confer enhanced UV sensitivity *in vivo* also resulted in a deficiency in NER *in vitro*. These data indicate that Rpa is an essential component of the NER machinery in *S. cerevisiae*, as it is in mammalian cells. Nevertheless, not all protein-protein interactions appear to be conserved in these two highly homologous systems. I showed that yeast Rpa could interact with the acidic domain of Rad2, as was the case for XPG, the human counterpart of Rad2, which interacts with human RPA. However, using several different approaches, I failed to detect a significant interaction between yeast Rpa and Rad14. This finding is unexpected because the homologous human proteins, human RPA and XPA, have been demonstrated to interact with each other in several studies, and both yeast and human RPA are required for the early steps of NER.

NER can be divided into two subpathways: global genome repair, which is involved in the repair of both active and inactive genes, and transcription-coupled repair. In the latter subpathway, lesions in the transcribed strands of active genes are more rapidly repaired, but contributions of the transcription machinery to this repair pathway are poorly understood. To determine whether the RNA polymerase elongation factor SII plays a role in excision repair, I examined the effect of deleting the *SII* gene of *Saccharomyces cerevisiae*. Lack of SII activity enhanced UV sensitivity, but only in the absence of *RAD7/16*-dependent global genome repair. This increased UV sensitivity was also seen with RNA polymerase II mutants defective in their response to SII. UV sensitivity was also conferred by other RNA polymerase II mutations that do not affect SII function. Indeed, enhanced UV sensitivity can be achieved simply by decreasing the steady-state level of RNA polymerase II. My results indicate that compromising the activity of yeast RNA polymerase II can increase UV sensitivity when the highly efficient global genome repair pathway is compromised. These data suggest that a primary defect in transcription may underly the phenotypes seen in individuals with Cockayne syndrome, a human disease associated with a defect in transcription-coupled repair.

Another pathway of excision repair, base excision repair (BER), involves DNA glycosylases that recognize and excise specific types of base damage in DNA, including uracil and thymine glycol. Human uracil DNA glycosylase (UDG) was recently found to interact with the 34 kDa subunit of human RPA. To investigate whether RPA plays a role in BER in yeast and human cells, I set up *in vitro* BER systems using yeast and HeLa cell extracts. Both of these systems appear capable of performing efficient BER in the presence of several types of lesions. I fractionated HeLa extracts to deplete endogenous RPA and I will test the ability of these fractions to perform BER. To approach the same question for *S. cerevisiae* cells, extracts will be made from mutant yeast strains with C-terminal deletions in the Rpa2 polypeptide. These truncations span the region of the yeast polypeptide that corresponds to that part of the human polypeptide shown to interact with UDG. These two complementary approaches should provide a means to assess the functions of RPA, if any, in BER.

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When I first joined this laboratory, I thought my Masters training should have prepared me to a certain extent for the long journey of a phD program. I was wrong! In Jim's laboratory, I have learned and experienced so much not only about science, but about life itself that I truly felt that a "Doctor of Philosophy" is literally a well-worded title. Because of Jim's emphasis on creativity and independence, everyone that worked under him including myself is free to pursue and entertain his/her scientific ideas. This degree of freedom is certainly one of the important factors that contribute to the tremendous success enjoyed by many of Jim's students, both during and beyond the time under his supervision. In fact, we are encouraged to venture into fields other than transcription which has been the dominating area of Jim's research until several years ago. This foresight has proved to serve us very well as we moved into the hot and exciting areas of DNA repair. The opportunity for me to pursue projects relating to all these fundamental cellular processes is a true learning and stimulating experience, providing me a unique perspective for appreciating the interconnection between different cellular pathways. Jim's perfectionism certainly does not make life any easier in an already challenging lab. A manuscript is not acceptable until proofread and revised almost 10 times by him and by that time, Jim is basically editing a lot of his own sentences! A normally presentable slide would not be acceptable to Jim until every single "flaw", including small imperfection undetectable to a typical unaided human eye, is corrected. Certainly, it is also this very attitude of perfectionism towards science that makes Jim one of those scientists that goes for "quality" rather than "quantity", an increasingly rare trait considering the current competitive atmosphere of basic biological science. The years in Jim's lab has definitely engendered in me this desire of aiming for the highest standard and vigor in experimentation.

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APPENDIX I: Nucleotide Excision Repair in *Saccharomyces cerevisiae* Whole Cell Extracts

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List of Abbreviations

bp - base pair
DTT - dithiothreitol
EDTA - ethylenediaminetetraacetic acid
EGTA - ethylene glycol-bis[b-aminoiethyl ether] N,N,N',N'-tetraacetic acid
HEPES - N-[2-Hydroxymethyl]piperazine - N'[2-ethanesulphonic acid]
kD - kiloDalton
min - minute(s)
PCNA - proliferating cell nuclear antigen
rpm - revolution per minute
SCID - severe combined immunodeficiency disease
SDS-PAGE - sodium dodecyl sulfate-polyacrylamide gel electrophoresis
ssDNA - single-stranded DNA
SV40 - simian virus 40
Tris - Tris [hydroxymethyl] aminomethane
UV - ultraviolet
XRCC - X radiation cross complementary

CHAPTER I

Introduction

Preface

Both prokaryotic and eukaryotic organisms have evolved several DNA repair pathways to maintain the integrity of their genetic material. Disruption of these pathways can have serious consequences, such as the fixation of mutations and tumorigenesis. The past few years have seen enormous growth in our understanding of DNA repair, leading to the realization that inactivation of various repair pathways can predispose humans to various types of cancer. For instance, mutations in the nucleotide excision repair (NER) pathway are linked to the human diseases of xeroderma pigmentosum and the Cockayne syndromes, whereas mutations in the mismatch repair pathway predispose to a familial form of colon cancer.

Because my thesis research encompassed several repair pathways, I will outline the major repair pathways in the Introduction of my thesis. I will emphasize NER, because it has been the main focus of my work. In Chapter II, I report the development of an *in vitro* NER system using yeast extract and its utilization in demonstrating the role of yeast replication protein A in this repair pathway. In Chapter III, I have used a genetic approach to assess the role of the transcription elongation factor SII and RNA polymerase II in NER *in vivo*. Finally, in Chapter IV, I describe the establishment of *in vitro* systems capable of carrying out base excision repair using both yeast and human extracts. Using these systems, I hope to assess whether there is any role for replication protein A in base excision repair.

I. Overview of DNA Repair Pathways

The genetic material is constantly subject to damage caused by environmental insults, including UV light and chemical mutagens, and toxic metabolic side products such as reactive oxygen species. Furthermore, changes in the genetic information can occur due to errors in the fidelity of DNA replication. Hence, organisms have evolved multiple pathways of DNA repair to process and eliminate effectively different types of DNA lesions and alterations. The four major repair mechanisms that are conserved in various organisms are double-strand break repair, DNA mismatch repair, base excision repair and nucleotide excision repair.

Double-strand break repair safeguards the genome against assault from ionizing radiation, which generates breaks in DNA. DNA breaks can also arise from cellular processes such as meiosis and replication; the latter also generates errors as a result of misincorporation by DNA polymerases. Fortunately, the fidelity of DNA replication is drastically improved by another repair system, DNA mismatch repair, which mainly serves to remove mismatches introduced during replication. The two excision repair pathways differ mainly in the types of DNA damage they recognize. Whereas base excision repair removes relatively small lesions that do not substantially distort the double helix, nucleotide excision repair acts on helix-distorting bulky lesions. Failure to carry out these repair processes due to mutational disruption can cause cellular death or transformation as exemplified in several human disorders, including xeroderma pigmentosum and hereditary nonpolyposis colorectal cancer. Because each of these four repair pathways will be discussed in detail in later sections, I will briefly describe here several other alternative DNA repair mechanisms that are of significance.

One can envisage that the simplest way to repair a damaged DNA residue is the direct reversal of the modified residue to the original chemical form. Indeed at least two such direct repair systems have been described. The enzyme DNA photolyase is a highly specialized enzyme involved in the repair of cyclobutane pyrimidine dimers (CPD) induced by ultraviolet light (Sancar, 1994; Sancar, 1990). In this process of photoreactivation, the photolyase recognizes and binds to the cyclobutane dimer, and subsequent absorption of a photon from the visible light

spectrum then provides energy to convert the dimerized pyrimidines to their monomeric forms (Sancar, 1994; Sancar, 1990). Photoreactivation has been observed in numerous organisms, including bacteria, yeast, invertebrates and various vertebrates, including fish and marsupials (Sancar, 1990; Todo, 1993; Yasuhira and Yasui, 1992; Kato et al, 1994). However, placental animals, including humans, do not appear to possess photolyase activity (Li YF et al, 1993; Ley, 1993). More recently, another class of photolyase specific for the UV-induced 6-4 photoproduct was discovered (Todo et al, 1993). As this 6-4-specific photolyase has been found in *Drosophila, Xenopus,* rattle snake, and *Arabidopsis,* it is likely to be widespread (Todo et al, 1993; Kim et al, 1996a, b; Nakajima et al, 1998). Interestingly, a human cDNA clone with 48 % sequence similarity to the *Drosophila* gene was identified, raising the possibility that humans might also possess a photolyase specific for 6-4 photoproducts but not CPDs (Todo et al, 1996). However, since recombinant proteins expressed from this and another related human cDNA did not possess photolyase activity, these human proteins may only function as blue-light photoreceptors (Hsu et al, 1996).

Another case of a direct reversal of DNA damage involves the repair of guanine alkylated at the O^6-position by alkylating mutagens such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). O^6-alkylguanine is a lesion of considerable biological significance since the degree of carcinogenicity of alkylating agents correlates with the amount of O^6-alkylguanine induced in DNA (Newbold et al, 1980). Hence, various cells, including those of bacteria, yeast and humans, are protected from such lesions by a specialized repair enzyme, O^6-methylguanine-DNA methyltransferase (MGMT), which removes the alkyl group by transferring it to a cysteine residue in its own active site (Pegg, 1990). The importance of MGMT in preventing carcinogenesis can be appreciated in experiments demonstrating that overexpression of the enzyme in thymus or liver of transgenic mice reduced their susceptibility to tumor formation after treatment with carcinogens such as N-methyl-N-nitrosourea and dimethylnitrosamine (Dumenco et al, 1993; Nakatsuru et al, 1993). Since this enzyme is inactivated irreversibly during the repair process, MGMT has been widely known as the "suicide repair protein". Another interesting

feature of this system is that expression of the MGMT gene is inducible by a variety of forms of genotoxic stress (Mitra and Kaina, 1993; Rafferty et al, 1996; Wilson, 1993). This induction is at the transcriptional level and requires the expression of wild-type functional forms of the tumor suppressor protein p53 (Grombacher et al, 1998; Rafferty et al, 1996). Dubbed "the guardian of the genome" (Lane, 1992), p53 plays an important role in cell cycle checkpoint control and the response to DNA damage (Smith and Fornace Jr, 1997; Agarwal et al, 1998).

Direct measurement of the O^6-ethylguanine residues formed and repaired in specific genes of rat cells treated with N-ethylnitrosourea showed that the mutagenic lesions were preferentially repaired within transcriptionally active DNA (Thomale et al, 1994). Immunocytological studies also provided evidence that MGMT is localized in speckles that correspond to sites of active transcription by RNA polymerase (Ali et al, 1998). At first glance, this localization is reminiscent of transcription-coupled repair, a major subpathway found in nucleotide excision repair (NER). However, unlike the lesions that are repaired by NER, O^6-alkylguanine residues in DNA do not arrest transcription by RNA polymerase II (Gerchman and Ludlum, 1973). The direct presence of MGMT at sites of active transcription could simply mean that a more open chromatin structure is associated with transcription, allowing better access of MGMT to actively transcribing regions of the genome. Regardless of the mechanistic detail of MGMT targeting to active genes, this form of transcription-repair coupling is fundamentally different from that in NER.

II. Double-Strand Break Repair

Chromosomal DNA breaks can be generated by exogenous agents such as ionizing radiation or endogenously produced free radicals. Breaks can also arise as a result of normal cellular processes, such as replication, or as intermediates in V(D)J recombination during lymphoid maturation. Accurate repair of these genotoxic lesions is essential for the prevention of chromosomal rearrangements such as translocations and deletions. Failure to do so would lead to genomic instability that

frequently precedes cellular transformation. To repair DNA double-strand breaks (DSB), eukaryotic cells have developed two distinct pathways: homologous recombination, which employs segments of homologous DNA as template to replace a region containing a double-strand break, and nonhomologous DNA end-joining (NHEJ), in which the two ends of a broken chromosome are religated directly without the need for extensive homology.

1. Non-Homologous End Joining

Apart from being one of the pathways responsible for repairing DSR, NHEJ is also utilized by lymphocytes to assemble coding regions of the variable domains of immunoglobulin and T cell receptors (Jeggo et al, 1995). As a result, cells that are defective in NHEJ exhibit increased X-ray sensitivity and deficiency in V(D)J recombination (Jeggo et al, 1997). Rodent cell lines defective in this form of DSB repair, such as those derived from the *scid* mouse, have been instrumental in the identification of genes involved in the NHEJ repair pathway (Thompson and Jeggo, 1995; Jeggo et al, 1995). So far, four genes, corresponding to complementation groups *XRCC4-7*, are known to encode proteins involved in NHEJ and V(D)J recombination. *XRCC4* (*LIF1* in *S. cerevisiae*) encodes a novel protein that complexes with DNA ligase IV, the *in vitro* and *in vivo* activity of which is enhanced by this interaction (Grawunder et al, 1998; Grawunder et al, 1997; Critchlow et al, 1997). This enzyme is probably responsible for catalyzing the ligation step in both NHEJ and V(D)J recombination in humans (Wilson et al, 1997; Schar et al, 1997; Teo et al, 1997). The remaining three genes of the *XRCC* complementation groups encode components of the human DNA-dependent protein kinase (DNA-PK). DNA-PK is a nuclear serine-threonine protein kinase and consists of a catalytic subunit, DNA-PKcs (encoded by *XRCC7*), and a heterodimeric DNA end-binding component, Ku, which is made up of 2 subunits termed Ku70 and Ku80 (encoded by *XRCC6* and *XRCC5*, respectively) (Lees-Miller, 1996). The Ku heterodimer binds to double-stranded DNA ends without sequence specificity. It appears to recognize various DNA structures including gapped and hairpin molecules (Blier et al, 1993;

Falzon et al, 1993). Homologs of the two subunits of Ku have also been identified in *Saccharomyces cerevisiae,* suggesting that the process of NHEJ is also present in lower eukaryotes (Feldmann et al, 1993; Milne et al, 1996). Ku is the DNA-targeting component of the catalytic subunit (DNA-PKcs) whose activity is stimulated by DNA ends. Sequence anaylsis of DNA-PKcs revealed that its kinase domain belongs to a subfamily of the phosphatidylinositol 3-kinase (PI3-K) family (Hartley et al, 1995). Members of this subfamily possess only protein kinase and not lipid kinase activity (Hunter, 1995). In addition, almost all members, including ATM and MEC1, appear to function at relative early steps in the cell cycle checkpoint in response to DNA damage (Keith et al, 1995).

Despite the evidence that DNA-PK is required for the NHEJ pathway of DSB repair, relatively little is known about the mechanistic details of this process. A direct role for Ku in the repair process has been suggested by its ability to bind DNA ends and by imaging studies showing that two molecules of DNA-bound Ku can associate to form DNA loops (Cary et al, 1997). The recent demonstration that Ku protein can stimulate DNA end-joining and that this effect is specific to eukaryotic ligases strongly argues for a direct role for the processing and rejoining of DNA breaks (Ramsden et al, 1998). Ku may also act as a DNA damage sensor and signal other cellular events by recruiting and stimulating the activity of DNA-PKcs. For this reason, much research has been focused on the identification of phosphorylation substrates for DNA-PKcs. Interestingly, the catalytic subunit of DNA-PK itself was found to undergo an *in vitro* autophosphorylation which inhibits kinase activity by dissociating the DNA-PKcs from the Ku-DNA complex (Chan et al, 1996). This autophosphorylation might serve to feedback regulate the functions of DNA-PK in DNA repair and other cellular processes affected by this kinase. Another potential physiological substrate for DNA-PK is the 34 kDa subunit of RPA, which is a trimeric complex involved in DNA replication and repair. Normally, RPA becomes hyperphosphorylated following ionizing irradiation of cells. This phosphorylation of the 34 kDa subunit of RPA was reported to be diminished in SCID cells which lack DNA-PK activity, suggesting that RPA might indeed be an *in vivo* substrate for DNA-PK (Boubnov and Weaver, 1995). However,

this finding has not been reproduced in another study (Fried et al, 1996). Clearly, more work is needed to clarify the role of phosphorylation on RPA function and its relationship with DNA-PK and the related ATM kinase. Another proposed function for DNA-PK, that of a cell cycle checkpoint regulator, originated from the observation that p53 is a good *in vitro* phosphorylation substrate for DNA-PK. However, neither a checkpoint defect nor changes in phosphorylation pattern of p53 was detected in DNA-PK-defective cells (Fried et al, 1996). It should also be noted that many other *in vitro* substrates for DNA-PK have been identified: SV40 large T antigen, serum response factor, c-jun, c-fos, Oct-1, Sp1, myc, progesterone receptor and the C-terminal domain (CTD) of RNA polymerase II (Anderson, 1993). That most of the these substrates are DNA-binding proteins involved in transcriptional regulation, argues a role for DNA-PK in controlling gene expression. Caution should be taken, however, regarding the actual cellular functions of DNA-PK, especially when many of the *in vitro* substrates have not been shown to be phosphorylated by DNA-PK *in vivo*.

Further insight into the mechanism of Ku-dependent DSB repair came from genetic analysis of both Ku deletion mutations as well as mutations in genes belonging to the *RAD52* epistasis group. Double mutants harboring a deletion in one of the Ku genes in addition to a deletion of *RAD50* displayed end-joining defects comparable to either of the single mutants, indicating that Ku operates in the same genetic pathway as *RAD50* (Milne et al, 1996). It is known that *RAD50, MRE11* and *XRS2* comprise a single epistasis group and that the three gene products function as a complex, with MRE11 having a 3' to 5' exonuclease activity (Dolganov et al, 1996; Paull et al, 1998). Thus, broken DNA ends might be processed by this complex before being presented as substrates for subsequent DNA-PK-dependent steps during NHEJ. The importance of the RAD50-MRE11-XRS2 complex in repair and V(D)J recombination is evidenced by the recent recognition of *XRS2* as the gene defective in the cancer-predisposing and immunodeficient disorder, Nijmegen breakage syndrome (Featherstone et al, 1998).

2. Homologous Recombination

Double-strand break repair by homologous recombination results in the precise repair of the DNA lesions, since the process uses the homologous sequence as the template for repair (Stahl, 1996; Haber, 1995). This pathway is utilized primarily by *S. cerevisiae* to repair chromosomal breaks (Haber, 1995). Yeast genes that are involved in homologous repair belong to the *RAD52* epistasis group. Many of them, including *RAD51*, *RAD52* and *RAD54*, have been found to have homologs in mammalian cells (Petrini, 1997). Because yeast have an efficient homologous recombinational repair pathway, nonhomologous recombination events can be detected only if homologous recombination is abrogated by a *rad52* null mutation (Kramer et al, 1994; Mezard et al, 1994; Moore and Haber, 1996). Thus, yeast mutants defective for Ku70 or Ku80 expression show wild-type levels of radiation sensitivity. However, in the presence of a *rad52* null mutations, mutations in the yeast Ku homologs confer elevated sensitivity to DNA break-inducing agents when compared to the single mutants (Milne et al, 1996; Siede et al, 1996 ; Boulton et al, 1996). This observation is consistent with the finding that NHEJ plays only a minor role in repairing DSBs in yeast.

Unlike yeast, mammalian cells were originally thought to utilize NHEJ as the predominant mechanism for DSB repair. Recent analyses have suggested, however, that both homologous recombination and NHEJ are utilized to similar extents in mammalian cells (Liang et al, 1998; Liang et al, 1996; Jasin, 1996). The reason for this difference in preferential usage of the two DSB repair pathways could be related to differences in genome organization. The genome of *S. cerevisiae* is very compact, with only a few short introns, and with few repetitive sequences. Thus, nonhomologous repair in yeast is potentially mutagenic because it frequently leads to small deletions or insertions. Homologous recombination, on the other hand, ensures precise repair of breaks with no changes in genetic information (Stahl, 1996; Haber, 1995) This situation contrasts with that found in mammals, where nonhomologous repair is much less likely to create deleterious alterations in a genome with plenty of large introns and repetitive sequences. Homologous

recombination may predominate if sister chromatids are available, as when cells have passed through S phase in the cell cycle. However, NHEJ may be a favored process in the absence of sister chromatids, since recombination between homologous pairs of chromosomes could be deleterious. Somatic recombination between alleles in homologous chromosomes is thought to be a major mechanism contributing to loss of heterozygosity in tumor cells (Moynahan et al, 1997). It is therefore possible that the two DSB pathways are utilized to different degrees, in a cell cycle-dependent manner.

Genetic studies in *S. cerevisiae*, an organism that preferentially uses homologous recombination in repairing breaks, have yielded much information about the identity of genes involved in recombinational repair. Genes belonging to the *RAD52* epistasis group, such as *RAD51, RAD52, RAD54, RAD55*, and *RAD57*, are believed to function in this particular repair pathway (Game 1993; Shinohara and Ogawa, 1995). Of these genes, *rad51, rad52* and *rad54* mutants exhibit more severe repair defects, suggesting that these three genes encode the core components of the recombination repair pathway.

Cloning of the yeast *RAD51* gene and its human counterpart revealed that they are homologs of the *E. coli* RecA protein. Like RecA, both yeast and human RAD51 proteins can form nucleoprotein filaments on single-stranded and double-stranded DNA and possess DNA-dependent ATPase activity (Sung, 1994; Sung and Robberson, 1995; Benson et al, 1994; Baumann et al, 1996; Gupta et al, 1997; Ogawa et al, 1993). In addition, both proteins can catalyze strand-exchange reactions between single-stranded DNA and homologous double-stranded DNA *in vitro* (Sung and Robberson, 1995; Namsaraev and Berg, 1997; Gupta et al, 1997; Baumann et al, 1996; Baumann and West, 1997). However, the rate of this reaction is slower than that with the *E. coli* RecA protein. In both yeast and human cells, the efficiency of strand-exchange can be increased by the addition of replication protein A (RPA) and RAD52, and RAD52 has been shown to interact with RPA. (New et al, 1998; Shinohara and Ogawa, 1998; Sugiyama et al, 1997; Baumann et al, 1996; Baumann and West, 1997; Benson et al, 1998). Addition of yeast RAD55 and RAD57 can also stimulate strand-exchange reactions *in vitro* (Sung , 1997); however, human

homologs of these two yeast genes have not yet been identified. This multitude of interactions and modulations of activity by different proteins encoded by the *RAD52* epistasis group is consistent with the finding that they may all interact in a multiprotein complex dubbed the "recombinosome" (Hays et al, 1995).

Cloning of the human and mouse *RAD54* genes identified them as members of the *SNF2/SWI2* family of DNA-dependent ATPases (Kanaar et al, 1996). This protein family is involved in chromatin remodelling and transcriptional regulation (Eisen et al, 1995). The role of *RAD54* in homologous recombination has not yet been characterized except that both yeast and human RAD54 are known to interact directly with RAD51 from its cognate species (Clever et al, 1997; Jiang et al, 1996; Golub et al, 1997). Human RAD54 can perform functions similar to the yeast protein, as the human gene can complement the methyl methane sulfonate (MMS) sensitivity of a yeast *rad54* mutant (Kanaar et al, 1996). Cell lines lacking functional Rad54 protein are highly sensitive to ionizing radiation and MMS, and have reduced levels of homologous recombination; these phenotypes are shared by yeast *rad54* mutant cells (Essers et al, 1997). These findings provide strong evidence that the general functions of the *RAD52* epistasis group are conserved between yeast and higher eukaryotes. In contrast to the situation with *RAD54*, it has proven impossible to establish mouse embryonic stem cells homozygous for a *rad51* null mutation (Lim and Hasty, 1996; Tsuzuki et al, 1996). This finding and the observation that *RAD51* is indispensable for mouse early embryonic development indicate that*RAD51* has an essential role in cellular viability and/or proliferation, in addition to its role in repair (Lim and Hasty, 1996; Tsuzuki et al, 1996). This possibility of *RAD51* having a role in cellular growth control has gained credence from the finding that *RAD51* and the tumor suppressor p53 interact physically and genetically (Buchhop et al, 1997; Sturzbecher et al, 1996; Lim and Hasty, 1996).

In addition to its interaction with p53, evidence for a connection of *RAD51* to human cancer biology has also come from the field of breast cancer research. The gene products of the breast cancer susceptibility genes BRCA1 and BRCA2 have been shown to interact with human RAD51 (Zhang et al, 1998b; Vispe and Defais, 1997). In the developing mouse embryo, the patterns of *RAD51, BRCA1* and *BRCA2* gene

expression are very similar, suggesting that all three proteins participate in similar cellular processes (Rajan et al, 1997; Sharan et al, 1997). A functional link between RAD51 and BRCA1 is also supported by their co-localization in nuclear foci (Scully et al, 1997a). Moreover, their nuclear localizations undergo similar alterations in response to DNA-damaging agents (Scully et al, 1997b). This nuclear relocation following exposure to DNA damaging agents has also recently been reported to occur for BRCA2 (Chen J et al 1998). In fact, BRCA1 and BRCA2, along with RAD51, appear to coexist in the same biochemical complex in immunoprecipitation experiments (Chen J et al, 1998). The case for BRCA2 having a role in DNA repair has also gained significant support from two recent reports which demonstrated a direct DNA repair defect in cells derived from mice homozygous for a truncated allele of *BRCA2* (Patel et al, 1998; Connor et al, 1997). Like *Rad51* null mutants, cells from these embryos exhibit hypersensitivity to ionizing radiation. Taken together, these data strongly suggest that at least one of the cellular functions of BRCA proteins is in a RAD51-dependent recombination repair pathway. Since assays measuring strand-exchange activity mediated by RAD51 and other proteins have been developed (Gupta et al, 1997; Baumann et al, 1996; Baumann and West, 1997), it may be possible to determine whether this activity can be modulated by the addition of BRCA proteins. Biochemical characterization of BRCA proteins will undoubtedly shed light on the molecular functions they may have in DNA repair. This line of research may also reveal a role for the large BRCA proteins in other cellular processes, for it has been suggested that both BRCA1 and BRCA2 may act as transcriptional activators or coactivators (Ouchi et al, 1998; Somasundaram et al, 1997; Scully et al, 1997c; Zhang et al, 1998a; Milner et al, 1997).

III. Mismatch Repair

The process of mismatch repair (MMR) is mainly concerned with repair of mismatched base pairs which arise during DNA replication and which have escaped the proofreading mechanism of DNA polymerases (Modrich and Lahue, 1996; Kolodner, 1995). The pathway has been studied extensively in *E. coli,* in which the basic repair mechanism has been elucidated in considerable detail. *In vitro*

reconstitution experiments with purified proteins and templates containing mispaired bases have demonstrated the requirements for MutH, MutL, MutS, and helicase II, DNA polymerase III holoenzyme, DNA ligase, single-strand DNA binding protein (SSB), and one or more of the single-stranded DNA exonucleases (Exo I, Exo VII, or RecJ). Recognition of mismatched base pairs is carried out by a homodimer of the MutS protein (Su et al, 1988; Grilley et al, 1989; Allen et al, 1997). MutL can bind to a MutS-mismatch complex, which leads to activation of a latent endonuclease associated with the MutH protein (Au et al, 1992). MutH is a sequence-specific endonuclease which incises only the unmodified strand 5' to a hemimethylated GATC sequence. This specificity provides the mechanism for the recognition of the newly synthesized strand with the replication error, since the sequence GATC is transiently unmethylated in the daughter strand. The single-strand break introduced by MutH directs the excision of that portion of the unmodified strand spanning the GATC seqeuence and the mispair (Lahue et al, 1989; Grilley et al, 1993). The excision reaction is dependent on MutS, MutL, DNA helicase II (MutU), and an exonuclease (Lahue et al, 1989; Grilley et al, 1993). The unwinding activity of helicase II can be stimulated by MutS and MutL and is required to unwind the incised strand to render it exonuclease sensitive (Yamaguchi et al, 1998). The identity of the exonuclease depends on whether the strand break is produced 3' or 5' to the mispair. When the strand break occurs 5' to the mismatch, excision requires RecJ exonuclease or exonuclease VII, both of which support 5' to 3' hydrolysis (Grilley et al, 1993; Cooper et al, 1993; Lovett et al, 1989). If the break occurs 3' to the mismatch, the 3' to 5' hydrolytic activity of exonuclease I is required (Lahue et al, 1989).

The first steps of mismatch repair in eukaryotes are similar to those in prokaryotes, although they are currently less well defined and appear to involve many more protein factors. Three known human MutS homologs, hMSH2, hMSH6(GTBP) and hMSH3, have been identified. Unlike that of *E. coli*, the human mismatch recognition complex is a heterodimer comprised of one molecule of hMSH2 and either hMSH6 or hMSH3 (Drummond et al, 1995; Palombo et al, 1995; Acharya et al, 1996, Palombo et al, 1996). The two recognition complexes have

different spectra of mismatch specificity. The hMSH2/hMSH6 heterodimer, referred to as hMutSα, recognizes mispaired bases and unpaired loops of 1 to 4 nucleotides (Acharya et al, 1996; Palombo et al, 1995; Drummond et al, 1995). On the other hand, the hMSH2/hMSH3 complex-termed hMutSβ-shows specificity for loops of more than 2 unpaired nucleotides (Acharya et al, 1996; Palombo et al, 1996). Like the bacterial and yeast MutS homologs, hMutSα possesses an intrinsic ATPase activity, and its binding to mismatches is ATP-dependent (Gradia et al, 1997; Iaccarino et al, 1998). The next step in the pathway involves the binding of hMutLα, the human homolog of bacterial MutL, to the hMutS-DNA complex. Like hMutS, hMutLα also exists as a heterodimer of hMLH1 and hPMS2, and both of which share sequence homology with bacterial MutL (Li and Modrich, 1995). Since no human homologs of MutH have been identified outside of gram-negative bacteria, the mechanisms of daughter strand recognition in gram-positive bacteria and in any non-human eukaryotes is presently unknown.

The identity of the eukaryotic exonuclease involved in MMR has not been definitively determined. However, genetic data obtained from yeast provided evidence that the *EXO1* genes of *S. cerevisiae* and *S. pombe* and the *RTH1* gene of *S. cerevisiae* (a 5' to 3' exonuclease) are strong candidates (Johnson et al, 1995; Tishkoff et al, 1997; Szankasi and Smith, 1995). The case for *EXO1* having a role in MMR is particularly strong. Not only did mutations in *EXO1* result in an elevated mutation rate, but its gene product was shown to interact physically with yeast and human MSH2 (Tishkoff et al, 1997; Szankasi and Smith, 1995).

The repair DNA synthesis step in human mismatch repair is believed to be carried out by polymerase δ, because extract depleted of this polymerase is defective in the synthesis step of MMR (Longley, 1997). It is noteworthy that the processivity of polymerase δ can be increased by PCNA, with which it interacts (Kelman, 1997). Recently, replication protein A was shown to be required for human MMR *in vitro* (Lin et al, 1998). This finding is consistent with the involvement of polymerase δ in MMR, since its activity can be stimulated by replication protein A (Podust and

Hubscher, 1993; Tsurimoto and Stillman, 1989). PCNA might have an additional role in mismatch repair other than its function at the step of repair synthesis (Gu et al, 1998). Several reports have demonstrated direct physical interactions between PCNA and various MutS or MutL homologs: between yeast PCNA and the MutSβ heterodimer (Johnson et al, 1996); between yeast PCNA and yeast MLH1 or MSH2 (Umar et al, 1996); and between human PCNA and human PMS2 (Umar et al, 1996). These protein-protein interaction studies strongly imply that, in addition to repair synthesis, PCNA may also function at or prior to the excision step. Genetic studies in yeast have also implicated a role for PCNA in MMR, since certain conditional *S. cerevisiae* PCNA mutants have a hypermutable phenotype that is dependent on yeast MSH2, MLH1 and PMS1 (Johnson et al, 1996).

The significance of the error removal functions of MMR was dramatically demonstrated when it was discovered that the molecular basis of hereditary non-polyposis colorectal cancer (HNPCC) involves genetic instability resulting from defective MMR (Lynch et al, 1996a, b; Peltomaki and de la Chapelle, 1997; Kinzler and Vogelstein, 1996). HNPCC accounts for 2%-4% of the total colorectal cancers in the western world (Lynch et al, 1996a, b). Mutation rates in tumor cells with MMR deficiency are two to three orders of magnitude higher than in normal cells (Eshleman et al, 1996; Bhattacharyya et al, 1994; Malkhosyan et al, 1996). Germline mutations in four mismatch genes (*hMSH2, hMLH1, hPMS1,* and *hPMS2*) have been identified in HNPCC kindreds, with mutations in *hMSH2* and *hMLH1* accounting for most of the mutations (Liu et al, 1996).

Although the mutator defect that arises from the MMR deficiency can affect any DNA sequence, microsatellite sequences are particularly sensitive to MMR abnormalities (Peltomaki et al, 1997; Kinzler and Vogelstein, 1996). Microsatellite instability is therefore a useful indicator of defective MMR. In addition to its occurrence in virtually all tumors arising in HNPCC patients, microsatellite instability is found in a small fraction of sporadic tumors with distinctive molecular and phenotypic properties (Marra and Boland, 1995; Eshleman and Markowitz, 1995). This finding suggests that some sporadic cancers may be due to acquired

mutations in MMR genes. However, not all of HNPCC or sporadic cancers with mutator phenotypes can be accounted for by known MMR genes. Consequently, there is great interest in identifying additional MMR genes.

IV. Base Excision Repair

The DNA lesions recognized by enzymes in the base excision repair (BER) pathway generally do not cause major helix distortions (Lindahl, 1997; Krokan et al, 1997; Seeberg et al, 1995). The damage may be caused by a variety of agents and processes, such as spontaneous deamination of bases, alkylating agents, radiation, oxidative stress or replication errors (Lindahl, 1997). Deamination of cytosine results in the formation of uracil, which mispairs with G to cause a transition mutation. Deamination appears to be a frequent process, giving rise to 100-500 uracil residues per mammalian genome per day (Mosbaugh and Bennett, 1994). Uracil may also result from misincorporation of dUMP during DNA replication. Reactive methylating agents, which can alkylate DNA directly, are continuously generated intracellularly from methyl donors such as S-adenosylmethionine (Rydberg and Lindahl, 1982). N-nitroso compounds formed endogenously or from cigarette smoking are mutagenic through alkylation of bases (Mirvish, 1995; Amin et al, 1996). High oxidative stress is caused by the formation of a variety of reactive oxygen species, including superoxide radical, hydroxyl radical, hydrogen peroxide and nitric oxide. They are formed during normal cellular metabolism but abnormally large amounts can be generated during inflammation (Wiseman and Halliwell, 1996; Conner and Grisham, 1996; Demple and Harrisson, 1994). These highly reactive substances can result in strand breaks, oxidized bases and sugars and apyrimidinic/apurinic (AP) sites (Demple and Harrisson, 1994). The susceptibility of the base-sugar bonds to hydrolysis means that AP sites represent a major source of damage. AP sites can inhibit DNA replication and are non-instructional for DNA polymerases, thus giving rise to base substitution mutations if misincorporation occurs (Gentil, 1992). Oxidation of bases such as cytosine results in 5-hydroxyuracil and isodialuric acid, whereas oxidation of guanine produces 7,8-dihydro-8-

oxoguanine (8-oxoG), a potent mutagen (Cunningham, 1997). The specialized enzymes of the BER pathway can eliminate a few types of mismatches as well as modified nucleotides and AP sites. However, most DNA mismatches, arising as a consequence of replication error, are largely removed by the mismatch repair system.

As its name implies, the first step in BER involves the removal of the damaged base by a class of enzymes known as DNA glycosylases (Fig. 1). In general, they are small, monomeric proteins, <30 kDa in size, with no requirements for cofactors (Krokan et al, 1997; Lindahl, 1979, 1982). The lack of a cofactor requirement has facilitated their detection in crude extracts since the addition of EDTA eliminates nonspecific nuclease activity. These enzymes catalyze the hydrolysis of the N-glycosyl bond linking the target base to deoxyribose, thus releasing a free base and resulting in an AP site in DNA. Hence, in addition to the processes described above, AP sites are generated during BER by glycosylases. Most DNA glycosylases, such as uracil DNA glycosylases and 3-methyladenine DNA glycosylases, are specific for a particular type or a few related forms of base damage. For instance, uracil DNA glycosylases act on uracil in DNA and on certain forms of oxidized cytosine and uracil, including 5-hydroxyuracil and isodialuric acid. In contrast, 3-methyladenine DNA glycosylases remove simple alkylation adducts of purines at the N-3 and N-7 positions; and they also remove hypoxanthine generated by deamination of adenine.

Cleavage at the resulting AP site involves an AP endonuclease which cleaves 5' to the AP site leaving a 3'-hydroxyl and a deoxyribose 5'-phosphate moiety; the latter is subsequently excised by a deoxyribophosphodiesterase (dRpase) to leave a single nucleotide gap. A dRpase activity has been detected in human extracts, although its identity has not been established (Price and Lindahl, 1991). In mammalian cells, the major AP endonuclease is called HAP1/APE/BAP1 (Robson and Hickson, 1991; Demple et al, 1991), which has also been isolated as Ref-1 (Rothwell et al, 1997), a nuclear redox factor that can regulate the activity of Fos-Jun by reduction of a specific cysteine residue in the transcription factor. Thus, HAP1 might also play a role in transducing cellular signals in response to oxidative stress.

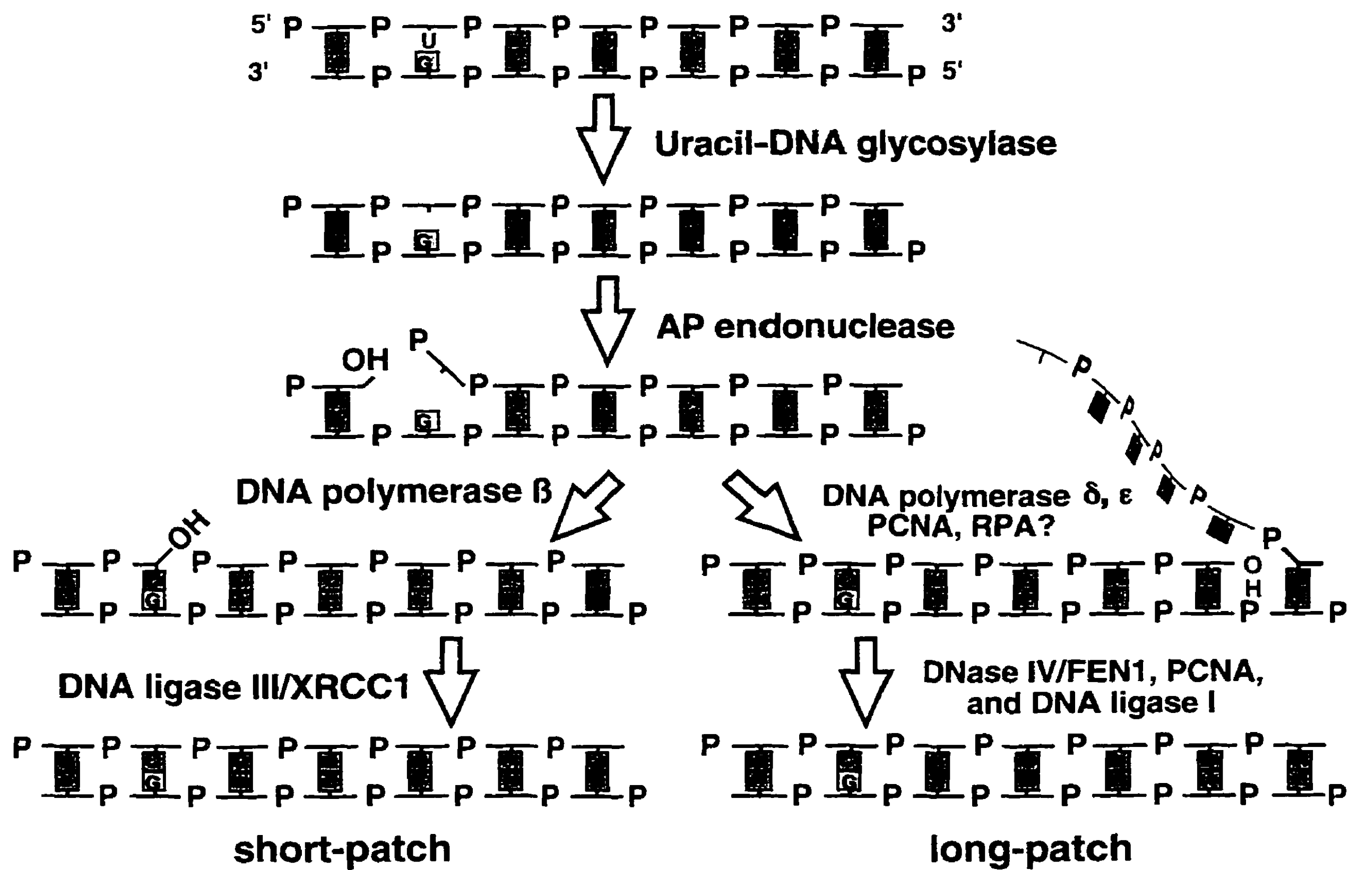

Figure 1. Steps in eukaryotic base excision repair

S. cerevisiae also has one major AP endonuclease encoded by the *APN1* gene. The importance of *APN1* in maintaining the integrity of the yeast genome can be appreciated by the phenotype of *apn1* deletion mutants. Loss of *APN1* function leads to hypersensitivity to oxidative and alkylating agents that damage DNA; *APN1*-deficient cells also have a higher rate of spontaneous mutation (Ramotar et al, 1991).

Another class of AP endonuclease, different from the type described above, is termed AP lyase. This enzyme cleaves 3' to the AP site by a β-elimination mechanism, leaving a nick with an unsaturated sugar-phosphate on the 3'-end and a 5'-phosphate. This AP lyase activity has been associated with DNA glycosylases such as thymine-glycol-DNA glycosylase, formamidopyrimidine-DNA glycosylase, and bacterial endonucleases V and VIII (Demple and Harrisson, 1994). Subsequent δ-elimination of the 3'-terminal sugar-phosphate produces 4-hydroxy-2-pentenal and 3'-phosphate, which must be further processed to generate a 3'-hydroxyl required for the polymerization activity of DNA polymerases. However, existing evidence-including the abundance of the AP endonucleases that cleave on the 5' side and genetic studies in yeast-does not support this AP lyase activity having an important role in BER (Robson and Hickson, 1991; Demple et al, 1991; Ramotar et al, 1991).

Following the cleavage at the AP site, two alternative pathways differing in the size of the repair patch may be utilized for completing the process of BER in eukaryotic cells (Fig. 1). Short-patch repair results in the incorporation of a single nucleotide residue during DNA repair synthesis, whereas long-patch BER generates a repair patch of 2-7 nucleotides. The presence of more than one pathway in BER may reflect the existence of a backup system. On the other hand, some lesions, such as oxidized or reduced AP sites, are not amenable to the short-patch pathway since they cannot undergo β-elimination, and can only be processed by the long-patch pathway (Klungland and Lindahl, 1997).

1. Short-Patch Repair

The major pathway of BER mainly involves the replacement of a single damaged nucleotide residue. These reactions have been reconstituted *in vitro,* first

with cell-free extracts from human and bovine cells, and later with purified recombinant human proteins (Dianov et al, 1992; Wiebauer et al, 1990, Singhal et al, 1995; Kubota et al, 1996). Using a uracil-containing oligonucleotide as substrate, Kubota et al successfully reproduced short-patch BER with human uracil-DNA glycosylase (UDG), AP endonuclease (HAP1), DNA polymerase β (Polβ) , the scaffold protein XRCC1 and either DNA ligase III or DNA ligase I (Kubota et al, 1996). This study and other findings, provides strong evidence that Polβ is the DNA polymerase used in the short-patch mode of repair synthesis during BER. Some of the key evidence includes studies of BER in mammalian extracts in the presence of various DNA polymerase inhibitors (Dianov et al, 1992; Wiebauer et al, 1990, Singhal et al, 1995), sensitivity of BER to a Polβ-specific neutralizing antibody (Wiebauer et al, 1990, Singhal et al, 1995), inability of rodent cells lacking Polβ to carry out BER and the ability of purified Polβ to rescue this defect (Sobol et al, 1996). Moreover, besides the gap-filling DNA polymerization activity, DNA polymerase β also has an intrinsic deoxyribophosphodiesterase (dRpase) activity which can catalyze removal of the 5'-terminal deoxyribose phosphate (dRp) residues at the incised AP site via a β–elimination mechanism (Matsumoto and Kim, 1995). Hence, the possession of this dRpase activity by Polβ implies that a separate hydrolytic dRpase, such as the one described by Price et al (Price and Lindahl, 1991), might not be necessary. The lack of a requirement for the addition of an exogenous dRpase in the reconstituted system further supports the conclusion that Polβ provides both the gap-filling and dRpase activities (Kubota et al, 1996). The importance of Polβ in conferring protection against DNA damaging agents through the BER pathway is also supported by the observation that rodent cells lacking Polβ exhibit hypersensitivity to DNA-alkylating agents such as MMS, but not to UV and ionizing radiation (Sobol et al, 1996). Moreover, upregulation of BER capacity by treatment of cells with various agents, including hydrogen peroxide and lipopolysaccharide, correlates with increased resistance to MMS and enhanced Polβ protein expression (Chen KH et al,

1998). Using several different approaches, Kubota et al showed that Polβ interacted directly with XRCC1 (Kubota et al, 1996), a protein that has no known catalytic activity but is implicated in mammalian BER through genetic studies (Thompson et al, 1990). The biological significance of this interaction lies in the ability of XRCC1 to inhibit the strand displacement activity of Polβ, thus limiting polymerization to one nucleotide (Kubota et al, 1996). To complete the repair process, the single nucleotide gap can be ligated by one of the five DNA ligase activities detected in mammalian cells. DNA ligases I, II, and IV have been cloned and the remaining two might represent proteolytic fragments of the three cloned ligases (Tomkinson and Mackey, 1998). In the *in vitro* reconstitution system, either DNA ligase I or ligase III can efficiently seal the nick; in fact, the reaction can even be completed by T4 DNA ligase (Nicholl et al, 1997; Kubota et al, 1996). However, the fact that XRCC1 also interacts with DNA ligase III (Taylor et al, 1998; Nash et al, 1997; Cappelli et al, 1997; Mackey et al, 1997; Caldecott et al, 1995; Caldecott et al, 1994), and that this complex formation is important to maintain the stability of DNA ligase III (Taylor et al, 1998; Cappelli et al, 1997; Caldecott et al, 1995; Caldecott et al, 1994), argues strongly that ligase III is probably the physiological enzyme.

The specific interaction between XRCC1 and two enzymes involved in the short-patch repair mode of BER implicates XRCC1 as a scaffold protein functioning to recruit other repair components and to promote a concerted reaction sequence, thereby suppressing unnecessary strand displacement. The recent observation that XRCC1 can interact with yet another enzyme believed to have a role in BER is therefore intriguing. XRCC1 was found to have a third distinct binding site for poly(ADP-ribose) polymerase (PARP) (Masson et al, 1998; Caldecott et al, 1996). The physiological role of PARP has been highly controversial: it has been implicated in virtually every major cellular function, including DNA repair, genome stability and apoptosis (Le Rhun et al, 1998; Lindahl et al, 1995). Eliminating PARP activity in mammalian cells or in mice resulted in increased sensitivity to alkylating agents or suppressed repair synthesis, implicating an *in vivo* role for PARP in BER (Lindahl et al, 1995; de Murcia et al, 1997; Kupper et al, 1995; Molinete et al, 1993). *In vitro*,

PARP has the ability to recognize and rapidly bind DNA containing strand breaks, including those arising during BER, thus resulting in inhibition of BER (Satoh et al, 1994). It therefore seems possible that PARP, through its interaction with XRCC1, may establish a repair complex at the AP site formed as a result of the actions of glycosylase and endonuclease. A recent breakthrough in the field came when it was discovered that cells derived from mice lacking PARP exhibited an elevated frequency of spontaneous sister chromatid exchanges, directly demonstrating a role for PARP in genomic recombination and stability (Wang ZQ et al, 1997). This result is also consistent with the notion that PARP functions as an anti-recombination factor at DNA ends and helps to suppress genomic instability (Lindahl et al, 1995; Satoh et al, 1994).

2. Long-Patch Repair

Studies using yeast and mammalian cell extracts showed that a circular plasmid molecule containing multiple AP sites was efficiently repaired by BER with a patch size of approximately 7 nucleotides per site of damage, pointing to the existence of a second mode of BER (Wang et al, 1992; Frosina et al, 1994). This observation corroborated the finding that BER occurred, not only via a Polβ-dependent pathway, but also by a PCNA-dependent pathway in a *Xenopus laevis* oocyte system. Since PCNA acts as a processivity factor for DNA polymerases δ and ε (Kelman, 1997), one or both of these two polymerases may have a role in long-patch BER. Indeed, the requirement for polymerase δ or ε in BER has been demonstrated in the yeast and *Xenopus* oocyte systems (Blank et al, 1994; Wang et al, 1993; Matsumoto et al, 1994).

In vitro reconstitution of the long-patch pathway confirmed that the initial BER reactions require the same molecular partners but that PCNA is required in the repair synthesis step (Klungland and Lindahl, 1997). In addition, it was found that the nuclease DNase IV/FEN-1, a "flap" endonuclease, was also essential for cleavage of the reaction intermediate produced by strand displacement in the 5' to 3' direction (Klungland and Lindahl, 1997). Here, the intermediate exists in the form of

a DNA flap composed of a double-stranded DNA and a displaced single-strand carrying a terminal deoxyribose 5'-phosphate moiety. It is this flap structure intermediate that the 5' single-strand/duplex junction-specific enzyme DNase IV/FEN-1 recognizes and cleaves. Hence, the addition of an extrinsic dRpase is not required. Moreover, the requirement for PCNA in this pathway might not reflect the participation of Polδ/ε since the activity of DNase IV/FEN-1 is also stimulated by PCNA (Wu et al, 1996; Li X et al, 1995). In an attempt to address the issue of the identity of the DNA polymerases involved in long-patch repair, Fortini et al employed Polβ-deficient mammalian cell extracts to assay the repair of a single abasic site present on a circular duplex (Fortini et al, 1998). Both short- and long-patch repair were found to occur even in the absence of Polβ. However, whereas the rate of long-patch repair was unaffected, the repair kinetics in short-patch BER were significantly slower than with Polβ-proficient extracts (Fortini et al, 1998). These data suggest that DNA polymerases other than Polβ, probably polymerases δ and/or ε, are involved in long-patch BER in mammalian cell extracts and act as backup polymerases in the short-patch pathway when Polβ is defective. The kinetic data also imply that Polβ–dependent short-patch repair is the pathway of choice for BER. However, long-patch repair, which appears to be the minor route of BER, might still be the preferential route for some lesions. Structurally distinct lesions may have different constraints on the choice of the route of BER. In this respect, it is known that reduced AP sites and tetrahydrofuran residues cannot be repaired by the Polβ-dependent short-patch pathway (Matsumoto et al, 1994) and that repair patches of 2-6 nucleotides in length were found after repair of reduced or oxidized AP sites (Klungland and Lindahl, 1997).

The involvement of PCNA and polymerases δ and ε in the long-patch mode of BER are reminiscent of their participation in repair DNA synthesis in NER. Thus, the possibility exists that factors such as replication protein A (RPA) may have a role in BER since polymerases δ and ε are known to be stimulated by RPA. In fact, the

activity of FEN-1, the nuclease involved in long-patch BER, can also be stimulated by RPA in addition to PCNA (Biswas et al, 1997). Moreover, a potential role for RPA in the early steps of BER has been suggested by the observation that uracil DNA glycosylase can interact with the C-terminus of the 34 kDa subunit of RPA (Nagelhus et al, 1997). More recently, in an *in vitro* reconstituted system, RPA was shown to stimulate the long-patch mode of BER by assisting the repair synthesis step (DeMott et al, 1998). In Chapter IV of my thesis, I will describe my preliminary attempts to assess the role of RPA in BER using a combined biochemical and genetic approach. Briefly. I have set up *in vitro* BER assays using mammalian and yeast extracts which will allow me to test the effect of depleting RPA from the extract and the effects of RPA mutations on BER activities.

V. Nucleotide Excision Repair (NER)

The nucleotide excision repair pathway is one of the most versatile systems of DNA repair and is capable of dealing with a diverse array of structurally unrelated lesions. In general, the types of damage on which NER operates most efficiently are those which cause perturbation of the helical structure of the DNA helix (Van Houten, 1990). These lesions include UV-induced cyclobutane pyrimidine dimers and (6-4) photoproducts, various classes of chemical adducts, such as those introduced by conjugation of aminofluorene derivatives, and DNA crosslinks, including those induced by the chemotherapeutic drug cisplatin. The main feature of this evolutionarily conserved pathway of repair is that the damaged base is removed by two endonucleolytic incisions made on the damaged strand on either side of the site of damage. Hence the lesion is removed as part of an oligonucleotide of a defined length.

1. NER in *E. coli*

The most well understood NER system is the Uvr repair pathway of *E. coli* , consisting of six proteins (UvrA, B and C, helicase II, DNA polymerase I and ligase) plus other factors, such as Mfd (TRCF) and photolyase, which modulate the reaction (Van Houten, 1990; Lin and Sancar, 1992a; Van Houten and McCullough, 1994).

Damage recognition is believed to proceed when two molecules of UvrA dimerize in solution and interact with UvrB to form a A_2B complex in an ATP-dependent reaction (Orren and Sancar, 1990; Orren and Sancar, 1989; Oh et al, 1989). The resulting heterotrimer binds DNA nonspecifically; activation of the latent ATPase/helicase activity in UvrB allows the complex to translocate along the DNA searching for lesion-induced DNA distortions (Oh and Grossman, 1986, 1987, 1989). Once the structural abnormality is encountered, the helicase activity is inhibited while at the same time UvrB induces a conformational change in the DNA as evidenced by a bend of 130°C in the DNA axis, local unwinding and kinking (Shi et al, 1992; Hsu et al, 1994; Oh and Grossman, 1986; Takahashi et al, 1992; Lin et al, 1992; Lin and Sancar, 1992a). These changes, in turn, enhance the binding affinity of UvrB to the damaged region, forming the preincision complex (Oh and Grossman, 1989). Hence, specific recognition of the damage occurs at two stages: the initial damage recognition is carried out by the damage-specific UvrA protein, whereas the second stage of damage recognition exploits the enhanced affinity of UvrB for the distorted DNA. Formation of the UvrB-DNA complex then leads to the dissociation of UvrA, allowing the next player, UvrC, to bind and initiate the incision steps (Oh and Grossman, 1989). Incisions are carried out by UvrB and UvrC, which make the 3' and 5' incisions, respectively (Lin et al, 1992; Lin and Sancar, 1992b). The 4th to 6th phosphodiester bond 3' to the damaged site is first hydrolyzed (Sancar and Rupp, 1983; Beck et al, 1985; Svoboda et al, 1993a) and this reaction is also the rate-limiting step in the overall reaction (Zou et al, 1997). Subsequently, the UvrC protein cuts the 8th phosphodiester bond 5' to the damage (Orren and Sancar, 1989, Lin and Sancar, 1992b). The incision reactions require ATP binding but not hydrolysis (Orren and Sancar, 1990). The release of the 12-13 mer containing the lesion and the turnover of both UvrB and UvrC is assisted by the actions of both helicase II (also called UvrD) and DNA polymerase I, which also fills the gap (Caron et al, 1985; Orren et al, 1992). DNA ligase seals the nick and completes the reaction.

Studies with mammalian cells and *E. coli* showed that NER occurs preferentially in actively transcribed genes. Furthermore, this preferential repair is restricted to the transcribed strand of the active genes. The mechanism of

transcription-coupled repair is best understood in *E. coli* and is known to require the *mfd* gene product. Transcription-coupled repair in various systems will be discussed separately in detail, in later sections of this Chapter.

Aside from the core components essential for NER in *E. coli*, several factors have been found to be capable of modulating the repair reaction. One such class of proteins belongs to a group of heat shock proteins. The heat-labile UvrA protein is known to be stabilized by heat shock proteins of the DnaK, DnaJ, and GrpE families *in vitro* (Zou et al, 1998). These molecular chaperonins also allow UvrA to undergo multiple cycles of UvrB loading, leading to increased incision efficiency (Zou et al, 1998). Coupled with the observation that a DnaK-deficient mutant strain exhibited decreased survival and repair efficiency upon UV irradiation (Zou et al, 1998), the above observations clearly suggest that molecular chaperonins have a role in NER, at least in *E. coli*. It would be of interest to determine if the same phenomenon exists in eukaryotes, including yeast and humans, in which the process of NER has also been extensively studied.

Cyclopyrimidine dimer (CPD) photolyase, the enzyme involved in direct removal of CPD during photoreactivation, is another protein known to modulate the efficiency of NER in *E. coli*. (Sancar et al, 1984). Interestingly, stimulation of NER by photolyase occurs even in the absence of photoreactivating light (Sancar et al, 1984), suggesting that the effect is mediated by interaction between photolyase and components of the NER machinery. Since enhancement of NER efficiency by photolyase was also observed in the yeast system (Sancar and Smith, 1989), this phenomenon appears to be quite general, and suggests that photolyase should be regarded as an accessory protein in the NER pathway.

2. NER in Eukaryotes

Because I will be discussing the individual steps of eukaryotic NER in considerable detail in the next section of this Chapter, I will now present only a brief outline of the repair pathway (Fig. 2). The first step of NER, recognition of DNA damage, is believed to be performed by XPA (with RPA) in humans or Rad14 in yeast. Both proteins have been found to bind preferentially to damaged DNA.

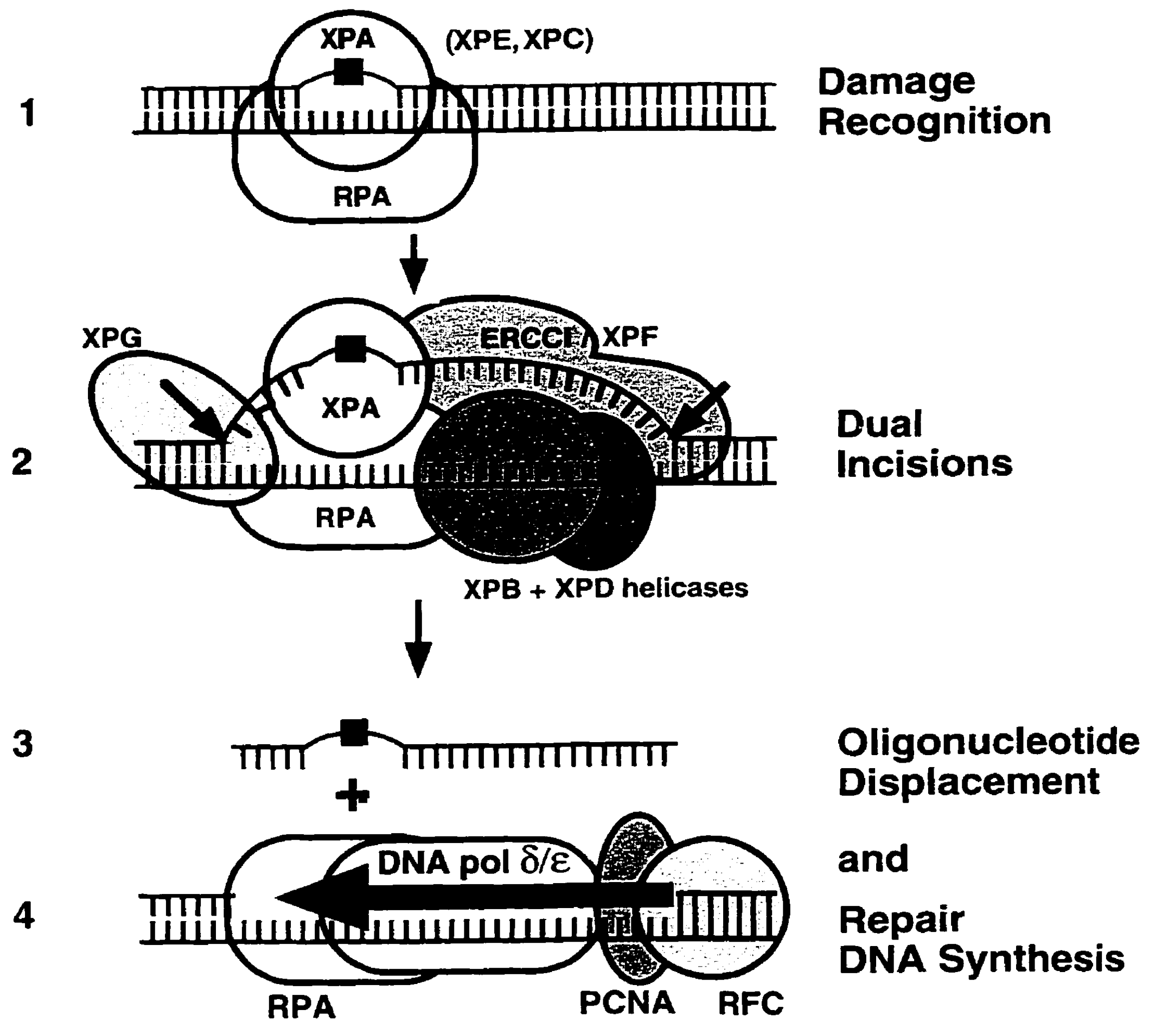

Figure 2. Steps in mammalian nucleotide excision repair

Damage recognition is followed by recruitment of proteins involved in executing the dual incisions on the damaged strand. Two endonucleases, XPG (Rad2 in yeast) and XPF-ERCC1 (Rad1-Rad10 in yeast), are responsible for introducing the incisions flanking the lesion. This incision/excision process is assisted by other factors, including XPC(Rad4) and TFIIH, the latter a multisubunit complex with two different DNA helicase activities. Removal of the cut strand, and DNA polymerization to fill in the single-strand gap, is then carried out by a multitude of factors in an analogous fashion to DNA replication. This last step requires the participation of proteins such as PCNA, replication protein A, DNA polymerases and ligases.

The importance of NER in humans is dramatically illustrated by the fact that three hereditary diseases, xeroderma pigmentosum (XP), Cockayne's syndrome (CS) and trichothiodystrophy (TTD), are caused by mutations in NER genes (Lehmann, 1987; Nance and Berry, 1992; Cleaver and Kraemer, 1989; Bootsma et al, 1998). Most afflicted individuals are hypersensitive to sunlight and have developmental abnormalities and neurological dysfunction, including mental retardation. XP patients are roughly 1000-fold more likely to develop skin cancer in sun-exposed regions of the body. The hypersensitivity of XP patients to sunlight implies that the major function of NER in humans is to repair damage induced by UV light. However, the fact that some patients slowly show progressive neurological degeneration suggests that NER may also be important for removal of DNA damage caused by carcinogens and toxic metabolic byproducts. Complementation analysis with cells derived from various patients has led to the identification of seven XP complementation groups, named XPA through XPG, corresponding to seven genes involved in NER. Repair analysis showed that cells from XP patients are deficient in NER, suggesting that the corresponding gene products represent core elements in the repair pathway. The exception to this generalization is the XPC complementation group. Cells derived from XPC patients are deficient in NER of the genome overall, but not in NER of actively transcribed genes. On the other hand, CSA and CSB mutant cells display a phenotype opposite to that of XPC; they

are deficient in the preferential repair of active genes while having normal global genome repair.

Another line of research involving rodent cells has also yielded a significant amount of knowledge in the field of NER. Many rodent cell lines sensitive to UV light have been isolated by screening mutagenized cell cultures for sensitivity to UV. Eleven known rodent cell complementation groups have been isolated, and human genes that complement their repair defects have been identified for many of the rodent groups. These *ERCC* (excision repair cross complementary) genes overlap considerably with those designated as *XP* and *CS* genes. Hence, many NER genes have had more than one name depending on whether they were isolated by complementing human XP cells or rodent UV-sensitive cell lines. For instance, *XPB* and *XPD* correspond to *ERCC3* and *ERCC2*, respectively (Table 1). Another complication regarding nomenclature in the NER field came from the realization that significant homology exists between corresponding genes in budding yeast and mammalian cells, and that much of the genetics and biochemistry of NER in *S. cerevisiae* has parallels in mammals. In yeast, most of the NER mutants were isolated as UV-sensitive *rad* mutants. Amongst the yeast *rad* strains are some that exhibit hypersensitivity to γ-radiation but not to UV light. These γ-ray-sensitive *rad* mutants have defects in other repair pathways but not in NER. The *rad* mutants relevant to NER belong to the "*RAD3* epistasis" group, and *RAD* genes that correct the UV-sensitive phenotype of yeast mutants have functional counterparts in the highly homologous mammalian system (Prakash et al, 1993). Because many of the mammalian genes are often described in conjunction with their yeast counterparts, the corresponding proteins often have more than one or even up to three different names (Table 1). For example, the yeast homologs of the mammalian NER proteins XPB(ERCC3) and XPD(ERCC2) are called Rad25 and Rad3, respectively. The high conservation of the NER systems between yeast and humans also means that information gained from biochemical and genetical studies in yeast is often directly applicable to humans. At the very least, knowledge gained in yeast has provided and

Human	Human genes complementing mutant rodent cell lines	*S. cerevisiae*	Properties/Functions
XPA		Rad14	binds damaged DNA; interacts with RPA
XPB	ERCC3	Rad25 (Ssl2)	DNA helicase (3' to 5'); TFIIH subunit
XPC	ERCC2	Rad4	binds damaged DNA; interacts with HHR23B/Rad23
XPD		Rad3	DNA helicase (5' to 3'); TFIIH subunit
XPE			binds damaged DNA
XPF	ERCC4	Rad1	subunit of DNA endonuclease for 5' side of damage
	ERCC1	Rad10	subunit of DNA endonuclease for 5' side of damage
XPG	ERCC5	Rad2	DNA endonuclease for 3' side of damage
CSA	ERCC8	Rad28	contains WD repeats; essential for TCR
CSB	ERCC6	Rad26	DNA-dependent ATPase; essential for TCR

Table 1. Nomenclature and properties of eukaryotic NER proteins

will continue to provide insight into the molecular mechanisms of NER in humans.

It should be pointed out that, although the basic mechanism of NER is similar in prokaryotes and eukaryotes, the details of the two systems are really quite different. The prokaryotic and eukaryotic NER pathways are similar in the sense that dual incisions on the damaged strand are made in an ATP-dependent manner during the process of repair. However, none of the excision repair proteins from yeast and humans shows any significant homology to the Uvr proteins required for NER in *E. coli*. In addition, many more polypeptides are needed in eukaryotes to carry out NER than are needed in *E. coli*. Hence, NER appears to represent an excellent example of convergent evolution that is so efficient in repairing bulky DNA lesions that it has arisen independently in organisms that diverged very early in the process of evolution.

VI. Steps of NER in Eukaryotes

1. Damage Recognition

The first and rate-limiting step of NER is the recognition of DNA damage among the vast excess of undamaged DNA in the genome. This step is thought to be mediated by Rad14 in *S. cerevisiae* and its homolog, XPA, in humans. Both these proteins are known to be essential for the process of NER, since inactivation mutations result in complete elimination of NER activity and a dramatic increase in cellular sensitivity to UV light. Rad14 and XPA are zinc metalloproteins and display preferential binding to DNA damaged by UV irradiation or chemical agents, including cisplatin and AAAF. Replication protein A (RPA), first discovered as a factor essential for SV40 DNA replication *in vitro*, is another protein capable of preferential binding to various types of DNA damage (He et al, 1995; Burns et al, 1996; Clugston, 1992). It is a heterotrimeric protein that binds to single-stranded DNA tightly and only weakly to double-stranded DNA. For this reason, RPA is also called human single-stranded DNA-binding protein (HSSB). RPA associates tightly with XPA to form a heterodimeric complex, and the interaction appears to be

mediated by the 70 kDa and the 34 kDa subunits of RPA (RPA1 and RPA2) and the middle and N-terminal regions of XPA (Stigger et al, 1998; Saijo et al, 1996; He et al, 1995; Li L et al, 1995; Matsuda et al, 1995; Lee et al, 1995). This XPA-RPA complex appears to be the functional form of the damage recognition entity as it displays higher affinity for damaged DNA than does either protein alone (He et al, 1995; Li L et al, 1995). Whether this interaction and synergism between XPA and RPA is conserved in other organisms such as *S. cerevisiae* has not yet been determined. In Chapter II of this thesis, I provide evidence that Rad14, the yeast homolog of XPA, may not interact directly with yeast RPA. The implication of this observation with respect to both evolution and the mechanistic process of yeast NER will also be discussed.

Studies attempting to define the molecular functions of the *XPC* gene product suggested that XPA-RPA might not be the earliest damage recognition complex to initiate NER. The *XPC* gene product, in conjunction with HHR23B, one of the two mammalian homologs of *S. cerevisiae* Rad23, was found to act at a step earlier than XPA (Sugasawa et al, 1998). Coimmunoprecipitation and DNase I footprinting data clearly demonstrated that XPC-HHR23B binds in a specific manner to lesions induced by UV, cisplatin and AAAF (Sugasawa et al, 1998). These findings are consistent with earlier reports showing that XPC-HHR23B acts at an early stage of NER (Mu et al, 1997). It therefore appears that damage recognition in eukaryotes, much like that in *E. coli*, involves a two-stage process with the XPC-HHR23B complex possibly being the primary damage detector, followed by damage verification by XPA-RPA. Similarly, Rad4-Rad23, the yeast homolog of XPC-HHR23B, was recently found to preferentially bind damaged DNA (Jansen et al, 1998).

Another protein that has the ability to recognize damaged DNA is a DNA damage-binding protein, denoted UV-DDB. The activity of this protein is missing in some cell lines established from the XPE complementation group (designated DDB$^-$ cells) (Chu and Chang, 1988; Kataoka and Fujiwara, 1991; Keeney et al, 1992). This DNA-binding activity has been purified as a 127 kDa protein and also as a complex of two peptides of 127 and 48 kDa (Abramic et al, 1991; Keeney et al, 1993). The DNA

repair defect in DDB$^-$ but not in DDB$^+$ cells can be corrected by microinjection of purified UV-DDB (Keeney et al, 1994). Mutations have been detected in the cloned gene for p48 but not in p127 (Nichols et al, 1996). The recent demonstration that p48 could activate the ability of p127 to bind damaged DNA, and that previously identified mutations in p48 abolished this activation, provide the strongest evidence to date that p48 mutations are the causative defects in the DDB$^-$ variants of XPE (Hwang et al, 1998). It was also reported that RPA could rescue the repair defect in XPE cell extracts and that RPA interacts with UV-DDB, raising the possibility that RPA might be involved in the underlying repair defect associated with XPE (Kazantsev et al, 1996; Otrin et al, 1997). However, careful analysis of the relationships of RPA and UV-DDB to the defect in XPE cells has not reproduced the findings reported by Kazantsev et al (Otrin et al, 1998). The repair-stimulating effect of RPA reported by Kazantsev et al and previously noted by Coverley et al (1991 and 1992) was found not to be specific for XPE cell extracts; it was also present in normal cell extracts (Otrin et al, 1998). These findings, and the fact that RPA appears to be present in normal amounts in XPE cells (Otrin et al, 1998) and that no mutation has been found in any of the RPA subunits (Kazantsev et al, 1996), make it very unlikely that RPA is primarily disregulated in XPE. Regardless of the identity of the factors underlying XPE , the protein(s) involved do not appear to be required for the NER reaction. These factors may only play an accessory modulating role in NER, and may not be essential for repair activity, as seems to be also the case with another factor named IF7 (Aboussekhra et al, 1995). The non-essential function of the putative XPE factor is also consistent with the observation that XPE mutant cells have 50% of normal repair capacity and that XPE patients are only mildly affected (Cleaver, 1989).

The human damage-specific binding protein XPA, the yeast XPA homolog, Rad14, and UV-DDB each display higher affinity for (6-4) photoproduct than for CPD (Jones and Wood, 1993; Reardon et al, 1993; Hwang and Chu, 1993; Abramic et al, 1991, Treiber et al, 1992). This property is in agreement with the observation that (6-4) photoproducts are eliminated *in vivo* much more efficiently than CPDs (Mitchell 1988a, b). More rapid repair of (6-4) photoproducts has also been observed *in vitro* with human extracts (Szymkowski et al, 1993). If XPC-HR23B provides the first level

of damage discrimination equally efficiently for all types of lesions, faster repair of (6-4) photoproducts might imply a tighter interaction or more efficient recognition by the damage verifier XPA-RPA. Alternatively, the XPC-HR23B complex itself may exhibit differential affinity for various lesions.

2. Dual Incision/Excision

One of the main features of NER that distinguishes it from other forms of repair rests in the way the lesion is removed. Two incisions flanking the DNA lesion are made in the damaged strand, which is eventually released as an oligonucleotide containing the damage. The size of the excised fragment ranges from 24 to 32 nucleotides in eukaryotes, with the actual incision sites varying according to the type of damage (Svoboda et al, 1993a, b; Mu et al, 1995) and the sequence context (Huang and Sancar, 1994). For instance, a DNA substrate containing either a cyclobutane pyrimidine dimer or a psoralen monoadduct is incised at the 5th to 6th phosphodiester bond 3' and at the 22nd to 24th phosphodiester bond 5' to the lesion, giving rise to a released single-stranded fragment of 27 to 32 nucleotide in length (Svoboda et al, 1993b). Hence, it appears that, regardless of the sites of incision, the length of the released fragment is always approximately 24-32 nucleotides (Huang and Sancar, 1994; Moggs et al, 1996; Mu et al, 1995; Mu et al, 1996; Matsunaga et al, 1995; Svoboda et al, 1993a, b; Huang et al, 1992; Hansson et al, 1989). Perhaps, the excinuclease is assembled in such a topological way that the exact distance between the two incisions is relatively constant.

The two endonucleases responsible for the incisions are XPG, which makes the 3' incision, and XPF-ERCC1, which makes the 5' incision. The yeast system is analogous to that in humans, with the corresponding nucleases named Rad2 and Rad1-Rad10. XPG and Rad2 have significant amino acid sequence homology to the human flap endonuclease (FEN1), and they all utilize as substrates a Y structure called a DNA flap (O'Donovan et al, 1994; Harrington and Lieber, 1994). In other words, they are structure-specific endonucleases that cut at the border between single-stranded and duplex regions. Structure-specific junction-cutting activity is

also possessed by the yeast Rad1-Rad10 complex and its human counterpart, XPF-ERCC1 (Bardwell et al, 1994a; Matsunaga et al, 1996; Park et al, 1995a; Bessho et al, 1995b). However, in this case, the polarity of the activity is opposite to that of XPG/Rad2. The data obtained with undamaged model substrates in the form of a bubble or flap structure established that XPG/Rad2 and XPF-ERCC1/Rad1-Rad10 were the endonucleases responsible for the 3' and 5' incisions, respectively. This conclusion was later supported by studies utilizing damaged DNA and specific antibodies in a reconstituted system. Using a DNA duplex containing a cholesterol moiety, an excellent synthetic substrate for the human excinuclease, and antibodies specific to XPG, Matsunaga et al (1995) concluded that XPG makes the 3' incision since anti-XPG antibodies inhibited 3' incision without affecting the 5' incision. In addition, in the absence of XPF-ERCC1, a partial repair complex containing XPG can produce normal levels of 3' incision (Mu et al, 1996). Similar experiments have confirmed that XPF-ERCC1 is the endonuclease making the 5' incision (Mu et al, 1996; Matsunaga et al, 1995). Kinetic experiments revealed that the 3' incision precedes the 5' incision (Mu et al, 1996; Matsunaga et al, 1995), although other time course studies showed that both incisions are made in a near-synchronous manner (Moggs et al, 1996). The specific protein requirement for dual incisions has been addressed in reconstituted systems using purified repair factors in the human and highly analogous yeast systems (Mu et al, 1995; Aboussekhra et al, 1995; Guzder et al, 1995c). In addition to the two endonucleases described above, four other factors are required: XPA(Rad14), RPA, TFIIH and XPC-HHR23B (Rad4-Rad23). The requirement of RPA for the 5' and 3' incisions is consistent with its role in damage recognition as a complex with XPA. However, RPA appears to have a more direct role in the incision steps, because it was found to dramatically stimulate the junction incision activities of XPF-ERCC1 and XPG (Matsunaga et al, 1996; Bessho et al, 1997b). This effect of RPA may be mediated by direct protein-protein interaction since RPA has been found to bind to both XPG and XPF (He et al, 1995; Bessho et al, 1997b; Li L et al, 1995). Moreover, XPA can also interact with XPF-ERCC1, mainly through the ERCC1 subunit (Bessho et al, 1997b; Li et al, 1994; Nagai et al, 1995; Park and Sancar, 1994). Taken together, these observations suggest that XPA, in

conjunction with RPA, may act, not only as a damage-recognition complex, but also to nucleate the formation of a properly oriented nuclease complex for dual incisions. This notion is strengthened by the recent observation that RPA binds ssDNA with a defined polarity and that this polarity is crucial for the proper positioning of the two nucleases (de Laat et al, 1998). Some of the protein-protein interactions observed in the human system are also conserved in yeast. For instance, Rad14, like its human homolog, appears to interact with Rad1-Rad10 to form a ternary complex (Guzder et al, 1996). My own experiments (Chapter II) show that the interaction between XPG and RPA in humans is paralled by a Rad2-Rpa interaction in yeast.

Another essential factor for the excision nuclease activity in NER is TFIIH, a multisubunit factor originally identified as a basal transcription initiation factor for RNA polymerase II (Conaway et al, 1993; Zawel and Reinberg, 1995). The additional requirement for TFIIH in DNA repair indicates that this factor should be considered as a dual function repair/transcription factor (Drapkin and Reinberg, 1994a, b). Two of the subunits of human TFIIH, XPB and XPD, and their yeast counterparts, Rad3 and Rad25, possess 3'-5' and 5'-3' DNA helicase activities, respectively (Sung et al, 1988; Harosh et al, 1989; Sung , Bailly et al, 1993; Guzder et al, 1994; Qiu et al, 1993). Genetic studies in yeast showed that, whereas the Rad3 helicase activity is required for NER but not transcription, the helicase activity of the Rad25 protein is required for both transcription and NER (Sung et al, 1988; Guzder et al, 1994; Qiu et al, 1993). In transcription, the ATP-dependent helicase activity in TFIIH is used to catalyze opening of a 10-20 bp region around the promoter (Holstege et al, 1996), and an analogous reaction is thought to occur to promote the unwinding of dsDNA around a DNA lesion (Schaeffer et al, 1993; Weeda et al, 1990). This proposed function of TFIIH has recently gained experimental support from permanganate footprinting analysis around the site of a cisplatin-DNA adduct in mutant and wild-type human extracts (Evans et al, 1997a, b). Open complex formation around the lesion creates a structure conducive to cleavage by XPG (Evans et al, 1997a). Mutations in XPB or XPD, the DNA helicase subunits of TFIIH, completely abolished DNA strand opening and the incision reactions (Evans et al, 1997b). The requirement for

hydrolysis of ATP for full DNA opening is also consistent with the requirement for the two ATPase/DNA helicase activities of XPB and XPD (Evans et al, 1997a, b). Another study from the laboratory of Aziz Sancar using purified factors led to the same conclusion-that the helicase activities of TFIIH are responsible for DNA unwinding prior to the dual incisions (Mu et al, 1997). In addition, they identified three reaction intermediates which may represent the sequence of events during NER (Mu et al, 1997). The formation of the first stable complex requires XPA, RPA, XPC-HHR23B and TFIIH, as well as ATP hydrolysis. In this complex, the DNA was unwound on either side of the lesion. The incorporation of XPG resulted in a tighter, more stable complex, even when XPG had lost its nuclease activity. Finally, binding of XPF-ERCC1 to the second complex required the presence of XPG, independent of its nuclease function. This observation is consistent with an earlier findings that an active-site mutant XPG protein with total loss of nuclease activity was still required for 5' incision by the XPF-ERCC1 nuclease (Wakasugi et al, 1997). Binding of XPF-ERCC1 completed the assembly of a complex containing all 6 repair factors and immediately led to dual incisions and release of the excised oligomer of 24-32 nucleotides (Mu et al, 1997). Overall, the findings from this study were consistent with those seen in antibody inhibition and reconstitution experiments in which repair factors were systematically omitted (Mu et al, 1996; Matsunaga et al, 1995). Interestingly, specific binding to DNA lesions by XPA or RPA, or by the XPA-RPA complex, could not be detected, even by sensitive methods such as permanganate footprinting (Mu et al, 1997). These results suggest that formation of a more stable complex requires collaboration of multiple repair proteins. In this respect, it is noteworthy that protein-protein interactions have also been observed between XPA and TFIIH (Park et al, 1995b), TFIIH and XPG or Rad2 (Iyer et al, 1996; Mu et al, 1995; Bardwell et al, 1994b), and TFIIH and XPC or Rad4 (Drapkin et al, 1994; Bardwell et al, 1994b).

Another factor required for the formation of the human excinuclease complex is XPC and its stably associated protein, HHR23B. Recent data indicating a role of XPC-HHR23B in NER in damage recognition was discussed in section VI-1. Briefly, it was suggested that XPC-HHR23B may be the earliest damage recognition

complex in the process of NER (Sugasawa et al, 1998), before the damage recognition step carried out by XPA-RPA. Other studies showed that the formation of the first stable complex requires XPA, RPA, XPC-HHR23B and TFIIH as well as ATP hydrolysis (Mu et al, 1997). Although these data are consistent with XPC-HHR23B functioning as damage recognition protein, other data indicate that XPC-HHR23B may have additional functions in NER. In reconstitution experiments, omission of XPC led to degradation of the damaged strand, presumably by the nonspecific nuclease activity of XPG or XPF-ERCC1 (Mu et al, 1996). Thus, a possible role of XPC may be to stabilize the unwound state generated by TFIIH, preventing further unwinding past the normal cut sites; XPC may bind to the damaged strand in the process, and protect it from nuclease attacks. Wakasugi and Sancar (1998) reported that XPC dissociated from the preincision complex upon the entry of XPG followed by XPF-ERCC1. Hence, whatever the role of XPC is in NER, it seems to have fulfilled all its functions before the two incisions are made. Such an early requirement of XPC in NER is compatible with its proposed role in the earliest step of damage recognition (Sugasawa et al, 1998), and with observations indicating that damage binding by XPC is a prerequisite for the subsequent actions of other factors, including those that promote DNA opening. Finally, it should be mentioned here that, under some conditions, XPC is not required for NER to occur. Such conditions are fulfilled in the case of actively transcribed genes and in experiments employing an artificial "bubble" template which mimicks that of an elongating RNA polymerase II transcription complex. The relationship of XPC and transcription-coupled repair will be discussed in detail in sections VII-1 and VII-2-ii-c.

3. Repair Synthesis

All of the protein factors implicated in DNA replication, except DNA polymerase α, also have a role in filling the single-stranded gap in DNA introduced by the action of the NER excinuclease. The two processes, DNA replication and repair synthesis, are thus analogous but not identical. Strong evidence from several lines of research has led to the conclusion that DNA polymerases δ and ε are the two

enzymes capable of repairing the single-stranded gap after the excision step. Using specific inhibitors selective for different DNA polymerases, it was concluded that repair DNA synthesis was catalyzed by DNA polymerase δ and/or ε (Coverley et al, 1992; Popanda and Thielmann, 1992; Hunting et al, 1991, Dresler et al, 1986; Nishida et al, 1988). Other studies using more definitive approaches, such as biochemical fractionation and antibody-inhibition experiments, arrived at the similar conclusion that either DNA polymerase δ or ε can act as the repair polymerase (Syvaoja et al, 1990; Zeng et al, 1994). Genetic studies in yeast also suggest that DNA polymerases δ and ε are both capable of performing repair synthesis, and that, in the absence of one, the other can efficiently substitute (Budd and Campbell, 1995). The involvement of these two polymerases in DNA repair is further strengthened by the finding that PCNA, which can enhance the activities of DNA polymerases δ and ε, is required for repair synthesis in cell-free extracts (Shivji et al, 1995; Nichols and Sancar, 1992; Shivji et al, 1992). Furthermore, many immunostaining studies showed that PCNA can be detected in association with the chromatin of nonproliferating cultured cells after UV irradiation (Prosperi et al, 1993; Stivala et al, 1993; Jackson et al, 1994; Celis and Madsen, 1986; Toschi and Bravo, 1988). These experiments strongly suggest that PCNA relocates to sites of repair *in vivo* and forms a tight complex with chromatin. Importantly, this relocation did not occur in UV-irradiated *XPA* and *XPG* cells, indicating that the association with chromatin of PCNA requires NER (Miura et al 1992a, b; Aboussekhra and Wood, 1995). The increased UV sensitivity of yeast cells expressing a conditional PCNA mutant also provided *in vivo* evidence for a role of PCNA in repair (Ayyagari et al, 1995).

The participation of PCNA in NER also implicates replication factor C (RFC) in NER. RFC functions to load the PCNA clamp onto DNA, thereby recruiting DNA polymerases to the site of DNA synthesis (Mossi and Hubscher, 1998). That RFC is also required for NER has been demonstrated in experiments involving reconstitution of NER with human cell extracts or with purified components (Aboussekhra et al, 1995; Shivji et al, 1995). Furthermore, the ability of PCNA mutations to suppress the DNA repair defects of mutations in the RFC gene

provides *in vivo* evidence that RFC functions in repair through its interaction with PCNA (McAlear et al, 1996).

Apart from its role in damage recognition, RPA may also participate in the repair synthesis step of NER. Using purified components, Shivji et al (1995) concluded that polymerase ε is most suited to the task of creating NER patches only when PCNA, RFC and RPA are also included. RPA may stimulate, possibly through direct interactions, the activities of DNA polymerases δ and ε, both of which are implicated in repair synthesis (Podust and Hubscher, 1993; Tsurimoto and Stillman, 1989; Lee et al, 1991). Other evidence for the involvement of RPA in NER came from the isolation of yeast mutants harboring mutations in the *RFA1* and *RFA3* genes, which encode the largest and the smallest subunits of RPA, respectively. These yeast mutants display increased sensitivity to UV, which is indicative of an *in vivo* defect in NER (Longhese et al, 1994; Smith and Rothstein, 1995; Firmenich et al, 1995; Maniar et al, 1997). In Chapter II of my thesis, I describe the development of an *in vitro* NER system using yeast whole cell extracts and its use in demonstrating the requirement of yeast RPA in NER. I will also report that two yeast mutant strains harboring mutations in the *RFA2* gene establish an *in vivo* role for yeast RPA in NER. After repair synthesis, one of the three known human ligases can seal the nick that is left behind (Tomkinson and Mackey, 1998). The most likely candidate is ligase I as mutations in its gene result in hypersensitivity to DNA damaging agents, including UV light and alkylating agents (Barnes et al, 1992). *In vitro*, ligase I can also efficiently provide the nick-sealing activity during repair synthesis (Shivji et al, 1995; Aboussekhra et al, 1995).

The involvement in NER of polymerases δ and ε, PCNA, RFC and ligase I is also supported by the observation that they are all present in a high molecular weight multiprotein complex termed the "repairosome", isolated from HeLa extract by He and Ingles (1997). The isolation of such a complex also argues that at least some of the repair factors can exist in a preassembled form in the absence of damaged DNA. Repairosome complexes have also been isolated from yeast (Svejstrup et al, 1995); however, the particular complex described by Svejstrup et al

differs from the human preparation in that critical factors such as RPA and DNA polymerase are missing; this complex is, therefore, incapable of supporting the complete reactions of NER (unpublished results cited in Svejstrup et al, 1995). In addition, another group, using somewhat different chromatographic conditions, failed to detect the existence of an assembled repairosome complex in yeast, although they did report the isolation of several different multiprotein subcomplexes (Guzder et al, 1996). The human repairosome complexes described by He and Ingles (1997), on the other hand, can support all the steps of NER *in vitro* and thus may represent a *bona fide* active repair entity. It should be pointed out, however, that only 20 % of the repair factors, at most, were assembled as part of the human repairosome (He and Ingles, 1997). The existence of such high molecular complexes does not, of course, necessarily exclude the possibility that some, or even most NER reactions are initiated by sequential assembly of repair factors around DNA lesions.

VII. Two Subpathways of NER

1. Global Genome Repair

As in *E. coli*, transcriptionally active genes in eukaryotic cells are repaired faster than inactive genes and non-transcribed sequences. This more rapid repair of active genes is attributed to the preferential repair of transcribed strands over the nontranscribed strands. This phenomenon of transcription-coupled repair (TCR) will be dealt with later in this Chapter. The other branch of NER, termed global genome repair, is particularly important for the removal of lesions in non-transcribed DNA, which is not subject to transcription-coupled repair and, hence, is repaired at a slower rate. Yeast genes have been identified that are specifically involved in global genome repair. Repair of the silent mating-type locus *HMLα* and the nontranscribed strand of the active gene *RPB2* are completely abolished by disruption of the *RAD7* or *RAD16* gene (Verhage et al, 1994). In these mutants, repair of the transcribed strand is not affected, suggesting that, at least when the gene

is transcribed, the function of *RAD7* and *RAD16* is restricted to repair of the nontranscribed strands. However a contribution of *RAD7* and *RAD16* to repair of the transcribed strand was also revealed by Verhage et al. (1994). These investigators performed a careful analysis of yeast mutants with alterations in global genome and transcription-coupled repair by combining *rad7* or *rad16* deletion mutations with a null mutation in the *RAD26* gene, which is required for transcription-coupled repair (van Gool et al, 1994; Verhage et al, 1996). The repair rate of the transcribed strand of the *RPB2* gene was found to be slower in mutants that were deficient in *rad7* and *rad26* than it was in the *rad26* single mutants defective only in TCR (Verhage et al, 1996). Thus, the two NER subpathways of global genome repair and transcription-coupled repair are partially overlapping. The fact that repair of all types of DNA sequences, transcribed or not, appears to contain a *RAD7/16*-dependent component indicates that global genome repair truly reflects repair of the overall genome, as its name implies. Global genome repair should also be distinguished from the core NER machinery in that the latter represent the basic components essential for the reaction of NER (e.g. Rad14, the TFIIH components).

The cloned Rad7 sequence did not reveal any significant sequence similarity to other domains or proteins except that it contains regions of marked hydrophobicity and 12 leucine-rich motifs which could be involved in protein-protein interactions (Perozzi and Prakash, 1986; Schneider and Schweiger, 1991). *RAD16* encodes a protein with two potential zinc finger DNA-binding domains and the seven DNA-dependent ATPase motifs found in members of the SWI2/SNF2 protein family (Bang et al, 1992). The SWI/SNF complex, the prototypic member of this family, appears to be involved in chromatin remodelling to facilitate the binding of transcription factors to nucleosomal DNA (Eisen et al, 1995). By analogy, it is possible that Rad16 may be involved in remodelling chromatin to allow the NER machinery to gain access to lesions within chromatin. Furthermore, the identification of Sir3, a protein involved in chromatin repression, as an interacting partner with Rad7 in a two- hybrid interaction study also pointed to a chromatin connection regarding the proteins involved in global genome repair (Paetkau et al, 1994). In fact, recent studies showed that Rad7 and Rad16 form a stable complex *in*

vitro and *in vivo* (Guzder, 1997; Wang Z et al, 1997). Complex formation between the two proteins is also consistent with the observation that extracts from *rad7* and *rad16* strains did not complement each other *in vitro* (Chapter II of this thesis; Wang Z et al, 1997). This *in vitro* finding contradicts the proposed role of Rad7-Rad16 in chromatin remodelling, since the DNA templates used in the experiments reported in Chapter II and those of Wang Z et al (1997) were plasmid DNAs not pre-assembled into chromatin. Although unlikely, it is possible that some of the DNA templates used in these studies with crude extracts were inadvertently assembled into nucleosomal DNA. However, Rad7 and Rad16 are dispensable for NER even in a defined reconstituted system using purified proteins (Guzder et al, 1995c), arguing against a role involving chromatin. Recent data are beginning to clarify the biochemical functions of the Rad7-Rad16 complex in NER. In the presence of ATP, the Rad7-Rad16 complex was found to exhibit highly specific binding to UV-damaged DNA (Guzder et al, 1997). Importantly, inclusion of this complex in a reconstituted NER system resulted in marked stimulation of damage-specific incision (Guzder et al, 1997). It therefore seems that Rad7-Rad16, like XPA-RPA and XPC-HHR23B in humans, may be involved in recognition of DNA lesions, possibly in all types of DNA sequences *in vivo*, resulting in overall stimulation of NER.

Interestingly, identification of the human homologs of *RAD7* and *RAD16* has not been reported so far. The phenotype of defective global genome repair is manifested, however, by cells derived from the XP complementation group C. Like *rad7* and *rad16* mutant strains, *XPC* cells are defective in removal of DNA lesions on nontranscribed sequences and the nontranscribed strand of active genes, but are proficient in transcription-coupled repair (Venema et al, 1990b,1991). Furthermore, the residual repair activity in *XPC* mutant cells can be abolished by treatment of the cells with α-amanitin, an inhibitor of transcription, suggesting that transcription-coupled repair is the only operational NER pathway in these cells (Carreau and Hunting, 1992). *In vivo*, most of human XPC was found to complex with HHR23B, but not with HHR23A, which shares significant homology with HHR23B (Masutani et al, 1994). Both HHR23A and HHR23B are human homologs of the *S. cerevisiae*

repair protein Rad23 (Masutani et al, 1994) and both have been shown to stimulate the repair activity of XPC (Sugasawa et al, 1996; Sugasawa et al, 1997). As in human cells, Rad23 in *S. cerevisiae* cells appears to complex with Rad4, the yeast counterpart of human XPC (Guzder et al, 1995b). More recently, both XPC-HHR23B and Rad4-Rad23 complexes were shown to be essential for the incision steps of NER in defined reconstituted systems (Aboussekhra et al, 1995; Guzder et al, 1995c). These observations argue against a role for XPC-HHR23B and Rad4-Rad23 only in global genome repair, and suggest that they may have a more fundamental role in the core reaction of NER. However, it is now known that certain kinds of lesions, usually those that cause large DNA distortions, can be repaired without XPC *in vitro* (Mu et al, 1996; Mu and Sancar, 1997b). That large, bulky adducts in DNA may produce sufficient unwinding of local helical structure to mimick a transcription bubble, thus subsuming the requirement of XPC, has important implications for the mechanism of transcription-coupled repair; this finding may also explain the proficiency of TCR in human *XPC* mutant cells (see section VII-2-ii).

2. Transcription-Coupled Repair (TCR)

i. TCR in *E. coli*

In 1989, Mellon and Hanawalt showed that induction of the *E. coli* lactose operon selectively increased repair of its template strand (Mellon and Hanawalt, 1989). This efficient repair of the transcribed strand was hypothesized to have resulted from a direct coupling between a stalled RNA polymerase complex at a lesion and the repair machinery. It turned out that an additional factor was required to mediate coupling between the transcription and repair machineries. A factor was purified by nature of its ability to restore preferential repair of the transcribed strand in a highly defined system (Selby and Sancar, 1991). This factor was designated transcription-repair coupling factor (TRCF) and later identified as the product of the *mfd* (for mutation frequency decline) gene (Selby et al, 1991; Selby and Sancar, 1993; Selby and Sancar, 1994).

The sequence of the gene encoding TRCF/Mfd revealed the presence of conserved ATPase/helicase motifs near the central region (Selby and Sancar, 1993). However, the purified protein does not show any helicase activity but has a weak DNA-dependent ATPase activity (Selby and Sancar, 1993; Selby and Sancar, 1994; Selby and Sancar, 1995b). This ATPase activity is important for TRCF to mediate displacement of a stalled *E. coli* RNA polymerase from a lesion, a step requiring hydrolysis of ATP (Selby and Sancar, 1995a). The exact mechanism of this displacement step is not known. However, several observations suggest that specific protein-protein interactions between TRCF and RNA polymerase are probably involved. First, displacement of RNA polymerase by TRCF occurs only with *E. coli* but not T7 RNA polymerase (Selby and Sancar, 1993). Second, TRCF does not show any preferential binding to damaged DNA or nucleic acid structures inherent to a transcription elongation complex, including a premelted "bubble" structure (Selby and Sancar, 1995a). Third, TRCF can bind directly to free RNA polymerase and DNA-bound initiation and elongation complexes (Selby and Sancar, 1995a). However, stimulation of repair by TRCF does not occur in the promoter region and can be detected only 15 nucleotides downstream of the start site, suggesting that TRCF exerts its effect only on elongating RNA polymerase (Selby and Sancar, 1995b). This coupling effect of TRCF is due to increased delivery of UvrB to the damage site through interaction with the UvrA subunit of the A_2B complex, an intermediate of the *E. coli* excinuclease (Selby and Sancar, 1995b). A 140 amino acid domain near the N-terminus of TRCF with significant sequence similarity to cloned UvrB proteins is required for the UvrA interaction (Selby and Sancar, 1995a). In sum, the experimental findings are consistent with the model that, upon binding to an elongating RNA polymerase stalled at a lesion, TRCF releases the polymerase and the aborted transcript and recruits the A_2B complex to the damage site by binding to UvrA. UvrB then binds tightly to the damaged DNA and initiates strand incisions by recruiting UvrC. The remaining steps are the same as the basic steps of NER in *E. coli*.

In addition to TRCF, other factors may modulate strand-specific repair *in vivo*. Indeed, TCR does not occur in UV-irradiated cells lacking the mismatch repair

proteins, MutL or MutS (Mellon and Champe, 1996). However, not only did extracts made from *mutL⁻* or *mutS⁻* cells support wild-type levels of TCR, supplementing the mutant extracts with purified Mut proteins had no effect on repair (Selby and Sancar, 1995b). Thus, at least *in vitro*, mismatch repair proteins do not appear to have any role in TCR in *E. coli*.

ii. TCR in eukaryotes

The phenomenon of TCR was first shown in chinese hamster cells and later demonstrated in other mammalian cells, in yeast and in *E. coli*. Bohr and Hanawalt showed that the loss of cyclobutane dimers from the transcriptionally active DHFR gene of CHO cells was more efficient than in the genome overall (Bohr et al, 1985). This phenomenon, referred to as "preferential repair" of active genes, was subsequently shown to be the result of selective repair of the transcribed (template) strand (Mellon et al, 1987). That preferential repair is localized to the transcribed but not the nontranscribed strand of active genes argues against it being a consequence of differences in chromatin structure limiting the accessibility of NER machinery to active versus inactive genes. Other evidence that implicated a more intimate relationship between transcription and strand-specific repair includes the demonstration that TCR is strictly dependent on transcription. Different means of abolishing transcription also eliminated TCR in mammalian cellss e.g. treatment with α-amanitin, a potent inhibitor of transcription by RNA polymerase II (Christians and Hanawalt, 1992; Leadon and Lawrence, 1991), or the heat-inactivation of a temperature-sensitive RNA polymerase II in *S. cerevisiae* (Leadon and Lawrence, 1992). TCR in eukaryotes is complicated by the finding that the RNA polymerase II basal transcription factor TFIIH is also required for NER. That factors other than the core NER machinery also appear to be involved in the process of TCR in eukaryotes, as well as *E. coli*, is suggested by the lack of TCR in cells derived from the human hereditary disease Cockayne syndrome (see next two sections).

a. Connection between repair and transcription-TFIIH

The discovery that two of the subunits of TFIIH are encoded by the same genes mutated in XPB and XPD patients generated much excitement in both the DNA repair and transcription communities. For the first time, an important basal transcription factor, TFIIH, was directly linked to the important cellular process of DNA repair (Svejstrup et al, 1996). Direct demonstration of the participation of TFIIH in NER came from the demonstration that microinjection of TFIIH into *XPB* and XPD cells corrected their defect in NER (van Vuuren et al, 1994). *In vitro* experiments also showed that purified TFIIH could correct defective NER in cell-free extracts derived from XPB and XPD cells (van Vuuren et al, 1994; Schaeffer et al, 1994; Drapkin et al, 1994). Similar experiments with yeast also showed that defective NER in *rad3* and *rad25* mutant extracts could be corrected by purified yeast TFIIH (also called factor b) (Wang et al, 1994). Since the addition of purified Rad3 protein alone did not rescue the repair defect in an extract from *rad3* cells, it appeared that the whole TFIIH complex functions in NER (Wang et al, 1994). Subsequent experiments also showed that the yeast genes *SSL1, TFB1,* and *TFB2,* which encode subunits of TFIIH, and the human counterpart of the*TFB2* gene product, p52, were each required for both NER and transcription by RNA polymerase II (Wang et al, 1995; Marinoni et al, 1997; Feaver et al, 1997). Hence, TFIIH is now viewed as a dual function repair/transcription factor.

Biochemically, TFIIH has been described as a component of two large multiprotein complexes: 1) holo-TFIIH, which consists of TFIIH, and a kinase complex, termed TFIIK, capable of phosphorylating the C-terminal domain (CTD) of the largest subunit of RNA polymerase II (this holo-TFIIH is the form of TFIIH that is active in transcription); and 2) a large complex, termed the "repairosome", that is devoid of TFIIK but contains other gene products essential for NER, including Rad1, Rad2, Rad4, Rad10 and Rad14 (Svejstrup et al, 1995). However, other factors, such as RPA, DNA polymerases and ligase, are missing in this yeast repairosome. Hence, although it can complement repair-deficient mutant extracts missing one of the Rad proteins, the yeast repairosome, by itself, is incapable of supporting the complete reactions of NER (unpublished results cited in Svejstrup et al, 1995). The lack of

RPA in this preparation also means that this yeast repairosome is unlikely to support the incision/excision steps of NER. This situation is in contrast to the high molecular weight human repair complex purified using XPA as affinity ligand; this complex has been shown to support all steps of NER *in vitro* (He and Ingles, 1997). The possibility of a role for TFIIK in NER has been tested using a yeast strain with a temperature-sensitive mutation in the kin28 polypeptide, one of the subunits of TFIIK. These mutant cells displayed no significant increase in UV sensitivity at the nonpermissive temperature, suggesting that TFIIK is dispensable for NER (Valay et al, 1995). This *in vivo* finding is also corroborated by the demonstration that both yeast and human TFIIK are not required for NER in *in vitro* systems (Svejstrup et al, 1995; Mu et al, 1995; Aboussekhra et al, 1995; Guzder et al, 1995c).

Because TFIIH can exist in both a transcription-competent form and within a repairosome complex, it was suggested that the dynamic interchange between these two forms may provide a molecular mechanism to explain the phenomenon of TCR (Friedberg, 1996; Svejstrup et al, 1995). During transcription initiation, TFIIH exists as the TFIIK-containing holo-TFIIH, which possesses all enzymatic activities required for open complex formation and promoter clearance. On the other hand, when a transcription elongation complex arrests at a DNA lesion, TFIIH might be converted into the repair form in association with NER proteins, thus facilitating strand-specific repair. However, critical experimental evidence supporting this model is currently lacking. In fact, several studies have shown that TFIIH dissociated from the initiation complex after 30 ribonucleotides were synthesized, and was not present in the elongation complex (Dvir et al, 1997; Goodrich and Tijan, 1994; Zawel et al, 1995). Furthermore, this model does not take into account the involvement of other cellular factors, such as the *CSA* and *CSB* gene products, in TCR (see sections VII-2-ii-b, c below). All available evidence suggests that the dual functions of TFIIH in transcription and NER are the consequence of similar roles for TFIIH in two seemingly unrelated processes. Two of the TFIIH subunits (XPB/Rad25 and XPD/Rad3) are DNA helicases with opposite polarity (Svejstrup et al, 1996). It is now known that the two helicases of TFIIH are necessary for the local unwinding of duplex DNA around the site of damage, which in turn creates substrates for the two

structure-specific endonucleases involved in making the dual incisions. Hence, TFIIH-mediated local denaturation of DNA during NER is analogous to the "bubble" structure formed during the step of open complex formation in transcription. The same activities of TFIIH are therefore being utilized by eukaryotic cells in these two different processes.

b. Repair/transcription syndromes

As mentioned above, defective NER in humans has been established as the cause of xeroderma pigmentosum (XP). Two other hereditary diseases, Cockayne's syndrome (CS) and trichothiodystrophy (TTD), are traditionally classified as syndromes associated with defective NER (Lehmann, 1987; Nance and Berry, 1992; Cleaver and Kraemer, 1989; Bootsma et al, 1998). However, it is still not clear if defective repair is the primary defect underlying CS and TTD. CS patients differ from XP patients mainly in that they do not have an elevated risk of skin cancer even though they are photosensitive. However, CS patients often have major developmental and neurological abnormalities, including cachetic dwarfism, mental retardation, and skeletal and retinal abnormalities. Progressive neurological degeneration caused by demyelination usually results in death in most CS patients before the age of 20. Considering that CS cells have a NER defect restricted only to actively transcribed genes, it is paradoxical that CS patients exhibit many additional symptoms when compared to XP individuals with total deficiency in NER. From this observation, it could be concluded that a defect in NER may not be the only deficiency underlying CS. As in the case with CS, individuals with TTD have skeletal abnormalities and mental retardation, yet do not suffer from a higher incidence of skin cancer. However, unlike patients with CS, they also have ichthyosis and sulfur-deficient brittle hair, findings which are characteristics of this particular inherited syndrome.

Mutations in five genes can cause CS. Two genes, *CSA* and *CSB*, are associated with the pure forms of CS, i.e., those without the symptoms normally associated with XP. In addition, XP groups B, D and G include some patients with symptoms associated with both CS and XP. Similarly, some mutations in two *XP* genes, *XPB*

and *XPD*, give rise to TTD. A third complementation group, TTDA, is assigned to TTD patients carrying mutations in an as yet unidentified gene, whose product appears to be associated with subunits of TFIIH (Weeda et al, 1997). Hence there is overlaps between the three diseases associated with defective NER.

Analysis of NER in cells derived from patients with the hereditary disorder Cockayne syndrome (CS) revealed a severe deficiency in the preferential repair of the transcribed strands of active genes, while there was a normal rate of repair for the nontranscribed strands (Venema et al, 1990b; van Hoffen et al, 1993). In accordance with this observation, CS cells are moderately UV sensitive and show a delayed recovery of RNA synthesis after UV irradiation (Lehmann, 1982; Lehmann et al, 1993). Increased photosensitivity of the skin is also a common characteristic of CS patients. Thus, the evidence strongly suggests that the *CS* gene products, *CSA* and *CSB*, representing the two known complementation groups, are involved in the TCR subpathway of NER. Both *CSA* and *CSB* have been cloned, and mutations have been identified in CS individuals. The *CSA* protein contains five WD repeats, which are found in many proteins with diverse cellular functions (Henning et al, 1995). None of the known WD repeat-containing proteins has an enzymatic activity, and the repeats are now thought to mediate protein-protein interactions (Neer et al, 1994). Sequence analysis of the *CSB* (also called *ERCC6*) gene identified it as a member of the *SNF2/SWI2* superfamily of helicase/ATPases (Troelstra et al, 1990; Troelstra et al, 1992). None of the members of this particular subfamily has been shown to actually possess helicase activity (Carlson and Laurent, 1994; Eisen et al, 1995); however, DNA-dependent ATPase activity has been demonstrated for many members, including CSB (Selby and Sancar, 1997a). Since the Mfd protein, the transcription-repair coupling factor (TRCF) in *E. coli*, also has helicase motifs and is a DNA-dependent ATPase, it was originally thought that CSB would function in a way similar to that of Mfd. However, recent data have shown that CSB, unlike Mfd, cannot displace an arrested RNA polymerase (Selby and Sancar, 1997a). On the other hand, the presence of an arrested molecule of RNA polymerase II in eukaryotic cells does not seem to inhibit NER of a blocking lesion, indicating that removal of the RNA polymerase may not be necessary (Selby et al, 1997). Nonetheless, CSB may

function as a TRCF, recruiting the NER machinery to the site of damage without the need to remove the stalled elongation complex. This putative function of CSB is consistent with *in vitro* studies demonstrating the potential for direct interactions between CSB and NER proteins, including XPA, XPB and XPG (Iyer et al, 1996; Selby and Sancar, 1997a). Furthermore, Tantin (1998) recently showed that an RNA polymerase II elongation complex can indirectly interact with a TFIIH-like repair factor through CSB. Hence, CSB can apparently function like TRCF in bringing repair factors to a stalled polymerase II complex. Once the lesion is removed, other factors, such as TFIIS, may be required to allow resumption of elongation by the stalled RNA polymerase II.

Since the interior of the human body is shielded from UV irradiation, any model of CS that invokes defective TCR as primary cause of the disease would need to identify the endogenous source of DNA damage that presumably leads to neurological injury. One candidate for the source of the presumed spontaneous damage is free radicals generated as byproducts of oxidative metabolism (Wiseman and Halliwell, 1996; Conner and Grisham, 1996). Cells that are metabolically highly active, such as neurons and proliferating cells, are particularly vulnerable to this oxidative damage because they produce large amounts of free radicals. In this respect, it is interesting to note that some forms of oxidative damage are also subjected to TCR. Moreover, this TCR of oxidative damage was found to be defective in cells from CS and XPG/CS patients (Leadon and Cooper, 1993; Cooper et al, 1997).

CSB may also perform other cellular functions that may or may not be related to NER activity. Instead of functioning as a coupling factor like Mfd, CSB has also been postulated to be a repair-transcription uncoupling factor (van Oosterrwijk et al, 1996). In this view, NER and transcription are competitive processes as a consequence of the dual repair/transcription function of TFIIH (Bootsma and Hoeijmakers, 1993; Svejstrup et al, 1995). In other words, the sharing of a common factor, TFIIH, suggests that NER and transcription are normally in an equilibrium state. Upon DNA damage, some of the TFIIH molecules or its subunits need to be recruited for association with the NER machinery, and thus may shift the equilibrium in such a way that polymerase II transcription of certain genes may be

halted or slowed down. CS proteins may act as repair-transcription uncoupling factors to allow TFIIH to switch from repair back to the transcription mode (van Oosterrwijk et al, 1996). Mutations in the *CS* genes could affect this proposed uncoupling function, leading to defective transcription when most TFIIH molecules are recruited to repair complexes. According to this model, the combined XP/CS and TTD cases can be explained if specific mutations in *XPB* and *XPD* genes interfere with CS proteins, thereby impairing the conversion of TFIIH from a repair to a transcription function (van Oosterrwijk et al, 1996). The CS, XP/CS and TTD syndromes may therefore not be caused by defective NER, but may be the consequence of defective transcription. Such a hypothesis could explain the paradox that CS patients usually have many additional and more severe symptoms than XP patients who are totally repair-deficient. Deficient transcription of a subset of important genes at certain critical stages of post-natal development could explain the neurological abnormalities associated with CS, XP/CS and TTD patients. Some evidence that a competition occurs between transcription and NER has come from *in vitro* studies using yeast extracts that can support both RNA polymerase II transcription and NER (You et al, 1998). Transcription was shown to be inhibited by the simultaneous presence of active NER. Importantly, this inhibition of transcription was relieved by the addition of purified TFIIH and required the *RAD26* gene product, the yeast homolog of CSB (You et al, 1998) This finding suggests that Rad26 functions as an exchange factor to mediate the assembly and/or disassembly of the basal transcription machinery.

Other experiments in yeast also provided indirect evidence that defective TCR may not be responsible for CS. Deletion of the *RAD28* gene, the yeast homolog of *CSA*, leads to enhanced UV sensitivity, but only in the absence of global genome repair (Bhatia et al, 1996), as is also the case with deletion of *RAD26* (Verhage et al, 1996). However, whereas deleting *RAD26* by itself results in defective TCR in *S. cerevisiae* (van Gool et al, 1994; Verhage et al, 1996), a *rad28* deletion strain displayed no evidence of defective strand-specific repair (Bhatia et al, 1996). Therefore, there is no correlation between defective TCR and the UV sensitivity of yeast strains mutated in the *RAD26* or *RAD28* genes. Hence, at least in yeast, UV sensitivity,

which is also a hallmark of CS, need not be the consequence of defective TCR. Taken together, the evidence is most consistent with the notion that defective transcription is the primary cause of CS and related syndromes. One version of the "transcription hypothesis" for CS involves entrapment of TFIIH in repair complexes with a consequent defect in transcription. An alternative "transcription hypothesis" proposes that CS proteins are directly involved in transcription and that mutations in CS proteins impair this function, causing abnormal transcription of certain genes. In this version, defective transcription would be a direct consequence of mutations in the *CS* genes or in the *XPB/XPD* genes encoding subunits of TFIIH, itself an essential basal transcription factor. Support for this model has come from the recent demonstration that CSB can interact with the elongating transcription complex (Tantin et al, 1997) to enhance the elongation rate of RNA polymerase II, suggesting that it may function like other RNA polymerase II elongation factors (Selby and Sancar, 1997b). Furthermore, other studies have demonstrated a reduced level of RNA polymerase II transcription in CSB and CSA cells, both *in vivo* and in cell-free systems, in keeping with a transcriptional role for CS proteins (Balajee et al, 1997; Dianov et al, 1997). In addition, TTD, a disease caused mainly by mutations in *XPD* and *XPB*, is also best explained by a deficiency in transcription. The lethality of a null mutation in *RAD3*, the yeast homolog of *XPD*, can be rescued by a helicase-defective mutant XPD, indicating that the helicase activity is not essential for transcription and survival (Guzder et al, 1995a). However, the XPD variants containing TTD mutations failed to complement the inviability of a *rad3* null mutation (Guzder et al, 1995a). This finding implies that TTD mutations in *XPD* result in a defect in transcription. The results of my own experiments in yeast, which showed that compromising RNA polymerase II transcription could lead to enhanced UV sensitivity, also a hallmark of CS, are in keeping with the notion that a primary transcription defect underlies CS and other related syndromes like TTD (Chapter III of this thesis).

In addition to interacting with NER proteins, including XPA and XPG, CSB can also interact with the p34 subunit of TFIIE *in vitro* (Selby and Sancar, 1997a) and was found in stable association with RNA polymerase II in human extracts (van

Gool et al, 1997). Likewise, CSA can interact with CSB and the p44 subunit of TFIIH (Henning, 1995). It should be realized, however, that these interactions between CS proteins and the basal transcription machinery are consistent, in principle, with CS proteins being involved in either repair or transcription. For instance, interactions with general transcription factors could be important for a role of CSB as a transcription elongation factor or, alternatively, these interactions may be essential for CSB to function as a TRCF or an uncoupling factor, loading the NER machinery onto a stalled transcription elongation complex in a manner similar to Mfd in *E. coli.*

c. Other factors implicated in TCR

Examination of the phenotypes of XPC cells has shed some light on the mechanistic process of TCR. In XPC cells, the transcribed strand of active genes is repaired as efficiently as in wild-type cells, whereas no repair could be detected of the non-transcribed strand or of an inactive locus (Venema et al, 1991; Venema et al, 1990a). This observation is reminiscent of a *rad7* or *rad16* null allele in yeast in which global genome repair is abolished but TCR is not affected (Verhage et al, 1994). Further evidence that XPC cells have a fully functional TCR pathway, but are deficient in global genome repair, came from drug inhibition studies which showed that the residual level of repair in these cells was sensitive to α-amantin and was thus transcription-dependent (Carreau and Hunting, 1992). This proficiency of TCR in the absence of XPC suggests that a stalled transcription complex may exhibit some characteristics that bypass the requirement for XPC. Circumstantial evidence supporting this conjecture came from the finding that *in vitro* NER using a cholesterol moiety as substrate does not require XPC (Mu et al, 1996). It is thought that such a cholesterol moiety is sufficiently bulky to cause a major distortion in the DNA helix. Moreover, a model substrate consisting of a lesion within a non-complementary region of double-stranded DNA can be repaired independently of XPC (Mu and Sancar, 1997b). Hence it appears that XPC could normally function to

stabilize an NER intermediate with unwound DNA, and that this function is no longer needed when the DNA is already opened up by a transcription elongation complex. Curiously, this global repair function of XPC does not seem to be shared by Rad4, the yeast homolog of XPC. It was found that repair of both strands of the *RPB2* gene was completely abolished in a *rad4* mutant strain, suggesting that Rad4 is required for repair of all DNA (Verhage et al, 1994). This finding also suggests that Rad4 may not be the functional homolog of XPC, even though they do share limited sequence similarity (Henning et al, 1995; Legerski and Peterson, 1992). However, this difference between XPC and Rad4 activity in human and yeast cells, respectively, may imply that there is a structural difference in the stalled transcription complex that allows TCR to occur without XPC in humans but not without Rad4 in yeast. The successful development of a reconstituted yeast NER system should allow further experimentation in this area (Guzder et al, 1995c). Specifically, it would be of interest to determine whether a cholesterol moiety or a lesion within a bubble structure can be repaired in the absence of Rad4 in the yeast system, as seems to be the case in humans in the absence of XPC. Such experiments will undoubtedly shed more light on the issue of functional equivalence between the human and yeast NER pathways.

Proteins such as MutL and MutS, originally identified as factors involved in mismatch DNA repair, are also required for TCR in *E. coli* (Mellon and Champe, 1996). This observation provided the impetus for similar experiments in human cells and in yeast. The removal of UV-induced lesions from the transcribed strand of the *DHFR* gene was shown to be eliminated in mismatch-defective human cell lines with mutations in *hMSH2, hPMS2* or *hMLH1*, the eukaryotic homologs of bacterial MutS and MutL (Mellon et al, 1996). These results are in keeping with the observed TCR deficiency in mouse embryonic fibroblasts homozygous for mutations in the *MSH2* or the *PMS2* gene (cited as unpublished data in Mellon et al, 1996). More recent studies showed that, whereas defects in either *hMSH2* or *hMLH1* result in defective TCR of UV damage, only mutations in *hMSH2* lead to a deficiency in TCR of oxidative damage such as thymine glycols induced by ionizing radiation (Leadon and Avrutskaya, 1997). Mutations in *MSH2* also result in defective TCR of thymine

glycol in *S. cerevisiae* (Leadon and Avrutskaya, 1998). However, in striking contrast to humans, no defects in TCR of UV-induced damage were found in yeast cells with mutations in *MSH2, MLH1, MSH3* and *PMS1* (Leadon and Avrutskaya, 1998; Sweder et al, 1996). Thus, there is differential involvement of the mismatch repair proteins, *MLH1* and *MSH2,* in TCR of different types of damage in human and yeast cells. The involvement of mismatch repair proteins in TCR of some kinds of damage in *E. coli,* yeast and humans reflects the high degree of conservation, not only in the mismatch repair pathways, but also in TCR, hinting at a similar molecular mechanism underlying this special pathway of repair in different organisms.

In an attempt to explain the involvement of mismatch repair genes in TCR, Mellon and Champe (1996) proposed that MutS protein could bind to a "bubble" structure unique to a stalled transcription complex, causing the DNA around the lesion to loop out with the help of MutL. The resulting topological structure was hypothesized to enhance repair as a result of its higher affinity for the NER machinery. Other experimental findings provide some evidence for this hypothesis. The human mismatch binding protein hMsh2 and the complex denoted hMutSα, a heterodimer of hMsh2 and hMsh6, were found to exhibit some affinity for pyrimidine dimers and also for a particular type of cisplatin cross-link (Duckett et al, 1996; Mello et al, 1996). Interestingly, this affinity was significantly increased, at least for hMutSα, if the adducts were paired to non-complementary bases which might resemble a transcription bubble (Mu et al, 1997a; Yamada et al, 1997). However, a direct role of mismatch repair proteins in TCR has yet to be demonstrated. Extracts derived from mismatch repair-deficient cells carried out NER at a normal rate (Mu et al, 1997a, Moggs et al, 1997). Moreover, supplementing these extracts with the hMutSα dimer did not influence the repair reaction in any discernible way (Mu et al, 1997a).

By extrapolating from what is known about NER in *E. coli,* the current model for TCR in eukaryotes incorporates the notion that a transcription elongation complex stalled at a lesion somehow signals recruitment of the repair machinery

(Hanawalt, 1994). A cyclobutane pyrimidine dimer on the template strand is known to be an absolute block to RNA polymerase II (Donahue et al, 1994). However, unlike *E. coli* cells in which the Mfd protein utilizes its ATPase activity to dislodge the arrested polymerase, the putative human transcription-repair coupling factor, CSB, could not displace a stalled polymerase II (Selby and Sancar, 1997a). In addition, a blocked RNA polymerase II at a lesion did not appear to inhibit repair of the damage by NER (Selby et al, 1997). Coupled with the fact that eukaryotic transcripts are usually much longer than those of prokaryotes, these findings suggest that blocked RNA polymerase II remains bound to DNA at the site of damage. Because it would also seem inefficient to abort a partially completed transcript, it may be that other factors might assist arrested RNA polymerase II to regain its ability to support transcription elongation once the damage is removed by NER. The elongation factor SII (or TFIIS), which interacts directly with the largest subunit of RNA polymerase II, has been shown to facilitate polymerase II arrested at natural pause sites to resume elongation (Kassavetis and Geiduschek, 1993; Reinberg and Roeder, 1987; Reines et al, 1989). Unlike other general elongation factors such as elongin and TFIIF, which increase the overall rate of elongation by suppressing transient pausing, SII stimulates passage of RNA polymerase II through pause sites by preventing arrest of the polymerase (Reines et al, 1996). The process involves an SII-stimulated exonucleolytic cleavage of the transcript by a ribonuclease activity within RNA polymerase II (Reines, 1992; Reines et al, 1992; Izban and Luse, 1992; Rudd et al, 1994). Reiterative SII-dependent cleavage and resynthesis of the transcript presumably provides RNA polymerase II with multiple chances to translocate through pause sites (Gu et al, 1993). Similar reactions also occur when polymerase II is stalled at the site of a cyclobutane pyrimidine dimer (Donahue et al, 1994). These findings have led to the suggestion that SII might have a role in TCR *in vivo* (Hanawalt, 1994; Hanawalt, 1995). As described in Chapter III of this thesis, I used a genetic approach to test this hypothesis and found that, under certain conditions, the lack of SII activity can result in enhanced UV sensitivity, implying that NER activity was compromised, although SII itself is not required for TCR in *S. cerevisiae* cells (Verhage et al, 1997).

VIII. Replication Protein A - a Multifunctional Protein Complex

1. Roles in Repair, Replication, Recombination and Transcription

Although originally isolated as a component essential for DNA replication, replication protein A (RPA) is now known to be involved in all major DNA repair pathways (Chapter I and II of this thesis). In most cases, this function of RPA requires specific protein-protein interactions in addition to the general DNA-binding property of RPA. The essential functions of RPA can be arbitrarily divided into those that only exploit the ssDNA-binding ability of RPA and others that require more specialized functions of RPA involving protein-protein interactions. In order to give a complete account of the roles of RPA in the different cellular processes, a brief review of the structure and properties of RPA and its involvement in DNA replication, repair and transcription is given below.

RPA was originally purified from human cell extracts as a factor essential for simian virus (SV40) DNA replication *in vitro* (Wobbe et al, 1987; Wold and Kelly, 1988; Fairman and Stillman, 1988, Ishimi et al, 1988,). Human RPA is a heterotrimeric complex consisting of 70, 32 (34 in some literature) and 14 (11 in some literature) kDa subunits (Wold and Kelly, 1988; Fairman and Stillman, 1988), also referred to as RPA1, RPA2 and RPA3 in this thesis. Because RPA1 by itself is insoluble, whereas RPA2 and RPA3 can form a stable, soluble complex, it was suggested that the RPA2-RPA3 dimeric complex serves as a platform for RPA1 interaction during assembly of the trimeric complex (Henricksen et al, 1994). RPA binds as a heterotrimeric complex with high affinity to ssDNA and with at least three orders of magnitude lower affinity to dsDNA and RNA (Wold et al, 1989; Kim et al, 1992; Brill and Stillman, 1989). For this reason, human RPA is also called HSSB. Homologs of human RPA have been identified in *S. cerevisiae* and other eukaryotes, indicating that it is highly conserved throughout evolution (Wold, 1997). In yeast the genes for all three subunits of RPA are essential for survival, in agreement with their important roles in processes such as DNA replication (Brill and Stillman, 1991; Heyer et al, 1990, see below). Both human and yeast RPA display preferential binding to polypyrimidine sequences over polypurine sequences, with the affinity for the former being approximately 50-fold higher (Kim et al, 1992). The

affinity of RPA in binding to oligonucleotides is strongly dependent on the length of the DNA, with tight binding only seen with a minimum length of 30 nucleotides (Kim et al, 1994). However, under certain conditions, human RPA can also bind ssDNA in a different mode, displaying a binding site size of 8-10 nucleotides (Blackwell and Boroweic, 1994; Blackwell et al, 1996). The central region of the RPA1 polypeptide was shown to contain a high-affinity ssDNA-binding domain (Gomes et al, 1995; Lin, et al, 1996; Kim et al, 1996; Gomes et al, 1996). The three-dimensional structure of this central RPA1 domain clearly shows that it is composed of two tandem copies of a ssDNA-binding subdomain (Bochkarev et al, 1997). Both subdomains interact directly with ssDNA and share a very similar core structure, called an OB-fold, mainly consisting of beta strands. Philipova et al. (1996) showed, by deletion and photo-crosslinking analysis, that yeast Rpa1 is also comprised of two ssDNA-binding subdomains, each made up of approximately 120 amino acids. These findings are in agreement with those obtained from the crystal structure of human RPA1. Moreover, the yeast study also identified another ssDNA-binding domain in the Rpa2 subunit of yeast Rpa (Philipova et al, 1996), a feature also shared by the homologous human RPA2 subunit (Bochkareva et al, 1998).

Knowledge of the roles of RPA in DNA replication has mainly come from studies of the papova virus SV40, whose replication relies on cellular factors and the virus-encoded large T antigen (Li and Kelly, 1984; Waga et al, 1994; Waga and Stillman, 1994). Origin unwinding requires T antigen and RPA (Kenny et al, 1989; Wold et al, 1987; Borowiec et al, 1990; Melendy and Stillman, 1993); however, other SSBs can support this reaction, indicating that there is a non-specific role for RPA (Kenny et al, 1989; Wold et al, 1988; Gomes et al, 1996). Unlike T antigen-dependent unwinding, formation of the primosome that contains DNA polymerase α/primase specifically requires human RPA; other homologs and SSBs were non-functional in this step (Matsumoto et al, 1990 ; Kenny et al, 1989). Interactions between human RPA, T antigen and DNA polymerase α/primase are essential for the formation of the primosome and the subsequent steps of initiation of DNA replication

(Dornreiter et al, 1990; Dornreiter et al, 1992; Dornreiter et al, 1993). In addition to SV40 large T antigen, other viral replication proteins, such as the EBNA1 and E2 proteins from Epstein-Barr virus and bovine papilloma virus, respectively, also interact with RPA (Zhang D et al, 1998; Li and Botchan, 1993).

After primer synthesis and initiation of DNA synthesis, the elongation phase of DNA replication also requires RPA. Human RPA can stimulate DNA synthesis catalyzed by DNA polymerase α by increasing its processivity (Tsurimoto and Stillman, 1989; Melendy and Stillman, 1993; Braun et al, 1997). RPA also stimulates the activity of DNA polymerases δ and ε, possibly through direct interactions. (Podust and Hubscher, 1993; Tsurimoto and Stillman, 1989; Lee et al, 1991).

Under appropriate circumstances, initiation of DNA replication can also be stimulated by factors which normally function as transactivators. Thus, many transcription factors, including CTF (Cheng and Kelly, 1989; Bosher et al, 1990; Chen et al, 1990; Mermod et al, 1989), c-Jun (Murakami et al, 1991; Guo and DePamphilis, 1992) and GAL4, can activate DNA replication (Baru et al, 1991, Bennett-Cook and Hassell, 1991; Marahrens and Stillman, 1992). Similarly, the acidic activation domain of transactivator VP16, when fused to the DNA binding domain of GAL4 and positioned near the origin of replication of polyomavirus or SV40, can stimulate DNA replication (Bennett-Cook and Hassell, 1991; Guo and DePamphilis, 1992; Cheng et al, 1992). A possible molecular mechanism for this phenomenon of transactivator-stimulated DNA replication was provided by the observation that the acidic domains of transactivators, such as those found in VP16, GAL4 and p53 can all interact with RPA (He et al, 1993). Importantly, at least in the case of polyoma virus replication, a strong correlation exists between the ability of mutant VP16 activation domains to interact with RPA *in vitro* and the ability of chimeric GAL4-VP16 activators to activate polyomavirus DNA replication *in vivo* (He et al, 1993). The RPA1 subunit plays an important role in protein-protein interactions involved in DNA replication, since T antigen, EBNA1, VP16 and p53 all interact with RPA through the N-terminus of the RPA1 polypeptide (He et al, 1993; Braun et al, 1997; Zhang D et al, 1998; Lin et al, 1996).

In NER, interaction of RPA with XPA is important for damage recognition, whereas interactions with XPG and XPF-ERCC1 both recruit and orient the two endonucleases within the excinuclease complex and stimulate their endonuclease activities. In homologous recombination, an important mechanism for the repair of double-strand breaks, RPA is required for the strand-exchange activity mediated by RAD51; however, other single-stranded DNA-binding proteins can substitute for RPA. On the other hand, specific interaction of RPA with RAD52 is necessary for the latter to stimulate the RAD51-mediated strand exchange activity.

Unexpectedly, studies on the expression of several metabolically regulated genes in *S. cerevisiae* initially suggested that RPA might also be involved in the process of transcription. RPA was shown to be identical to a novel protein called BUF1, purified as a sequence-specific transcriptional repressor that binds to the URS1 element upstream of many genes involved in carbon, nitrogen and inositol metabolism (Luche et al, 1992; Luche et al, 1993). Independently, others also showed that RPA could bind to URS elements found upstream of more than 10 DNA repair and metabolism genes (Singh and Samson, 1995). Finally, RPA also appears to bind near the transcription start site of the human metallothionein genes and represses transcription *in vitro* (Tang et al, 1996). More recent data, however, has called into question the functional significance of RPA binding to URS elements in transcriptional repression. Although RPA can bind to URS sequences *in vitro*, it apparently does not bind to this site at detectable levels *in vivo* (Gailus-Durner et al, 1997). Moreover, conditional-lethal mutations in RPA did not have any effect on URS-mediated repression (Gailus-Durner et al, 1997). Therefore, the potential role of RPA in transcription should at best be considered as tentative; direct evidence for such a role is still lacking.

2. RPA as an Integrator of Cellular Process?

Post-translational modification in the form of phosphorylation provides a universal means to regulate protein functions. In the case of RPA, the 34 kDa subunit of human RPA (RPA2) is phosphorylated in a cell-cycle dependent manner (Fang and Newport, 1993; Din et al, 1990) and in response to DNA damage induced

by ionizing radiation or UV irradiation (Liu et al, 1993; Carty et al, 1994). Phosphorylation of RPA is efficient only in the presence of ssDNA, with several serine residues located in the first 35 amino acid residues of RPA2 being the sole phosphoacceptors (Fotedar and Roberts, 1992; Henricksen and Wood, 1994; Henricksen et al, 1996). This hyperphosphorylation of RPA2 was reported by Boubnov et al (1995) to be largely reduced in SCID cells which lack DNA-PK activity. Biochemical data also pointed to DNA-PK being the kinase phosphorylating RPA in cell extracts (Brush et al, 1994). This conclusion, however, was directly contradicted by a more recent study (Fried et al, 1996). Regardless of which kinase is responsible for RPA phosphorylation, the biological effect of phosphorylation on RPA is largely unknown. Several attempts to demonstrate an effect of RPA phosphorylation on various processes such as DNA replication and repair have been unsuccessful (Lee and Kim, 1995; Brush et al, 1994; Hehricksen and Wold, 1994; Pan et al, 1995). For instance, depleting DNA-PK from extracts abolished RPA phosphorylation with no effect on *in vitro* DNA replication (Brush et al, 1994). Similarly, an N-terminal deletion in RPA2 which eliminates its serine phosphorylation sites resulted in a mutant RPA complex with wild-type capacity for binding ssDNA, stimulating DNA polymerase and supporting *in vitro* SV40 replication (Lee and Kim, 1995; Henricksen et al, 1996). An analogous N-terminal deletion introduced in yeast RPA2 also had no effect on viability (Philipova et al, 1996). However, phosphorylation of RPA2 could modulate DNA replication indirectly, as the N-terminus of RPA2 is needed to overcome inhibitory effects of DNA-PK on other replication components *in vitro* (Henricksen et al, 1996). Another study using phosphatase inhibitors demonstrated a role for reversible protein phosphorylation in modulating NER, but this effect appeared to be independent of the phosphorylation state of RPA (Ariza et al, 1996). Taken together, these data suggest that phosphorylation of RPA is not essential for RPA functions *in vivo* and *in vitro*.

Despite the above conclusion, the possibility remains that RPA may serve as a central integrator coordinating various processes of DNA metabolism with other cellular functions. This notion is particularly appealing in view of the many central

roles played by RPA in various cellular processes involving DNA transactions. Indeed, recent studies in yeast have provided evidence that RPA may be involved in G1/S and DNA damage checkpoint controls (Longhese et al, 1996). Other studies showed that *MEC1*, an important checkpoint gene in yeast, is required for RPA phosphorylation during the normal cell cycle and in response to UV or ionizing radiation (Brush et al, 1996). It is noteworthy that MEC1 is similar in sequence to human ATM, the gene mutated in patients with ataxia-telangiectasia (AT) (Bentley and Carr, 1997; Weinert, 1997). Moreover, this similarity between MEC1 and ATM extends to their cellular functions as well, since AT cells exhibit a delay in RPA phosphorylation when exposed to ionizing radiation (Liu et al, 1993; Cheng et al, 1996). In addition, studies examining the connection between RPA and p53 also implicate a role for RPA in integrating various cellular processes in response to DNA damage. UV damage was found to greatly reduce the ability of RPA to bind to p53, partly because hyperphosphorylated RPA could not bind p53 (Abramova et al, 1997). Furthermore, this downregulation of the RPA-p53 interaction is dependent on a functional global genome NER pathway (Abramova et al, 1997). Interestingly, the RPA-p53 interaction, which appears to inhibit DNA binding by p53, is abrogated in the presence of single-stranded DNA (Miller et al, 1997). This provides a potential means to regulate p53 function in the event of DNA damage. One can envisage that the recruitment of RPA to sites of damage would release previously bound p53, freeing p53 to respond to cellular stress. As RPA is involved in the major DNA repair pathways, such as NER and DSB repair, and potentially also in MMR and BER, RPA is situated at a position to be a sensor of genomic integrity. Processes such as NER may result in the release of ssDNA fragments that are utilized as signals by RPA to determine its affinity for p53. Despite the appeal of this model, other experimental evidence does not support a role for RPA in the coordination of DNA repair and checkpoint control. Lieter et al (1996) showed that the transcriptional functions of p53, rather than its RPA interaction, were necessary for the growth inhibition function of p53. Furthermore, it was found that radiation-induced RPA phosphorylation in mutant AT cells could be uncoupled from the S-phase checkpoint, demonstrating that phosphorylation was not required for S-phase arrest

(Morgan and Kastan, 1997). Clearly, much more research is needed to dissect out any role for RPA in regulating checkpoint control and other cellular processes in response to various environmental insults.

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CHAPTER II

Assessing the Requirements for Nucleotide Excision Repair Proteins of *Saccharomyces cerevisiae* in an *in vitro* System

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My contributions to this work described in this Chapter were as follows: I set up the *in vitro* NER assay with Z He, and did the experiments described in Figs. 1, 2, 7(a), 8-12.

SUMMARY

Nucleotide excision repair (NER) is the primary mechanism by which both *S. cerevisiae* and human cells remove the DNA lesions caused by ultraviolet light and other mutagens. This complex process involves the coordinated actions of more than 20 polypeptides. To facilitate biochemical studies of NER in yeast, we have established a simple protocol for preparing whole cell extracts which perform NER *in vitro*. As expected, this assay of *in vitro* repair was dependent on the products of *RAD* genes such as *RAD14*, *RAD4*, and *RAD2*. Interestingly, it was also dependent upon proteins encoded by the *RAD7*, *RAD16*, and *RAD23* genes whose precise roles in NER are uncertain, but not the *RAD26* gene whose product is believed to participate in coupling NER to transcription. Replication protein A (RPA/Rpa), known to be required for NER in human cell extracts, was also shown by antibody inhibition and immunodepletion experiments to be required for NER in our yeast cell extracts. Moreover, yeast cells with temperature-sensitive mutations in the *RFA2* gene, which encodes the 34 kDa subunit of Rpa, had an increased sensitivity to UV and yielded extracts defective in NER *in vitro*. Extracts prepared from both *rfa2* mutant strains had a reduced content of the Rpa2 polypeptide. These data indicate that Rpa is an essential component of the NER machinery in *S. cerevisiae* as it is in mammalian cells. Affinity chromatography was used to examine interactions between proteins critical in the early steps of NER in yeast. Although yeast Rpa did not appear to interact directly with Rad14, as did human RPA with XPA, the acidic domain of Rad2, the yeast homolog of XPG was shown to bind yeast Rpa. These data suggest, that despite a high degree of functional conservation amongst the human and yeast NER components, certain of the specific protein-protein interactions seen in the human system may not be essential for NER in the evolutionarily distant species *S. cerevisiae*.

INTRODUCTION

Nucleotide excision repair (NER) is a versatile DNA repair strategy found ubiquitously in prokaryotes and eukaryotes. NER is capable of removing a broad spectrum of DNA lesions caused by physical and chemical mutagens (1-5). Failure to remove DNA lesions from the genome as a result of defective NER may lead to cancer-susceptibility, as exemplified by the hereditary human disease xeroderma pigmentosum (XP) (2-5). NER involves the concerted actions of several different enzymatic activities and can be arbitrarily divided into the following steps: damage recognition, incision and excision of the lesion and its flanking DNA, and repair DNA synthesis to fill in the resulting single-stranded gap. Many of the proteins encoded by XP genes and their evolutionarily conserved *RAD* gene homologs in *S. cerevisiae* are now known to function in the early steps of excision repair, participating in the removal of DNA lesions prior to the repair DNA synthesis step. Some of the proteins involved in the repair DNA synthesis step of excision repair also function in the replication of cellular DNA and include proteins such as proliferating cell nuclear antigen (PCNA) (6-8, 67) and replication protein A (9-14).

Both yeast and mammalian replication protein A (Rpa/RPA) are trimeric complexes consisting of polypeptides of approximately 70, 34 and 14 kDa (15-19). RPA, a single-stranded DNA binding protein, might function in the DNA synthesis step of NER as it does in cellular and viral DNA replication. Recent studies (11-13, 20-22) have suggested, however, that RPA participates in both the early (damage recognition, incision, excision) steps as well as the late (repair synthesis) step of NER. The human RPA complex is now known to interact directly with XPA (20-23), the human homolog of the yeast damage-recognition protein Rad14 (24-25). This interaction enhances the ability of the RPA-XPA complex to bind to damaged DNA (20, 21). Following the damage-recognition step, incisions flanking the lesion are made by two endonuclease activities, in humans XPG (26, 27) and the ERCC1-XPF complex (28-30), and in yeast Rad2 (31) and Rad1-Rad10 (32, 33). Two helicase activities encoded by the XPD and XPB genes (34, 35) in humans and *RAD3* and *RAD25* in *S. cerevisiae* (36-39) are also involved in the incision/excision steps prior

to repair synthesis. In addition to PCNA and RPA, repair DNA synthesis also involves the participation of replication factor C (RFC) (11,14), polymerase δ or ε (11, 14, 40) and one of the DNA ligases (11, 14).

Biochemical analyses of NER have depended largely on the availability of cell-free extracts that can support excision repair. The *in vitro* system developed by Wood and his colleagues (41) has proven to be instrumental in the dissection of the NER pathway in human cells. On the other hand, understanding excision repair in *S. cerevisiae* has relied mainly on the genetic analyses of *rad* mutants. The potential for biochemical analysis of NER in yeast cells has not yet been fully realized owing in part to the lack of a simple *in vitro* repair system. The only existing cell-free system from *S. cerevisiae*, that described by Wang *et al* (42-43), involves the preparation of separate nuclear and whole cell extracts. Hence, it seemed desirable to develop a simple *in vitro* preparation from yeast cells capable of NER and applicable for use with a variety of different yeast strains. Here we report the development of such a system using a whole cell extract prepared from *S. cerevisiae.* We have utilized this *in vitro* system to demonstrate a requirement for the products of the genes *RAD7*, *RAD16* and *RAD23*. Furthermore, we have also exploited this *in vitro* repair system to assess the role of yeast Rpa in NER. In addition, yeast strains with point mutations in the *RFA2* gene were constructed and characterized. Taken together, our biochemical and genetic results indicate that, as in human cells, Rpa plays an important role in the process of NER in *S. cerevisiae* cells.

EXPERIMENTAL PROCEDURES

Yeast Whole Cell Extracts

The *S. cerevisiae* strains used in this study were BJ2168 (from Dr. J. Segall, University of Toronto), LP2899, a gift of Dr. L. Prakash, and *rad* mutant strains, MGSC131 (*rad4Δ::URA3*), MGSC139 (*rad14Δ::LEU2*), MGSC104 (*rad7Δ::LEU2*), W303236 (*rad16Δ::URA3*), MGSC101 (*rad23Δ::URA3*), MGSC102 (*rad26Δ::HIS3*) and their isogenic parental strain W303-1B (44) (kindly provided by Dr. J. Brouwer, Leiden University), and EMY75 (*rad2Δ::URA3* from L. Prakash). The procedure for the preparation of NER-proficient yeast extract was a modification of protocols originally intended for transcription studies (45). A step-by-step description of this protocol has been published (57, see Appendix of this thesis). Yeast cultures were grown at 27°C in complete medium (YEPD; 1% yeast extract, 2% Bacto Peptone, 2% glucose) with vigorous shaking. Cells were grown to an OD_{600} of 2, chilled in ice water and then collected by centrifugation at 4000 rpm for 4 min in a Sorval H-6000A rotor. The cells were then washed once in ice-cold water and once in extraction buffer (0.2 M Tris, pH 7.5, 0.39 M $(NH_4)_2SO_4$, 10 mM $MgSO_4$, 20% (v/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol) containing various protease inhibitors (phenymethylsulfonyl fluoride, 1 mM; benzamidine hydrochloride, 2 mM; pepstatin A, 3.5 μg/ml; leupeptin, 1 μg/ml; bestatin, 0.35 μg/ml; and aprotinin, 10 μg/ml). The drained cell pellet was scraped into a syringe, then extruded directly into liquid nitrogen and stored at -70°C. Frozen cells were broken by manual grinding under liquid nitrogen using a ceramic mortar and pestle. Grinding continued until the material was reduced to powder. After grinding, the frozen powder of broken cells was mixed with one volume of cold extraction buffer supplemented with protease inhibitors and allowed to thaw at 4°C. The cell lysate was subjected to centrifugation at 120,000 X g for 2 hr at 4°C. The clear supernatant was recovered and $(NH_4)_2SO_4$ added to 2.94 M by the addition of 337 mg of solid

$(NH_4)_2SO_4$ per ml of lysate over the course of 1 h. The suspension was stirred for another 30 min, and the precipitated protein pelleted by centrifugation at 40,000 X g for 15 min. The pellet was resuspended in a small volume (approximately 50 µl/g of cells) of dialysis buffer (20 mM HEPES pH 7.5, 20 % (v/v) glycerol, 10 mM $MgSO_4$, 10 mM EGTA, 5 mM dithiothreitol) plus protease inhibitors. The sample was then dialyzed against the same buffer plus 1 mM phenymethylsulfonyl fluoride for 12 to 16 h. Any precipitated protein in the dialysate was removed by centrifugation and the resulting supernatant was collected and stored at -70°C until use. Protein concentrations were determined by the BioRad colorimetric assay using bovine serum albumin as standard.

***In vitro* Assay of Nucleotide Excision Repair**

A detail account of the preparation of DNA templates and the NER assay has been published (57 and Appendix of this thesis). Briefly, DNA from plasmids pUC18 (2.7 kb) and pGEM-3Zf(+) (3.2 kb) was isolated by alkaline lysis and CsCl-ethidium bromide equilibrium centrifugation. The pUC18 DNA was treated with N-acetoxy-2-acetylaminofluorene (AAAF) and repurified on a 5% to 20% sucrose gradient as described by Wang *et al* (42). Reaction mixtures (50 µl) contained 300 ng of AAAF-treated pUC18 and 300 ng of control pGEM-3Zf(+) DNA, 45 mM HEPES-KOH (pH 7.8), 70 mM KCl, 7.4 mM $MgCl_2$, 0.9 mM DTT, 0.4 mM EDTA, 20 µM each of dGTP, dATP, TTP, 8 µM dCTP, 2 µCi of [α-^{32}P]dCTP (3000 Ci/mmol), 2 mM ATP, 40 mM disodium phosphocreatine, 2.5 µg creatine kinase, 3.4% glycerol, 18 µg of bovine serum albumin, and 250 µg protein as yeast whole cell extract (typically 6-8 µl). Reactions were incubated at 28°C for 2 hr. Plasmid DNA was purified from the reaction mixtures, linearized by digestion with *Hin*dIII, analyzed on a 1% agarose gel, and autoradiographed as described by Wang *et al* (42). To quantitate the extent of repair synthesis, dried gels were exposed to a storage phosphor screen and the amount of radioactivity in each band was quantitated by phosphoimaging analysis and comparison to a set of known radioactivity standards. For the antibody

inhibition experiments shown in Fig. 4, the indicated amount of preimmune or anti-yeast Rpa anti-serum was preincubated with the cell extracts for 15 min at 28°C before addition of reaction buffer and plasmid DNAs.

Yeast Rpa Antibody and Immunodepletion

The polyclonal antibodies against yeast Rpa used in these studies were raised in a rabbit by injection of recombinant yeast Rpa protein. The depletion of yeast Rpa from whole cell extracts was effected using an immunodepletion procedure described by Adachi and Laemmli (46). Briefly, PBS-washed Protein A-Sepharose CL-4B matrix was incubated with equal amount of preimmune or anti-yeast Rpa serum at room temperature for 2 h. The matrices were washed and incubated with four volumes of yeast cell extract in the presence of an ATP-regenerating system (46) at 4°C for 1 h. The Sepharose was then briefly pelleted in an Eppendorf tube and the supernatant was collected as the depleted extract. As estimated by a Western blot, more than 90% of the yeast Rpa protein in the extract was removed (data not shown).

Expression of Recombinant Yeast Rpa

Plasmids pJM124, pJM223, and pJM329 contain, respectively, the *RFA1* gene, *RFA2* cDNA, and the *RFA3* gene under the control of the phage T7 promoter of plasmid pET11a (47) and are described in ref. 19. The T7 promoter, gene, and transcription terminator of these plasmids can be moved as a *Bam*H I/*Bgl* II cassette. The 1.2 kb *Bam*H I/*Bgl* II cassette of pJM223 was inserted into the *Bam*H I site of pJM329 to create pJM332. The resulting 1.9 kb *Bam*H I/*Bgl* II cassette of pJM332 was then inserted into the *Bgl* II site of pJM124 to create pJM126 containing the three *RFA* genes on a single plasmid, each with its own T7 promoter. pJM126 was transformed into BL21(DE3) for protein expression.

BL21(DE3) cells containing pJM126 were grown in LB containing 0.1 mg/ml ampicillin at 22-37°C to an OD of 0.5-0.8. IPTG was then added to 0.4 mM and the induction continued for 3 hours. Cells were harvested and the bacterial pellet resuspended in 1/10 volume A buffer (18) with 50 mM NaCl and 1 mg/ml

lysozyme. Following incubation at 4°C for 15 min, NP-40 was added to a final concentration of 0.1%. The lysate was subjected to a total of 3 freeze-thaw cycles using dry ice/ethanol and swirling in a 37°C bath. Chromosomal DNA was dispersed by sonication (3X1 min) in volumes of approximately 20 ml on ice. The extract was clarified by centrifugation at 20,000 x g for 20 min at 4°C. The extract produced from 1 L of culture was loaded on a 100 ml phosphocellulose column equilibrated in buffer A with 50 mM NaCl and the column washed with 300 ml buffer A with 50 mM NaCl. *E. coli* SSB is found in the flow-through of this column. Rpa was eluted with a 600 ml gradient from 50 mM to 800 mM NaCl in buffer A and assayed for unwinding activity as previously described (48). Rpa elutes at approximately 200 mM NaCl from this column. Active fractions were pooled, NaCl added to 500 mM, and the sample loaded onto a 5 ml ssDNA-cellulose column equilibrated in buffer A with 500 mM NaCl. This column was washed with 15 ml buffer A with 500 mM NaCl, 25 ml buffer A with 750 mM NaCl, and 25 ml buffer A with 1.5 M NaCl and 50% ethylene glycol. The protein peak from this last step contains approximately 1 mg of recombinant Rpa from a 1 L culture. Approximately 50 micrograms of recombinant Rpa was then loaded onto a 5 ml 15-35% glycerol gradient in buffer A containing 100 mM NaCl. A parallel gradient contained a similar amount of Rpa purified from yeast. The gradients were centrifuged in an SW50.1 rotor at 49,000 rpm for 21 hr. Fractions (130 μl) were collected from both gradients, assayed for unwinding activity using the assay described by Tsurimoto et al (48) and subjected to SDS-PAGE followed by silver staining. For experiments employing recombinant Rpa in the *in vitro* assays of nucleotide excision repair, recombinant Rpa protein was purified through an Affigel-blue column, an hydroxylapatite column as described for the purification of human RPA by Henricksen et al (49), and an ssDNA-cellulose column as described by Brill and Stillman (18).

Expression and Purification of Rad14 and Recombinant Fusion Proteins

Purification of Rad14 from an overexpression yeast strain (a gift of Dr. L. Prakash) was as described (58). To express recombinant yeast Rpa2, the coding sequence of *RFA2* was amplified by PCR and subcloned into pET19b (Novagen) expression vector which allows expression of Rpa2 as a fusion protein containing 10 histidine residues at the N-terminus. The Rpa2 fusion proteins (wild-type and mutant 214-15) were expressed in *E. coli* strain BL21(DE3) and were purified by one-step nickel-chelate (Qiagen) affinity chromatography according to manufacturer's instruction. To synthesize ^{35}S-labeled Rpa2, pET11a plasmid containing the*RFA2* coding sequence was transcribed *in vitro* and translated by a coupled transcription/translation system (Promega). To purify GST-Rad2, the acidic domain of Rad2 was expressed as a glutathione-S-transferase fusion protein from a pGEX vector (Pharmacia). The fusion protein was expressed and purified using glutathione-Sepharose 4B according to manufacturer's instruction (Pharmacia).

Isolation of *rfa2* Mutants

Details concerning the identification and characterization of *rfa2* mutants has been published (59). Briefly, the entire *RFA2* gene on plasmid pJM215 (19) was subjected to 35 cycles of amplification with *Taq* DNA polymerase in the presence of 1 mM $MnCl_2$ and the universal forward and reverse sequencing primers. The PCR product was digested with *Bam*H I and *Sal* I and ligated into the yeast centromeric plasmid pRS415. This library of mutagenized *RFA2* plasmid DNA was amplified in bacteria and introduced into strain SBY205 which carries a deletion of the *RFA2* gene but is kept alive by plasmid pJM218 (19) carrying the *RFA2* cloned into the yeast centromeric plasmid YCp50. Transformants were replica-plated to plates containing the drug 5-fluoroorotic acid and placed at 37°C and 25°C. Strains showing no growth after 2 days at 37°C were identified and the *RFA2* plasmid rescued from the corresponding 25°C colony by transformation into *E. coli*. Mutant plasmid DNA was then reintroduced into strain SBY205 (19) to confirm that the temperature-sensitive growth defect was plasmid-dependent. Strains passing this test were tested for loss of

viability in a liquid culture at 37°C. Typically these ts strains showed 10-20% viability following 4 hours exposure to 37°C. Two alleles, *rfa2-214-15* and *rfa2-210-3*, used in this study, have multiple amino acid changes: T3S/V74A/Q95L/I154V/K182R/Q183P/F197S/L255W/T264A for *rfa2-214-15*, and *L39S/L164V/C173G/K225R* for *rfa2-210-3*.

Analysis of UV Sensitivity

Cells were grown at 25°C overnight in YPD medium, diluted in sterile water, and plated in duplicate on YPD plates and immediately irradiated with a Stratalinker UV crosslinker (Stratagene). The plates were then incubated at 25°C for 3 days and surviving colonies were counted.

Western Blotting

For the experiment shown in Fig. 8, yeast extracts were prepared using a protocol that minimizes proteolysis (60). Briefly, yeast cells were washed and resuspended in 200 μl of 20% trichloroacetic acid. After addition of an equal volume of glass beads, cells were disrupted by vortexing. Glass beads were washed twice with 200 μl of 5% trichloroacetic acid, and the solution was spun for 10 min at 3, 000 rpm. The protein pellet was resuspened in 200 μl of gel loading buffer, neutralized by 100 μl of 1M Tris base, boiled and clarified by centrifugation as described above. After determination of protein content, aliquots from each extract corresponding to 50 μg protein, as well as purified His_{10}-Rpa2, were subjected to SDS-PAGE, after which proteins were electrotransferred onto nitrocellulose (Schleicher & Schuell) and probed with rabbit polyclonal antibodies raised against yeast Rpa. After incubation with alkaline phosphatase-conjugated secondary antibodies (Bio-Rad), the blots were developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Sigma) following the manufacturer's specifications.

Affinity Chromatography

The detailed procedures for affinity chromatography have been described elsewhere (61, 62). Rad14 or GST-Rad2 was coupled to Affigel-10 (Bio-Rad) at the indicated concentration. Yeast extract was prepared as described above except that the $(NH_4)_2SO_4$ precipitation step was omitted. For affinity chromatography with yeast extract, 20 μl micro-columns were loaded with 300 μl of yeast extract, washed with 10 volumes of affinity column buffer (ACB) (61, 62) containing 0.1 M NaCl, and eluted with 60 μl of ACB containing 1.0 M NaCl. Flow-through and bound fractions were analyzed for the presence of Rpa by western blotting. For affinity chromatography with yeast Rpa2, affinity columns (20 μl) containing 2 mg/ml immobilized Rad14 was loaded with either 2 μl of ^{35}S-labeled Rpa2 or 2 μg of purified His_{10}-Rpa2 in a total volume of 50 μl ACB containing 0.1 M NaCl. The columns were then washed 3 times each with 60 μl of the same ACB buffer and then eluted with 60 μl of with ACB containing 1.0 M NaCl. One half of each fraction was resolved by SDS-PAGE and protein bands detected by either silver staining or autoradiography.

RESULTS

Nucleotide Excision Repair in Yeast Cell Extracts

The availability of human cell-free extracts capable of performing nucleotide excision repair (41) has permitted major advances in our understanding of this pathway of DNA repair. The genetic analysis of the sensitivity to UV of *S. cerevisiae* has also provided an excellent approach for studying NER in eukaryotes (1, 2). However, the full potential of using *S. cerevisiae* and its many mutant *rad* strains in studies of NER has not been fully exploited. Biochemical analyses of NER in yeast may have been hampered by the lack of a simple method of preparing yeast extracts capable of repair *in vitro*. As the human extract system was developed originally for studying *in vitro* transcription, we tested whether yeast extracts capable of *in vitro* transcription (45) would also perform nucleotide excision repair.

In this protocol, yeast cells were quickly frozen and then ground in liquid nitrogen. After addition of minimal amounts of buffer, the thawed extract was then clarified by a centrifugation step, further concentrated by ammonium sulfate precipitation, solubilized and dialyzed (see "Experimental Procedures"). Nucleotide excision repair in the yeast cell extracts was monitored by the incorporation of radiolabeled nucleotides into plasmid DNA during repair DNA synthesis. DNA damage was introduced into plasmid substrates by treatment with N-acetoxy-2-acetylaminofluorene (AAAF) which forms DNA adducts known to be corrected by the excision repair pathway (50). An untreated plasmid was also included in each reaction to monitor repair-independent nucleotide incorporation. As shown in Fig. 1A, damage-dependent repair synthesis was detected in extracts made from three commonly used laboratory *S. cerevisiae* strains. The extent of damage-dependent repair synthesis increased with the amount of protein added. An amount of extract corresponding to 250 μg of protein in an assay volume of 50 μl produced good signals above the background level of incorporation of radiolabeled nucleotide into the untreated plasmid and was therefore routinely used in subsequent experiments. Using these assay conditions, extracts from wild type cells routinely incorporated

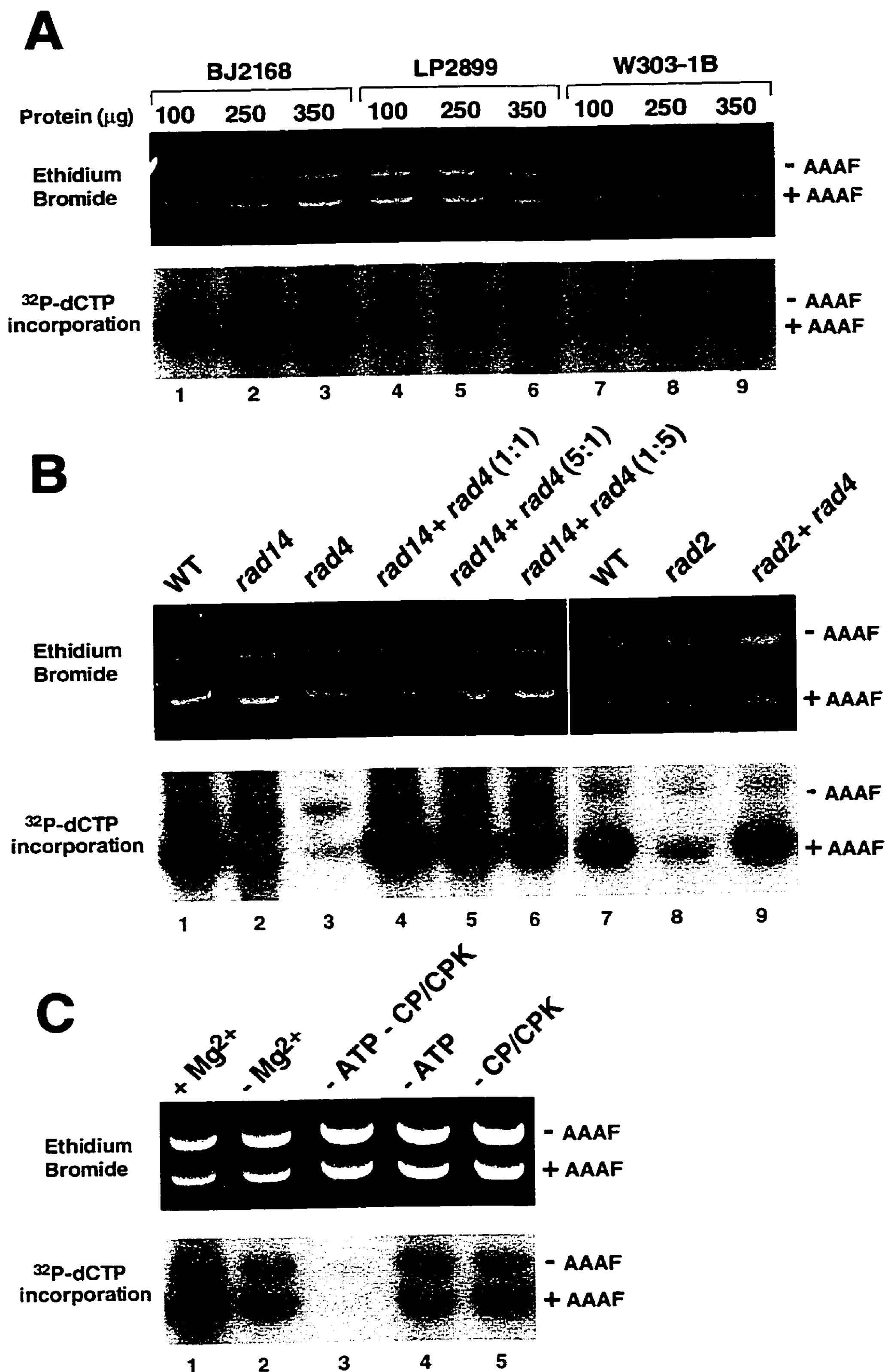

Figure 1. ***In vitro*** **NER in yeast whole cell extracts.** The NER activity of yeast cell extracts was assayed by detecting DNA repair synthesis. The incorporation of radioactively labeled deoxynucleoside triphosphate into control or AAAF-treated

DNA isolated from repair reaction mixtures was detected by autoradiography after agarose gel electrophoresis. In the upper panels the DNAs were visualized by ethidium bromide staining; the lower panels show the autoradiograms of the gels. (A) NER activity of extracts made from three different wild-type strains. The amount of protein used in 50 µl reactions is indicated. (B) NER activity in the extracts from the indicated isogenic wild-type and *rad* mutants as well as in mixtures of different *rad* mutant extracts. (C) Dependence of DNA repair synthesis during NER on $Mg2+$ (lanes 1, 2), ATP (lanes 3, 4) and an ATP-regenerating system (lanes 3, 5). The indicated components were omitted from the reaction mixtures in lanes 2-5.

approximately 140 fmols of dCMP into the AAAF-treated plasmid DNA during the 2 h incubation period. Background incorporation into the untreated control plasmid by these wild type extracts or into AAAF-treated plasmid DNA by *rad* mutant extracts was approximately 35 fmols. This *in vitro* NER activity, like that reported by Wang et al (42), was absolutely dependent on the addition of ATP (Fig. 1C, lanes 3 to 5). Omission of added Mg^{2+} ions in the assay also reduced incorporation significantly (Fig. 1C, lanes 1 and 2). The residual incorporation (lane 2) likely reflects the contribution of Mg^{2+} in the buffer used to prepare the cell extract.

We first established that the preferential incorporation of radiolabeled deoxynucleoside triphosphate into AAAF-treated DNA seen in the assays of Fig. 1A reflected *bona fide* nucleotide excision repair. To do this, we examined the dependence of our *in vitro* system on the products of *RAD* genes known to be involved in the excision repair pathway. The preferential incorporation of radioactivity into the AAAF-treated DNA was reduced to near background levels in extracts prepared from *rad14*, *rad4*, and *rad2* deletion strains (Fig. 1B, lanes 2, 3, and 8). Furthermore, when extracts prepared from *rad4* and *rad14*, or *rad2* and *rad4* mutant cells were mixed in different ratios, the defects in excision repair were corrected (Fig. 1B, lanes 4 to 6, and 9). This strict dependence of our cell-free system on known repair gene products, including one of the incision endonucleases, and the restoration of repair synthesis in the mixed extracts established that our simple preparation of *S. cerevisiae* extract is indeed capable of *in vitro* nucleotide excision repair.

We also assessed whether our yeast cell-free repair system was dependent upon the products of the yeast *RAD7*, *RAD16* and *RAD23* genes. The functions of the proteins encoded by these three *RAD* genes remain largely unknown, although *RAD7* and *RAD16* have been shown to be necessary for repair of the non-transcribed strand of transcribed genes and for repair of transcriptionally silent regions of the genome (44, 51). Rad23 may promote the formation of a repair complex (52). Fig. 2A shows that extracts made from the *rad7* and *rad16* deletion strains were completely defective for NER in our cell free system while the *rad23* deletion strain had a reduced ability to repair damaged DNA. In contrast to these results with *rad7*, *rad16*,

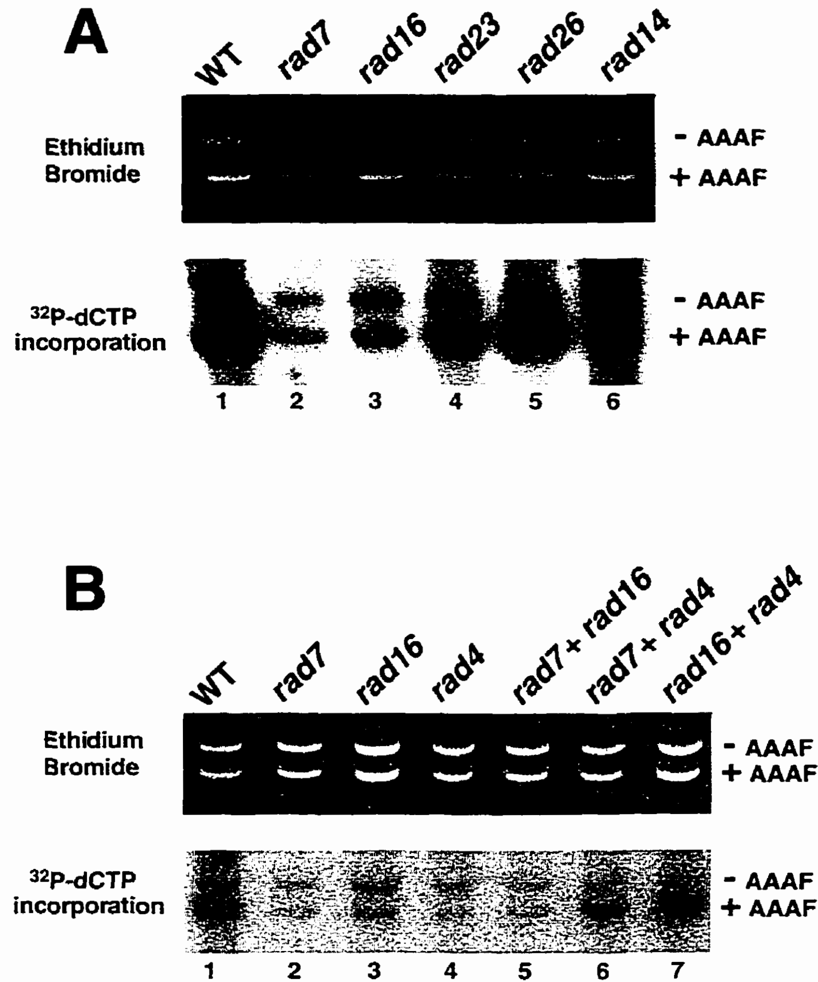

Figure 2. ***In vitro*** **NER activity in extracts prepared from** ***RAD7, RAD16, RAD23*** **and** ***RAD26*** **mutant strains.** (A) NER activity, detected by incorporation of radiolabeled deoxynucleoside triphosphate into DNA during repair synthesis, was assessed using extracts made from isogenic wild-type, *rad7, rad16, rad23, rad26,* and *rad14* deletion strains of *S. cerevisiae*. (B) Complementation of defective NER activity of *rad7* and *rad16* mutant extracts by the addition of *rad4* extract, and lack of complementation when rad7 and rad16 extracts were mixed in a 1:1 ratio. Top panels, DNA stained with ethidium bromide; bottom panels, the autoradiograms of the agarose gels.

and *rad23* strains, an extract prepared from a *rad26* deletion strain retained its ability to perform NER *in vitro*, an expected result given the evidence that the Rad26 protein functions exclusively in the subpathway of transcription-coupled repair (44, 53). To confirm that the deficient *in vitro* NER in the *rad7* and *rad16* extracts was not due simply to poor extract preparation from these particular strains, *in vitro* complementation experiments were also performed. When extracts from *rad4* and *rad7* or *rad16* cells were mixed, the defects in excision repair were corrected (Fig 2B, lanes 6 and 7). Curiously, however, when *rad7* and *rad16* extracts were mixed, *in vitro* NER was not restored (lane 5). Because the Rad7 and Rad16 proteins appear to function together in the repair of silent regions of the genome, extracts prepared from cells singly deleted for either function may also have acquired a deficiency in the other protein.

Overexpression and Purification of Recombinant Yeast Rpa

We and others have shown previously that human RPA functions at early steps in NER, and not exclusively at the DNA synthesis step of repair. In this study, we wished to address the role of yeast Rpa in nucleotide excision repair using the cell-free system described above. In order to facilitate these studies, we first expressed and purified recombinant yeast Rpa. The three subunits of yeast Rpa were co-expressed in *E. coli* using a T7 RNA polymerase-based expression system and the assembled trimeric Rpa complex was purified in four chromatographic steps to near homogeneity. To verify that recombinant yeast Rpa functioned like authentic yeast Rpa we characterized the purified protein after glycerol gradient sedimentation. Fractions from the gradient were assayed for the ability to cooperate with SV40 T-antigen to unwind plasmid DNA containing the SV40 origin of DNA replication. SDS-PAGE analysis of the glycerol gradient fractions revealed co-sedimentation of the three subunits indicating that the proteins exist in a complex (Fig. 3A). No free subunits were detected in this preparation and the sedimentation velocity of the complex was identical to authentic yeast Rpa run in a parallel gradient (data not

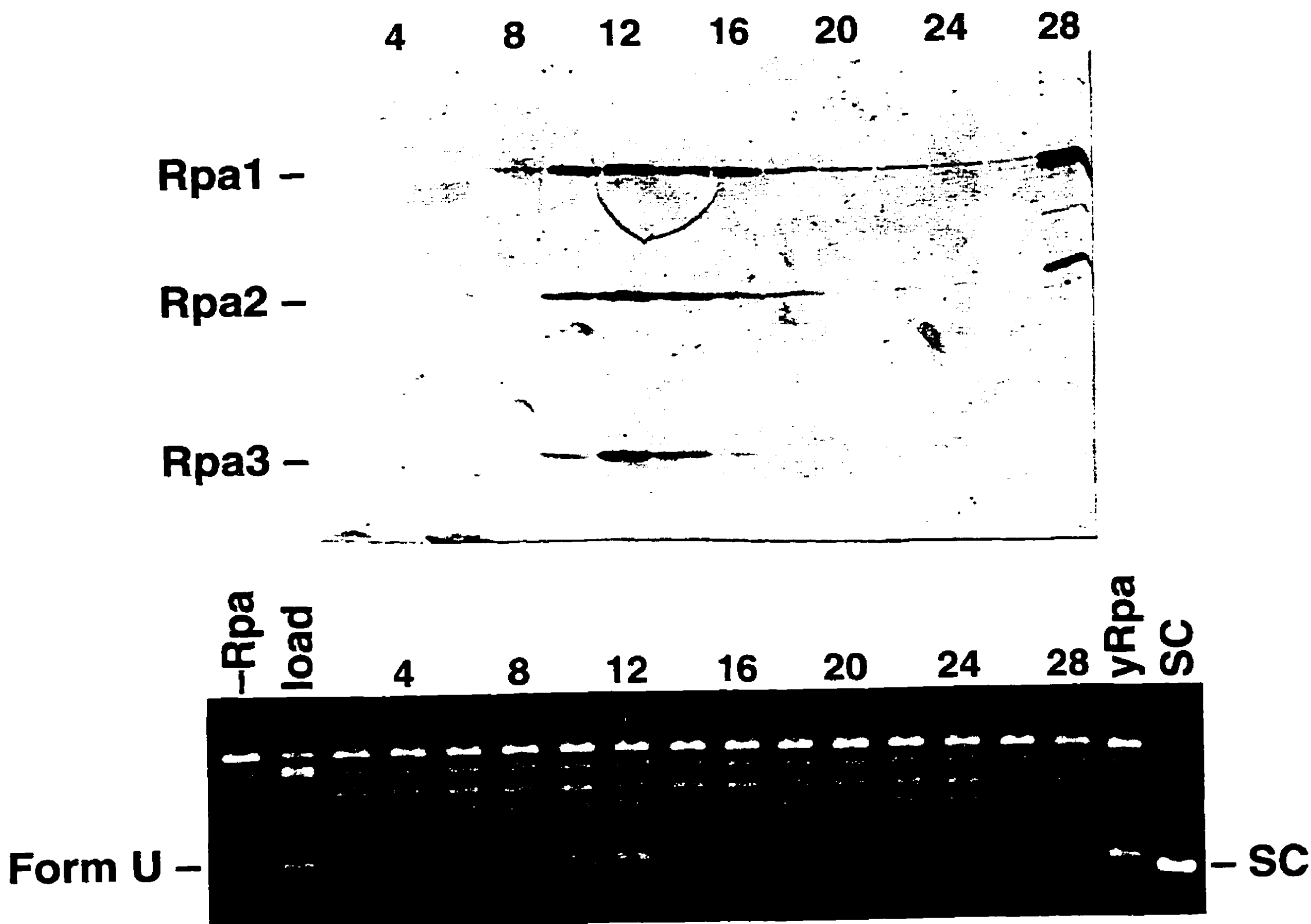

Figure 3. Recombinant yeast Rpa functions in the unwinding of the SV40 origin of DNA replication. Recombinant Rpa, purified as described in the "Experimental Procedures", was subjected to glycerol gradient sedimentation and alternate fractions were assayed for unwinding activity. (A). Silver stained SDS-PAGE of glycerol gradient fractions. Fraction 12 contains the peak of recombinant protein. Rpa purified from yeast showed similar sedimentation (data not shown). The material in lane 28 represents precipitated and pelleted material. (B). Unwinding assay (48) showing a peak of activity corresponding to fraction 12, as well as residual activity in the pellet fraction. The electrophoretic mobility of supercoiled (SC) and unwound plasmid DNA (form U) in the ethidium bromide-stained agarose gel is indicated. Assays without added protein, with an aliquot of the loaded material, and with purified Rpa from yeast cells are also shown.

shown). These fractions of recombinant Rpa supported the SV40 unwinding assay (Fig. 3B) as originally described for Rpa purified from yeast cells (18, 48). In addition, we also demonstrated that the purified recombinant yeast Rpa was able to bind single-stranded DNA in a mobility-shift assay (data not shown).

Requirement for Yeast Rpa in NER

To explore the role of yeast Rpa in excision repair, polyclonal antibodies raised against recombinant yeast Rpa were tested for their effects on DNA repair in our cell-free extract system. In Western blots these rabbit antibodies recognized the 70 Kd Rpa1 and the 34 Kd Rpa2 subunits of the trimeric Rpa efficiently, but bound to the 14 Kd Rpa3 subunit poorly (data not shown). Preincubation of yeast extracts with preimmune serum had little effect on their *in vitro* repair activity (Fig. 4A, lanes 2-4). However, addition of equal amounts of the rabbit anti-Rpa serum caused a marked inhibition of repair incorporation (lanes 5-7). Furthermore, when the extracts were preincubated with purified recombinant yeast Rpa prior to antisera addition, the inhibitory effects of the anti-Rpa antibodies were overcome (Fig. 4B, compare lanes 6 and 7 to lane 5). Addition of exogenous Rpa had minimal effect on the repair activity in the presence of preimmune serum (Fig. 4B, lanes 2-4).

To provide further evidence for the involvement of yeast Rpa in excision repair, we also used the anti-Rpa antibodies to deplete endogenous Rpa from our yeast extracts and then assayed for repair activity. As monitored by Western blotting (data not shown), more than 90% of the yeast Rpa was depleted from the extract by anti-Rpa antibodies immobilized on protein A beads, but not by control protein A beads preincubated with preimmune serum. The extracts depleted for Rpa failed to support efficient NER (Fig. 5, lane 2), whereas a mock-depleted extract efficiently performed NER (lane 1). Addition of recombinant yeast Rpa restored repair activity to the Rpa-depleted extract (compare lanes 4 and 6 to lane 2). Collectively, these data show that Rpa is required for nucleotide excision repair in yeast extracts as was the case for human RPA in human cell extracts (11-13, 20-22).

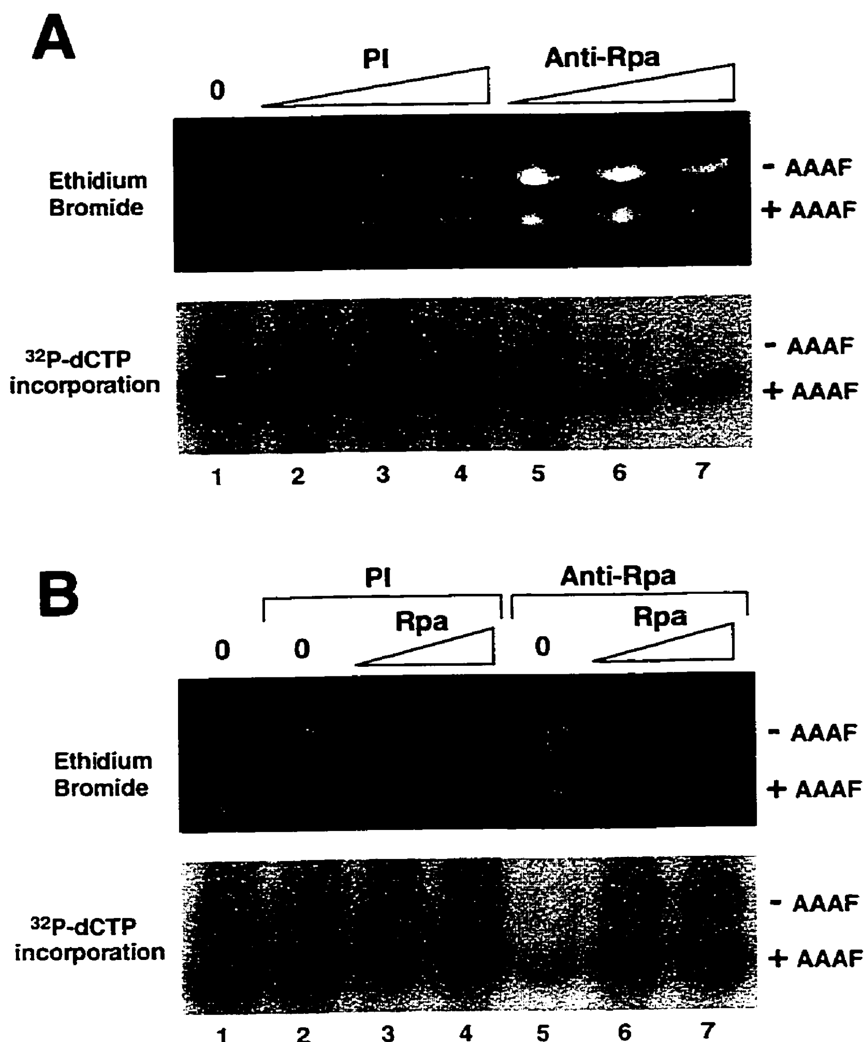

Figure 4. Inhibition of the *in vitro* NER activity of yeast extracts by anti-yeast Rpa antibodies. (A) DNA repair synthesis was assessed in yeast cell extracts after a preincubation with 0, 2, 4, or 6 µl of preimmune serum (lanes 1-4) or 2, 4, or 6 µl of rabbit anti-yeast Rpa antiserum (lanes 5-7). (B) Yeast cell extracts were preincubated with either 5 µl of preimmune serum (lanes 2-4), or 5 µl of anti-yeast Rpa antiserum (lanes 5-7) in the presence of 0 µg (lanes 2, 5), 0.5 µg (lanes 3, 6) or 1.0 µg (lanes 4,7) purified recombinant yeast Rpa, and then assayed for *in vitro* repair activity. Top panels, DNA stained with ethidium bromide; bottom panels, the autoradiograms of the agarose gels.

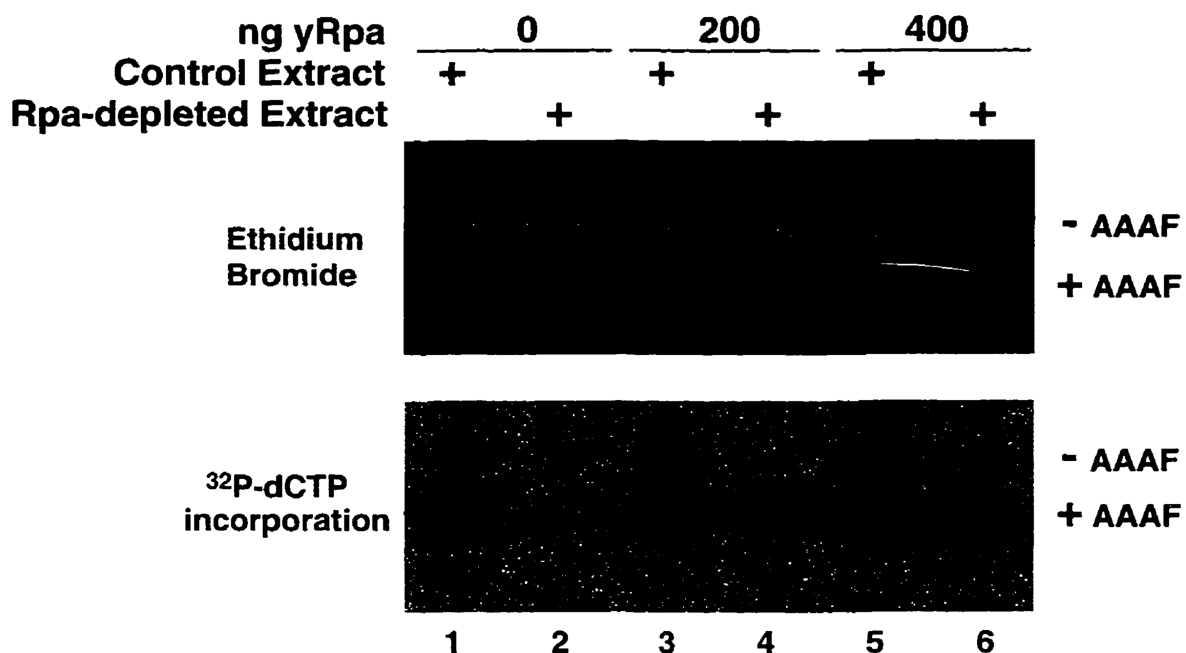

Figure 5. Reduced *in vitro* NER activity in an extract depleted of Rpa. Yeast Rpa was depleted using immobilized anti-RPA antibodies as described in the "Experimental Procedures". NER activities in mock-depleted (lanes 1, 3, and 5) or Rpa-depleted extracts (lanes 2, 4, and 6) were determined. As indicated, 200 ng (lanes 3 and 4), or 400 ng (lanes 5 and 6) of recombinant yeast Rpa was added to the depleted extracts. Top panel, DNA stained with ethidium bromide; bottom panel, the autoradiogram of the agarose gel.

Defective NER in *rfa2* Mutants

To provide *in vivo* evidence for the involvement of yeast Rpa in nucleotide excision repair, we assessed the DNA repair phenotype of yeast strains with conditional-lethal alleles of the *RFA2* gene, encoding the 34 kDa subunit of yeast Rpa. The *RFA2* gene was mutagenized using a PCR-based strategy and transformed back into yeast. Following removal of the wild type copy of *RFA2*, clones confering a temperature-sensitive phenotype were identified (59). Two alleles, *rfa2-214-15* and *rfa2-210-3*, lost viability quickly at 37°C and were chosen for further analysis. Stable mutant strains were constructed by integrating a single copy of these alleles into the yeast genome as the sole source of the 34 kDa subunit in these cells. The DNA repair capacities of these two *rfa2* mutant strains were examined, first by assessing their sensitivity to UV irradiation when grown at the permissive temperature. The UV dose-survival curves revealed that both of the *rfa2* mutant strains showed increased sensitivity to UV irradiation, and at doses of 50-150 J/m^2, they exhibited a 10 to 20 fold decrease in viability comparing to wild-type (Fig. 6). Neither strain was as sensitive to UV irradiation as the *rad4* mutant strain included for comparison. This heightened sensitivity to UV irradiation of these two *rfa2* mutant strains provided additional genetic evidence for the involvement of Rpa in the repair of UV-induced DNA damage.

To characterize excision repair in these *rfa2* mutant strains, we prepared cell-free extracts from each of the strains grown at 25°C and assessed them for their ability to perform *in vitro* DNA repair. Extracts from both *rfa2* mutant strains were deficient in our assay of excision repair (Fig. 7A, compare lanes 2 and 3 to the isogenic parental control in lane 1). As expected, addition of recombinant yeast Rpa to a defective extract restored *in vitro* repair activity (Fig. 7B). One possibility for the mutant phenotypes exhibited by these two strains could be that the mutations in *RFA2* led to instability of either the Rpa2 subunit itself or the trimeric RPA complex resulting in lower levels of active Rpa. To examine this possibility, yeast extracts were prepared from wild-type cells and the two *rfa2* mutant strains using a procedure that minimizes the possibility of proteolysis. Equal amounts of protein in

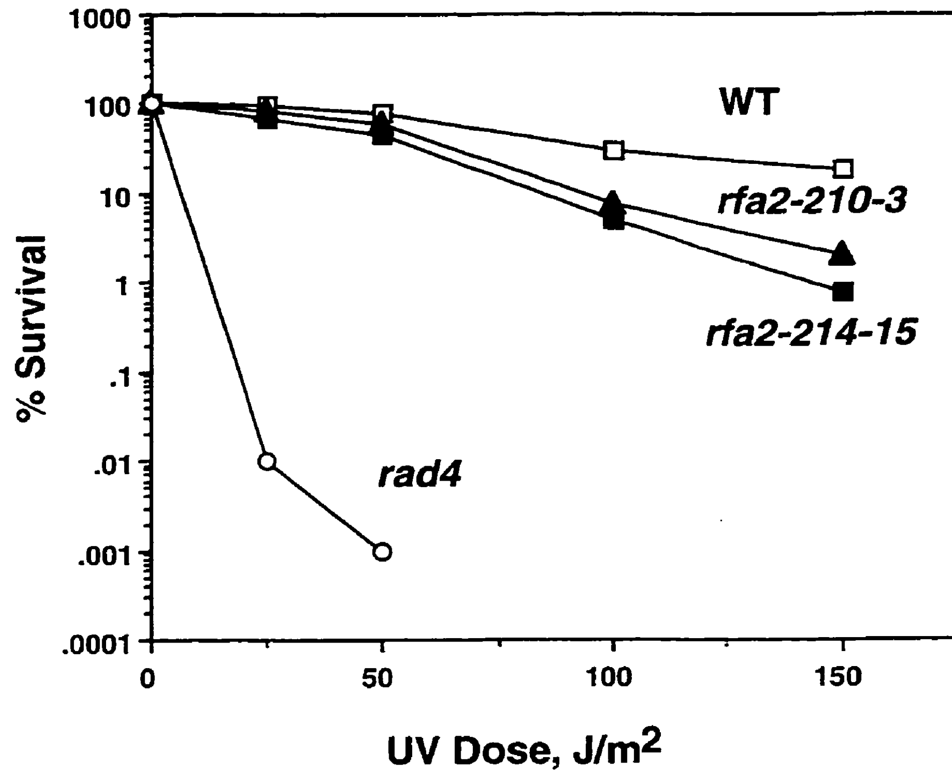

Figure 6. UV sensitivity of *rfa2* temperature-sensitive strains at the permissive temperature of 25°C. Aliquots of overnight yeast cultures were diluted and plated onto YPD plates. After exposure to the indicated UV dosage, the plates were incubated at 25°C for 3 days, and the surviving colonies were counted.□,wild-type cells W303-1B;▲,*rfa2-210-3* cells;■,*rfa2-214-15* cells;○,*rad4* cells.

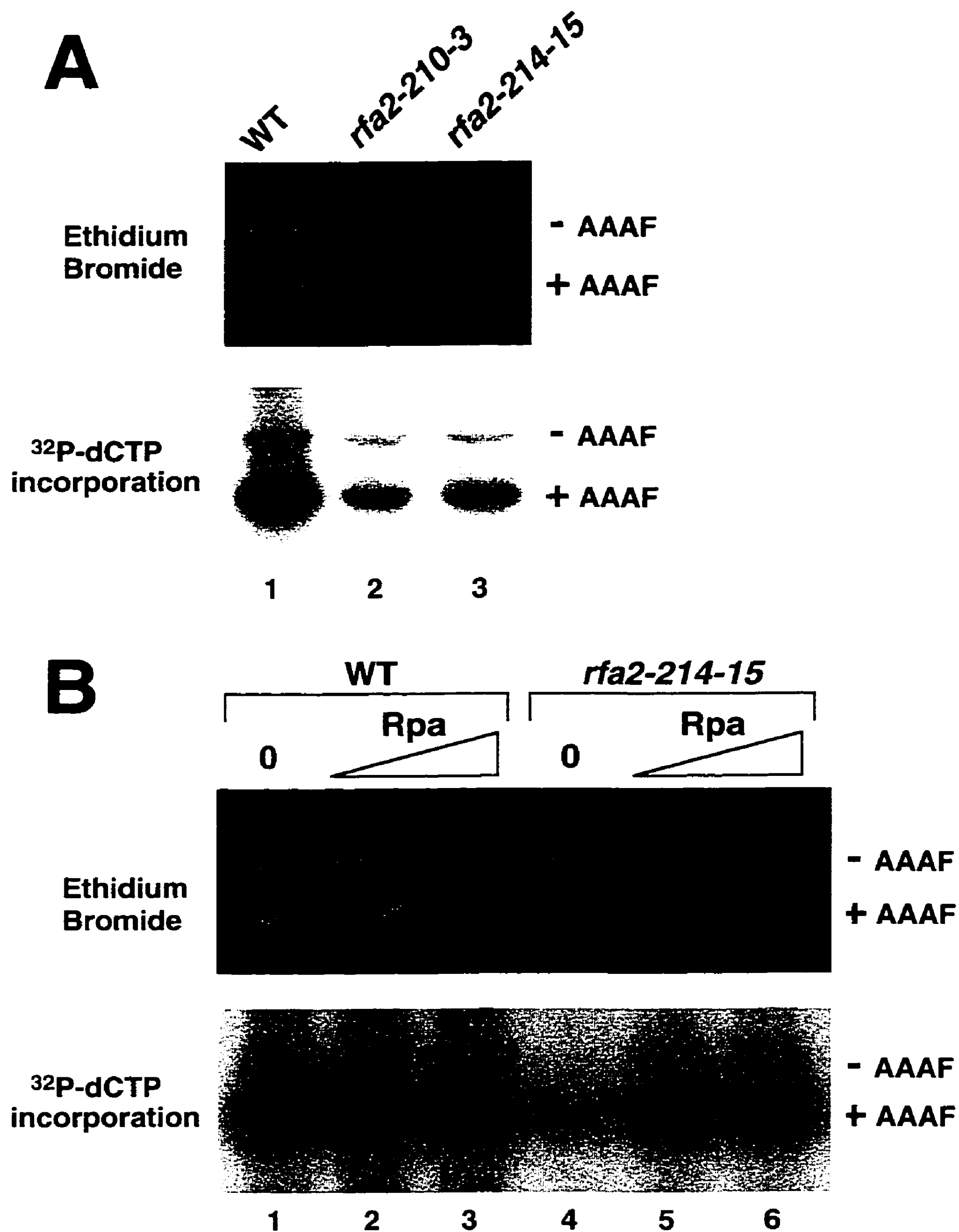

Figure 7. Defective *in vitro* NER in extracts from *rfa2* mutant strains. (A)*In vitro* NER activity in extracts prepared from wild-type (W303-1B), or two *rfa2* temperature-sensitive strains was determined. (B) The *in vitro* NER activity in extracts from wild-type (lanes 1-3) or *rfa2-214-15* (lanes 4-6) cells was measured in the presence of 0 ng (lanes 1, 4), 200 ng (lanes 2, 5), or 400 ng (lanes 3, 6) recombinant yeast Rpa.

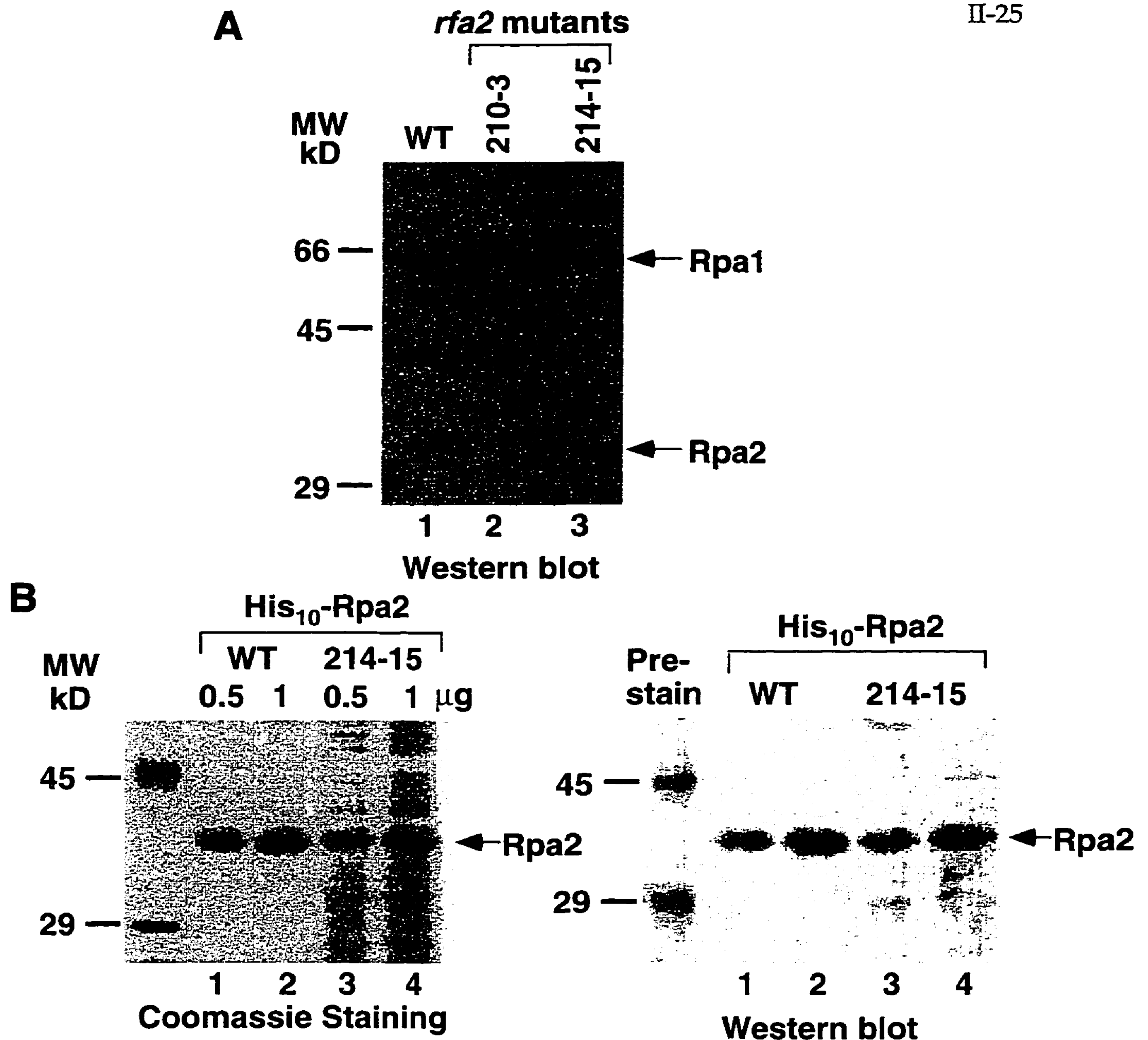

Figure 8. Extracts derived from *rfa2* mutant cells contain less Rpa2.

(A) Equal amounts of extracts (50 µg of protein content) derived from wild-type (W303-1B), or two *rfa2* temperature-sensitive strains were analyzed by western blotting using rabbit antibodies against yeast Rpa. (B) Equals amounts of purified recombinant wild-type and mutant 214-15 His_{10}-Rpa2 were fractioned on SDS gels and either coomassie-stained (Top panel) or transferred to nitrocellulose and probed with anti-Rpa antibodies (bottom panel) to determine if they are equally immunoreactive.

these extracts were resolved by SDS-PAGE and the amounts of endogenous Rpa monitored by western blot experiments with polyclonal antibodies capable of detecting each of the individual subunits of yeast Rpa. As shown in Figure 8A, all three extracts appeared to contain relatively similar levels of the Rpa1 subunit. However, there appeared to be markedly lower amounts of Rpa2 in the two mutant extracts. This difference in the Rpa2 levels in wild-type and mutant extracts was not the result of differences in immunoreactivity of the two polypeptides as both wild-type and mutant Rpa2 gave rise to comparable intensity of signals in a western analysis (Fig. 8B).

Since human RPA is known to interact with XPA, the human homolog of Rad14, we asked if this interaction is also conserved in yeast. Rad14 was purifed to homogeneity from an overexpression strain (Fig. 9A) and shown to be active in our NER assay as it could restore repair activity to an extract prepared from a *rad14* strain (Fig. 9B). To examine if yeast Rpa could interact with Rad14, yeast whole cell extract was applied to affinity columns containing an increasing concentration of immobilized Rad14 as affinity ligand. After loading and washing with buffer containing 0.1 M NaCl, the affinity columns were eluted with a high salt (1.0 M NaCl) buffer. The resulting eluates were resolved by SDS-PAGE and subjected to western blot analysis with anti-Rpa antibodies. The yeast Rpa1 and Rpa2 polypeptides were present only in the flow-through fractions but not in the eluates from both control columns containing immobilized GST and each of the columns containing immobilized Rad14 (Fig. 10). Hence, in contrast to the human NER proteins where an interaction was seen between RPA and XPA (20-23, 63), yeast Rpa does not appear to bind Rad14 under these conditions. In order to substantiate this result, I also tested if purified Rad14 could bind to recombinant yeast Rpa2. This second subunit of yeast Rpa was used because the homologous RPA2 subunit and the RPA1 subunit of human RPA are known to mediate the interactions between RPA and XPA (20-23, 63). Furthermore, unlike the individual subunits of human RPA (49), yeast Rpa2 was soluble by itself when expressed in *E. coli* (data not shown). Yeast Rpa2 was expressed and purified as a fusion protein with 10 N-

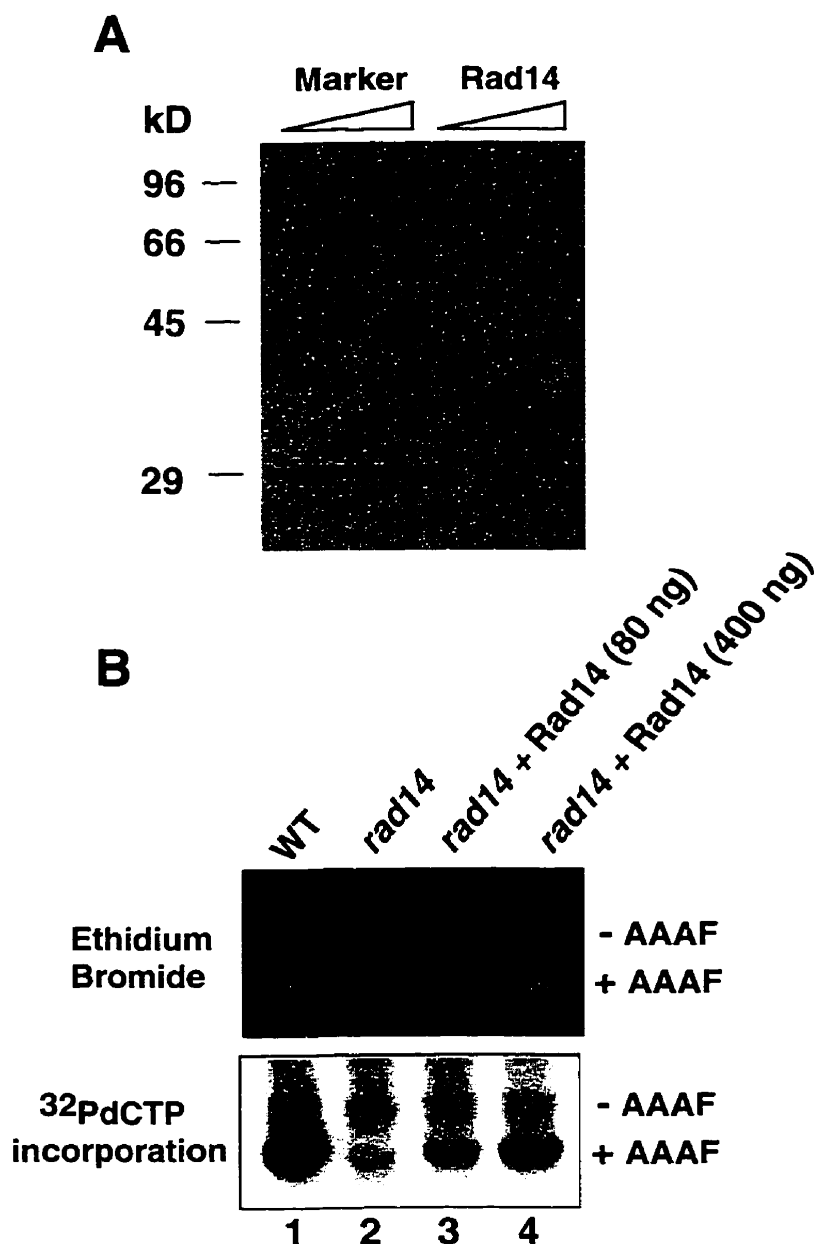

Figure 9. Purified yeast Rad14 can restore NER activity to *rad14* extract.

(A) Increasing amounts of protein markers (Bio-Rad) (0.5, 1, 2 µg) or purified Rad14 (0.5, 1, 2 µl) were resolved by SDS-PAGE and coomassie-stained (B) *In vitro* NER assay was performed with *rad14* extracts in the absence or presence of the indicated amounts of purified recombinant yeast Rad14. Top panels, DNA stained with ethidium bromide; bottom panels, the autoradiograms of the agarose gels.

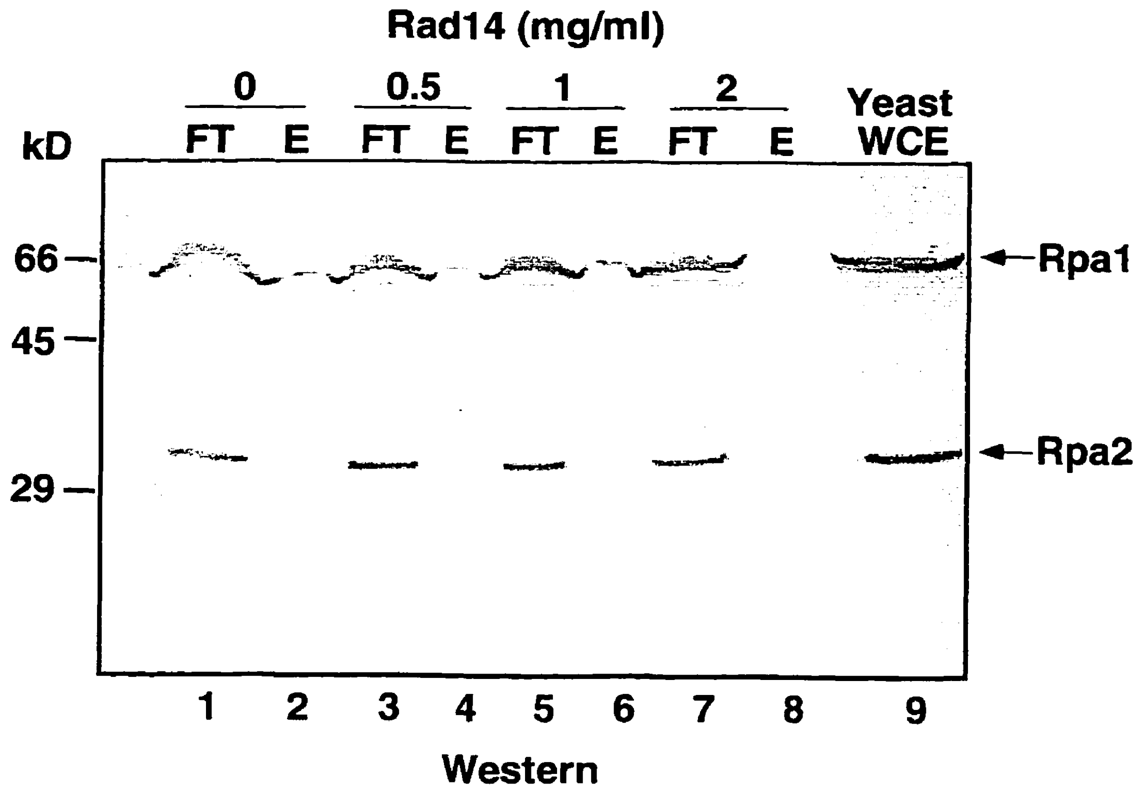

Figure 10. Rad14 does not bind Rpa from yeast extracts.

300 μl aliquots of yeast cell extracts were chromatographed on columns containing no coupled protein (lanes 1, 2), or the indicated concentrations of immobilized Rad14 (lanes 3-8). A 50 μl aliquot of the flow-through fraction (lanes 1, 3, 5, and 7) and a 30 μl aliquot of the 1.0 M salt eluate (lanes 2, 4, 6 and 8) from each column were resolved on a 12.5% SDS-polyacrylamide gel, transferred onto a nitrocellulose membrane, and blotted with polyclonal antibodies against yeast Rpa. A 50 μl of yeast extract was loaded in the rightmost lane.

terminal histidine residues (His_{10}-Rpa2) or synthesized as an ^{35}S-methionine-labeled protein using a rabbit reticulocyte lysate. His_{10}-Rpa2 and ^{35}S-labeled Rpa2 were both tested for binding to Rad14 by affinity chromatography. The flow-through, wash and eluate fractions were subsequently analyzed by SDS-PAGE and proteins detected by either silver-staining or autoradiography. Rpa2, as either the histidine-tagged polypeptide or as the ^{35}S-labeled polypeptide, was not retained by Rad14 and was only detected in flow-through and wash fractions (Fig. 11). This result, in keeping with that using yeast extracts, suggests that Rad14 may not interact directly with yeast Rpa.

In view of this result with Rpa and Rad14, I also asked if other interactions already detected between human NER proteins were conserved in yeast. Knowing that the acidic domain of XPG, the human counterpart of Rad2, can bind to human RPA (20), I tested if the equivalent region in Rad2 was capable of binding yeast Rpa. An affinity chromatography experiment using GST-Rad2 as ligand showed that Rpa present in a yeast cell extract was selectively retained by the GST-Rad2 column, but not by a control column (Fig. 12). Therefore, unlike Rad14, Rad2, like its human counterpart, XPG, does seem to interact directly with Rpa.

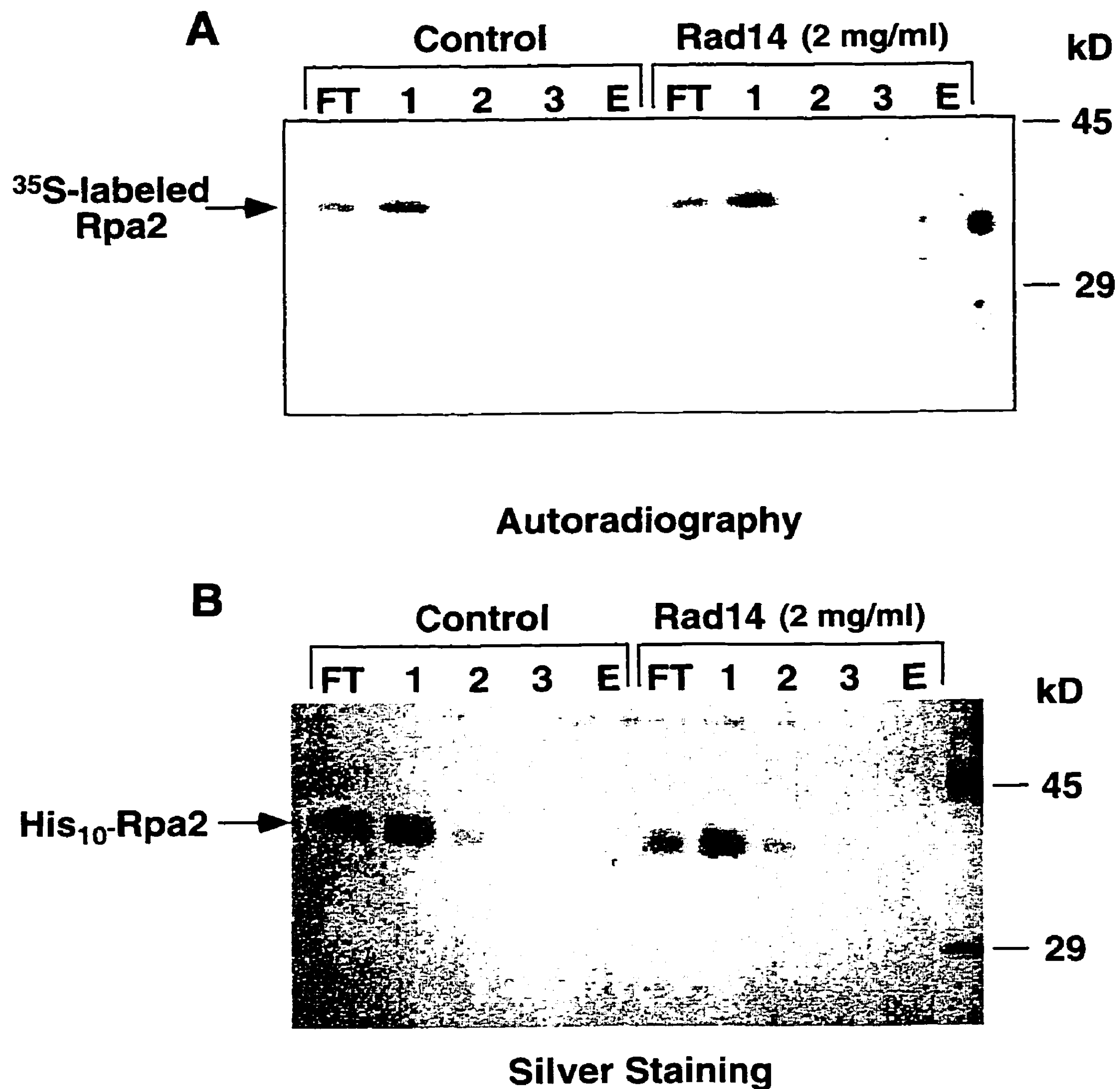

Figure 11. Rad14 does not bind recombinant yeast Rpa2.

(A) ^{35}S-labeled Rpa2 made in a reticulocyte lysate was chromatographed on 20 μl affinity columns containing either no bound protein or Rad14. Aliquots of the flow-through fraction (FT), each of the three 60 μl wash fractions (1-3), and the 1.0 M NaCl eluate fraction (E) were analyzed by SDS-PAGE and autoradiography. (B) 2 μg of purified His_{10}-Rpa2 was chromatographed on 20 μl affinity columns as described above. Fractions were analyzed by SDS-PAGE followed by silver-staining.

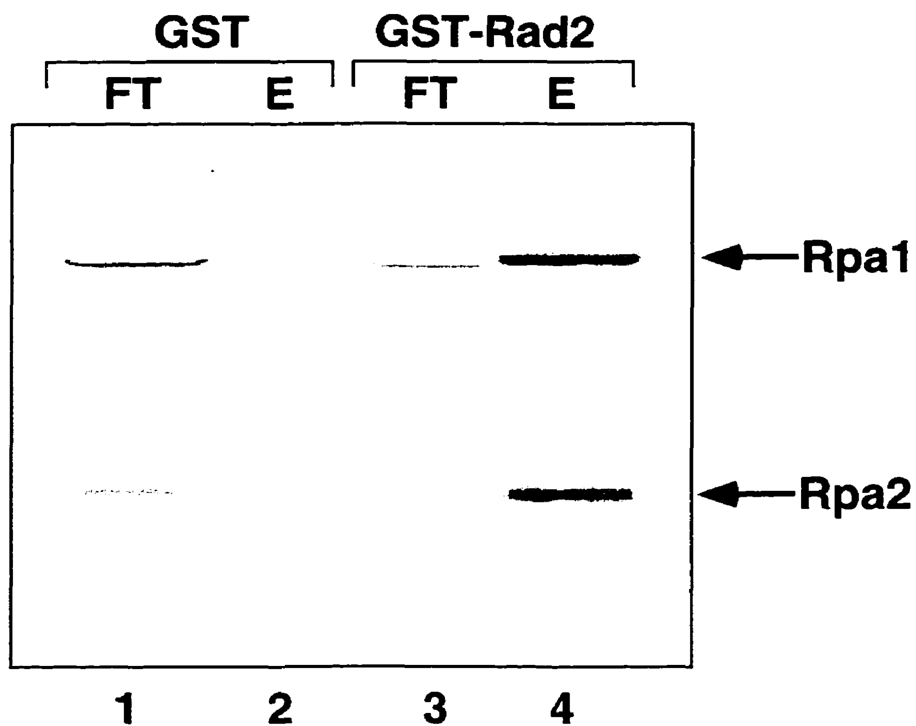

Figure 12. The acidic domain of Rad2 binds Rpa from yeast extracts.
300 μl aliquots of yeast cell extracts were chromatographed on affinity columns immobilized with either GST (lanes 1, 2) or GST-Rad2 (lanes 3, 4). A 30 μl aliquot of the flow-through fraction (FT) and a 30 μl aliquot of the 1.0 M salt eluate (E) from each column were resolved on a 12.5% SDS-polyacrylamide gel, transferred onto a nitrocellulose membrane, and blotted with polyclonal antibodies against yeast Rpa.

DISCUSSION

In this study, we have modified a protocol originally developed for assay of *in vitro* transcription by RNA polymerases I, II , and III (45) to make an NER-proficient yeast extract. Since these procedures appear to maintain the functional integrity of cellular components, this protocol may have overcome problems with the apparent lability of certain components such as the Rad2 protein which characterized the dual extract *in vitro* repair system described by Wang et al (42). We have demonstrated that our yeast extracts preferentially incorporate radiolabeled nucleotides into damaged DNA. This assay appears to detect *bona fide* excision repair activity since extracts made from mutant strains such as *rad14, rad4,* and *rad2,* known to be defective *in vivo* in yeast NER (13, 24), failed to preferentially incorporate nucleotides into the damaged DNA substrate. As these extracts support *in vitro* transcription by RNA polymerase II only with the addition of ribonucleotide triphosphates (data not shown), and do not require the *RAD26* gene product for NER, the signals detected in this study are not likely due to transcription-coupled repair.

This simple system using an easily prepared whole cell extract from a single strain of yeast may prove as useful in biochemical studies of NER as has the analogous mammalian whole cell extract. Although reconstitution of the initial incision/excision steps of NER with purified *S. cerevisiae* components has recently been reported (13), that system does not support the DNA synthesis step of NER. Our whole cell extract system supports the complete NER reaction. It may also facilitate detailed studies of the roles in excision repair of proteins such as the *RAD7, RAD16, RAD23,* and *RAD26* gene products. Using this system we have demonstrated that extracts made from *rad7, rad16,* and *rad23* strains failed to efficiently repair AAAF-induced DNA damage. In contrast, an extract made from a *rad26* deletion strain retained wild-type levels of *in vitro* excision repair, in keeping with the demonstrated role of *RAD26*, the homolog of the human Cockayne syndrome group B gene, in transcription-coupled repair (44, 54). Since our yeast transcription extracts also permit efficient transcription by RNA polymerase II, it

may be possible, by using a DNA substrate containing an RNA polymerase II promoter, to employ this system to study the role of the Rad26 protein in transcription-coupled repair.

The *in vitro* repair deficiency of the *rad23* extract is also consistent with the notion that the Rad23 protein might function as an assembly factor for a repair complex, mediating the association between Rad14 and TFIIH (52). When the incision step of NER was reconstituted with purified components, there was a requirement of Rad23 protein and/or the tightly associated Rad4 polypeptide (13). This reconstituted assay of incision, however, did not appear to require the Rad7 and Rad16 proteins. The *RAD7* and *RAD16* gene products have been shown to be absolutely essential for removal of UV-induced pyrimidine dimers from transcriptionally silent regions of the genome and from the nontranscribed strand of transcriptionally active genes in yeast (44, 51). These two proteins also contribute to the repair of the transcribed strands of active genes (51). The different requirements for Rad7 and Rad16 proteins in the two *in vitro* assays, incision or repair synthesis, may reflect the presence of additional factors in our whole cell extract system. One possibility is that, despite the use of the naked DNA substrates in our *in vitro* assay, this substrate is rapidly assembled into a nucleosomal array, thus necessitating proteins such as Rad7 and Rad16 to relieve a chromatin-dependent repression of NER. A role for the Rad7 and Rad16 proteins in chromatin remodelling *in vivo* has been suggested (55). The lack of complementation of repair activity between *rad7* and *rad16* extracts suggests that the two proteins may function together as a stable complex, a finding that has recently been confirmed (64, 65). Rad7-Rad16 complex was also shown to bind UV-damaged DNA and stimulate damage-specific repair (64). The use of our yeast extracts to dissect the different steps of NER may help further characterization of the precise functions in repair for the Rad7 and Rad16 proteins.

We have used this system to examine the involvement of yeast Rpa in NER. The antibody inhibition and Rpa depletion experiments, taken together with the demonstration of increased UV sensitivity for two mutant *rfa2* strains, argue persuasively for the involvement of Rpa in NER in yeast cells. Mutations in the

RFA2 gene may decrease the stability of the resulting polypeptide since extracts from the two mutant strains appear to contain less immunoreactive Rpa2 than do wild-type extracts. Since Rpa normally functions as a heterotrimeric complex, the consequence of a reduced content of Rpa2 could mean less active trimeric Rpa complex is present in the mutants. Thus, the number of trimeric Rpa molecules inside mutant cells may be too low to support efficient NER, leading to enhanced UV sensitivity. The exact step of NER affected as a result of these *RFA2* mutations has not been defined. Rpa is now known to function in the damage recognition and incision steps of NER, and likely plays a role in the later steps of repair DNA synthesis (11-13, 20, 21). That the two *rfa2* temperature-sensitive strains used in this study grow at near-normal rates at the permissive temperature of 25°C, and yet appear defective in NER at this permissive temperature both *in vivo* and *in vitro*, may indicate that the mutations in the Rpa2 polypeptide do not affect DNA synthesis but rather one of the early steps of NER. This is consistent with another report showing that a marked reduction of Rpa2 levels apparently has no effect on cell growth (66). Other mutations in *RFA2* and *RFA3* have now been reported to enhance UV sensitivity (59). In addition, a mutation in the yeast *RFA1* gene has also been shown to confer increased sensitivity to UV without affecting DNA synthesis (56). More recently, an N-degron protein degradation strategy was used to show that Rpa1 is required for NER *in vitro* (67).

The yeast Rpa complex may function, as human RPA appears to, at the early step(s) of damage-recognition and incision by making specific and direct contacts with other NER proteins. In this respect, yeast Rpa was shown to interact with the acidic domain of Rad2 as in the case for XPG, the human counterpart of Rad2 and human RPA. However, an interaction between yeast Rpa and Rad14 was not observed in our experiments. This is intriguing as the homologous human proteins, RPA and XPA, have been demonstrated to interact with each other in several different assays (20-23, 63). Importantly, this interaction between RPA and XPA appears to be biologically relevant as it confers synergistic binding of the complex to damaged DNA (20, 21, 63). Therefore, the finding of an apparent lack of interaction between Rad14 and Rpa needs to be further validated using additional

approaches before a definitive conclusion can be drawn. Specifically, immunoprecipitation experiments with yeast cell extracts should be conducted to test for interactions between endogenous Rad14 and Rpa. Affinity chromatography should also be performed in a reciprocal manner using purified yeast Rpa instead of Rad14 as column ligand, to eliminate the possibility that lack of Rpa binding to Rad14 ligand is not due to loss of Rpa binding sites in Rad14 as a result of chemical coupling to the column matrix. With these caveats in mind, the apparent lack of interaction between Rad14 and Rpa seen here raises several important issues. One might have expected this interaction be conserved in yeast which shares a highly homologous NER system. It is possible that the Rad14-Rpa interaction in yeast is too weak to be detected in solution; a stable Rad14-Rpa complex may form only on damaged DNA, in which case such an interaction would not have been detected in our assay. Secondly, the direct Rad14-Rpa interaction may not be required if interactions with other components of the NER machinery provide sufficient interactions to keep the repair complex together. This notion is particularly attractive in view of the findings that the NER machinery in both humans and yeast can exist as a preformed multiprotein complex even in the absence of damaged DNA (68, 69). Conceivably, within such a "repairosome", a multitude of different protein-protein interactions exist between various protein components. As such, the overall stability of such a complex might be dependent on the summation of all interactions between various components; individual interactions may contribute only modestly to the collective sum and there might be much less selective pressure for the evolutionary conservation of each of the individual protein-protein interactions. Our observation that yeast Rpa does interact with Rad2, as is the case with their human counterparts, XPG and RPA, also suggests that some but not all interactions are conserved throughout evolution. Finally, it is noteworthy that, even though the overall scheme of the NER pathway is highly homologous between humans and yeast, the specific details may well be different between the unicellular yeast and a metazoan animal like humans. There appear to be yeast NER genes that apparently do not have human counterparts. Examples of this include *RAD7* and *RAD16*, which are critical for global genome repair, and *MMS19*, which

may be involved in transcription and NER by indirectly regulating the activity of TFIIH (70). That yeast Rad4 is an essential core NER protein and its homolog, XPC, is dispensable for TCR (see Chapter I, section VII-2-ii-c of this thesis) is another illustration that differences exist between the otherwise highly homologous NER pathways in yeast and humans. In view of this, it will be of interest to systematically examine the various interactions that occur among the various yeast NER proteins; the results would permit a more complete assessment of the degree of functional conservation between the yeast and human NER systems.

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CHAPTER III

A Compromised Yeast RNA Polymerase II Enhances UV Sensitivity in the Absence of Global Genome Nucleotide Excision Repair

A version of this chapter will be submitted as a manuscript for publication

I did all of the experiments in this Chapter

SUMMARY

Nucleotide excision repair is the major pathway responsible for removing UV-induced DNA damage and therefore is essential for cell survival following exposure to UV radiation. Lesions in the transcribed strands of active genes are more rapidly repaired than those in the untranscribed strands or in nontranscribed DNA, but the contributions of the RNA polymerase II transcription machinery to this transcription-coupled repair pathway are poorly understood. To investigate whether the RNA polymerase elongation factor TFIIS (SII) might play a role in this form of excision repair, we examined the effect of deleting the *SII* gene of *Saccharomyces cerevisiae*. Lack of SII activity resulted in enhanced UV sensitivity, but only in the absence of global genome repair dependent on the *RAD7* and *RAD16* genes, a result seen previously with deletions of *RAD26* and *RAD28*, yeast homologs of the human Cockayne syndrome genes *CSB* and *CSA*, respectively. A *RAD7/16* -dependent reduction in survival after UV irradiation was also observed with RNA polymerase II mutants defective in their response to SII. *RAD26* appeared to contribute to UV resistance in a different manner than did *SII*, since *rad7* and *rad16* cells lacking both *RAD26* and *SII* had a greater sensitivity to UV than did these cells lacking either *RAD26* or *SII*. *RAD7/16*-dependent UV sensitivity, however, was also conferred by other RNA polymerase II mutations that do not affect the function of SII. Indeed, this enhanced UV sensitivity was achieved by simply decreasing the steady-state level of RNA polymerase II. Our results indicate that compromising transcription by yeast RNA polymerase II can increase the sensitivity of yeast to UV irradiation when the global genomic repair pathway is crippled. These results may have implications for the underlying pathophysiological defect of the human Cockayne syndrome disease.

INTRODUCTION

Multiple DNA repair pathways have evolved to repair different types of DNA lesions. Nucleotide excision repair (NER) is one of the most versatile of the DNA repair pathways as it is capable of removing a variety of helix-distorting lesions, including cyclobutane pyrimidine dimers and 6-4 photoproducts induced by UV radiation, as well as bulky chemical adducts (Wood 1997, 1996; Friedberg 1996; Sancar 1996). One distinctive feature of NER is its variability in the rate of repair across the genome. Damage in transcriptionally silent genes and in the non-transcribed strands of actively transcribed genes is processed by the global genomic repair subpathway of NER, which, in *Saccharomyces cerevisiae*, is dependent on both the *RAD7* and *RAD16* gene products (Verhage et al. 1994). On the other hand, damage in actively transcribed genes is often repaired more rapidly than it is in non-transcribed genes, as a result of preferential repair of the transcribed strand. This subpathway of NER, termed transcription-coupled repair (TCR), has been demonstrated in *E. coli* (Mellon and Hanawalt 1989) and yeast cells (Leadon and Lawrence 1992; Sweder and Hanawalt 1992), as well as in mammalian cells (Mellon et al. 1987).

To explain TCR at a molecular level, Mellon et al. (1987) proposed that RNA polymerase II stalled at a lesion might serve as a signal to direct repair to the transcribed strand of an active gene. Evidence supporting this model has come from work in *E. coli* where a coupling factor, the *mfd* gene product, has been identified as linking transcription and repair (Selby and Sancar 1993). This Mfd protein appears to function by displacing the stalled RNA polymerase from the template DNA, while at the same time recruiting the NER machinery through its interaction with the UvrA subunit of the excision nuclease (Selby and Sancar 1993; Selby and Sancar, 1995). Putative transcription repair coupling factors have also been identified in human and yeast cells. Human cells derived from patients with the hereditary Cockayne syndrome (CS) disorder exhibit a severe deficiency in enhanced repair of the transcribed strands of active genes, thus implicating the genes *CSA* and *CSB*, which represent two CS complementation groups, in TCR (van Hoffen et al. 1993).

Biochemical and genetic analyses of the *CSA* and *CSB* gene products and their

yeast homologs, designated *RAD28* and *RAD26* respectively, suggested that the molecular details of TCR in yeast and human cells may be more complicated and perhaps mechanistically different from those in *E. coli.* One surprise came from the observation that, although human CSA and CSB cells are more sensitive to UV irradiation than are normal cells (Friedberg 1996; Nance and Berry 1992), yeast *rad26* and *rad28* mutations confer UV sensitivity only when the *RAD7*- and *RAD16*-dependent subpathway of global genome NER was eliminated (van Gool et al. 1994; Verhage et al. 1996; Bhatia et al. 1996). Even more surprising was the observation that, in contrast to human CSA cells, yeast cells deleted for the *RAD28* gene did not manifest any deficiency in strand-specific repair (Bhatia et al. 1996). In addition, although the purified CSB protein possesses a DNA-dependent ATPase activity like that of the *E. coli* Mfd protein, it did not dissociate stalled RNA polymerase II from the template DNA (Selby and Sancar 1997a). It seems likely, therefore, that TCR in eukaryotic cells may be mechanistically different from that in bacteria.

Eukaryotic genes are normally much longer than prokaryotic genes, and other accessory factors may function to help RNA polymerase II resume elongation once the lesion is removed rather than aborting the incomplete transcript (Donahue et al. 1994; Hanawalt 1994). The elongation factor SII (or TFIIS), which interacts directly with the largest subunit of RNA polymerase II, has been shown to facilitate the resumption of elongation by RNA polymerase II arrested at natural pause sites (Kassavetis and Geiduschek 1993). SII mediates this process by stimulating cleavage of nucleotides from the 3′ end of the transcript by a ribonuclease activity residing within RNA polymerase II itself (Kassavetis and Geiduschek 1993). A similar reaction also occurs when RNA polymerase II is stalled at the site of a cyclobutane pyrimidine dimer (Donahue et al. 1994). These findings initially led to the suggestion that SII might have a role in TCR *in vivo* in conjunction with other factors such as the CS proteins (Donahue et al. 1994; Hanawalt 1994). In order to explore this hypothesis, we adopted a genetic approach used previously to characterize repair in *rad26* cells (van Gool et al. 1994). We first constructed *SII* deletion yeast strains in various genetic backgrounds and then analyzed their capacity to survive UV irradiation. Yeast strains carrying various RNA polymerase

II mutations, including those which affect the interaction of SII with RNA polymerase II, were also examined. During the course of our studies, deletion of the *SII* gene was shown by Verhage et al. (1997) to have no effect on the efficiency of transcription-coupled repair in yeast cells. Nevertheless, we found that compromising the activity of RNA polymerase II in a variety of different ways invariably led to a *RAD7* or *RAD16*-dependent enhanced UV sensitivity in these cells.

EXPERIMENTAL PROCEDURES

Growth Media and General Techniques

Yeast cells were grown in YPD or minimal medium supplemented with appropriate nutrients. Procedures including transformation, tetrad dissection, DNA isolation and polymerase chain reactions were performed according to standard protocols (Ausubel et al. 1994).

Construction of Strains

All *S. cerevisiae* strains used in this study are derived from the strain W303-1b and are listed in Table 1. The *SII* (*SII* is used here instead of the alternative names *PPR2* or *DST1*) gene was disrupted in W303 cells using a one-step gene disruption procedure (Rothstein 1983). Gene disruption was verified by PCR using specific primers. The plasmid used in making the *sII* null mutant contained the yeast *SII* gene disrupted by the marker *URA3* (plasmid pJD3, from Judy Davis through Caroline Kane and James Friesen). Yeast strains harboring deletions of either the *SII* or *RPB9* gene or various mutant alleles of *RPO21* were crossed to different *RAD* gene backgrounds to obtain double or triple mutant strains.

Analysis of UV Sensitivity

Cells were grown overnight at 27°C in YPD medium or minimal medium with the necessary amino acids, diluted in cold sterile water, and plated in duplicate on YPD or minimal medium plates. The plates were immediately irradiated with a Stratalinker UV cross-linker (Stratagene), then wrapped in aluminum foil and incubated at 27°C for 2-3 days until colonies were large enough to be counted. The number of colonies on irradiated plates was expressed as a percentage of colonies on unirradiated plates.

Strain	Genotype	Reference
W303-1b	*MATα ho can1-100 ade2-1 trp-1 leu2-3,112 his3-11, 15 ura3-1*	Rothstein (1983)
W303236	*rad16Δ::URA3*	Verhage et al. (1996a)
MGSC104	*rad7Δ::LEU2*	Verhage et al. (1996a)
MGSC102	*rad26Δ::HIS3*	van Gool et al. (1994)
MGSC106	*rad7Δ::LEU2 rad26Δ::HIS3*	Verhage et al. (1996a)
MGSC131	*rad4Δ::URA3*	Verhage et al. (1996b)
YCJ0073	*sIIΔ::URA3*	This study
YCJ0074	*rad7Δ::LEU2 sIIΔ::URA3*	This study
YCJ0075	*rad16Δ::URA3 sIIΔ::URA3*	This study
YCJ0076	*rad16Δ::URA3 rad26Δ::HIS3*	This study
YCJ0077	*rad7Δ::LEU2 rad26Δ::HIS3 sIIΔ::URA3*	This study
YCJ0078	*rad16Δ::URA3 rad26Δ::HIS3 sIIΔ::URA3*	This study
YCJ0079	*rad26Δ::HIS3 sIIΔ::URA3*	This study
YF1543	*rpo21Δ::LEU2* with *rpo21-18(I123TRARV) TRP1 CEN ARS*	Archambault et al. (1992a)
YCJ0080	YF1543*rad7Δ::LEU2*	This study
YCJ0081	YF1543*rad16Δ::URA3*	This study
YF2227	*rpb9Δ::HIS3*	D. Jansma & J. Friesen, unpublished
YF2234	*rpb9Δ::HIS3 sIIΔ::URA3*	D. Jansma & J. Friesen, unpublished
YCJ0082	*rad7Δ::LEU2 rpb9Δ::HIS3*	This study
YCJ0083	*rad7Δ::LEU2 rpb9Δ::HIS3 sIIΔ::URA3*	This study
YF2145	*rpo21Δ::HIS3* with *rpo21-30(H80Y) TRP1 CEN ARS*	I. Donaldson & J. Friesen, unpublished
YF2151	*rpo21Δ::HIS3* with *rpo21-36(C67, 70S) TRP1 CEN ARS*	I. Donaldson & J. Friesen, unpublished
YCJ0084	YF2145 *rad7Δ::LEU2*	This study
YCJ0085	YF2151 *rad7Δ::LEU2*	This study
YCJ0124	*rpo21-Δ88::URA3*	Allison et al. (1988)
YCJ0086	*rad7Δ::LEU2 rpo21-Δ88::URA3*	This study
YF1971	W303-1b with *pLEU2-RPO21 LEU2*	Archambault et al. (1996)
YCJ0087	YF1971 *rad16Δ::URA3*	This study

Table 1 List of yeast strains used

All strains are in W303-1b background

RESULTS

Disruption of SII Enhances UV Sensitivity in the Absence of Global Genome Repair

Human cell lines with mutations in the *CSA* and *CSB* genes show defects in TCR and display increased sensitivity to UV. Deletion of either of the yeast homologs of these two genes, *RAD28* and *RAD26*, however, has no effect on survival of yeast cells after UV irradiation unless the global genome repair pathway dependent on *RAD7* and *RAD16* is simultaneously crippled (van Gool et al. 1994; Verhage et al. 1996; Bhatia et al. 1996). As the RNA polymerase II elongation factor SII might play a role in the TCR subpathway of NER, yeast strains were constructed in which the endogenous *SII* gene was deleted. We also constructed double mutants lacking both *SII* and either *RAD7* or *RAD16*. As previously reported (Nakanishi et al. 1992), the yeast null mutant for the *SII* gene was viable. After irradiation with increasing doses of UV light, no enhancement of sensitivity to UV irradiation was observed in the *sII* strain, when compared to its isogenic wild-type parent (Fig. 1). We did find, however, that *SII* disruption, like deletion of *RAD26* or *RAD28*, (Verhage et al. 1996; Bhatia et al. 1996) led to a modest increase in sensitivity to UV light above that conferred by *rad7* or *rad16* alone (Fig. 1). For the purpose of comparison, we also tested the double mutants *rad7rad26* and *rad16rad26* in the same experiment and found that the enhanced UV sensitivity of *rad7* or *rad16* strains conferred by *sII* disruption was not as marked as that seen with a *rad26* deletion (Fig. 1). Although the increase in UV sensitivity conferred by the *SII* deletion was small, we reproducibly observed a 3-4 fold enhanced sensitivity to UV in these *rad7sII* and *rad16sII* strains compared to cells lacking only the global genomic repair pathway. With data from a typical experiment, statistical analysis using the Student's t test generated a P value of <0.0001 for the observed 3.9 fold difference in UV sensitivity between *rad7* and *rad7sII* mutant cells (Table 2). Thus, the transcriptional elongation factor SII does appear to have a small but significant contribution to UV survival when the global genomic repair pathway is abolished. We also wanted to determine if the SII protein, which functions in transcription elongation, influences UV survival by acting in the same genetic pathway as the

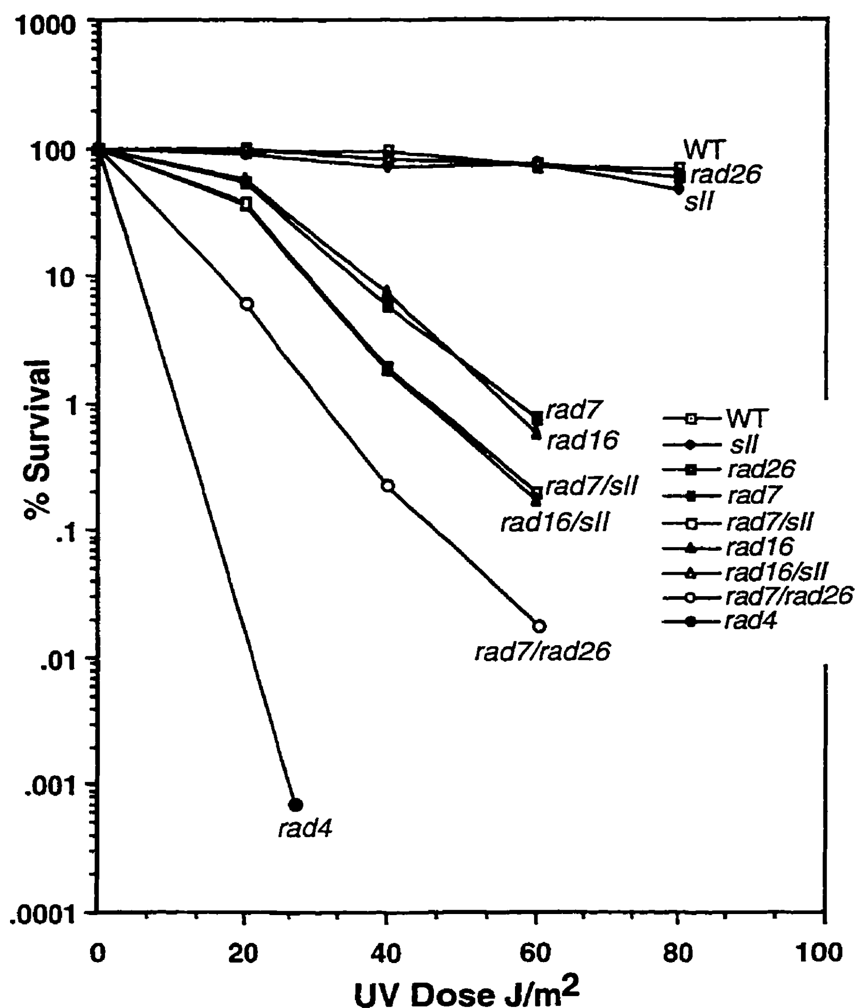

Fig. 1. Disruption of the *SII* gene leads to enhanced UV sensitivity in the absence of global genome repair. Yeast strains with the *SII* gene deleted in various *rad* backgrounds were irradiated with increasing doses of UV light. Appropriate dilutions of the cells were then spread on YPD plates and grown in the dark for 2-3 days. Colonies were counted and the results expressed as percentage survival relative to the unirradiated sample.

Mutant	UV dose (J/m²)	Average Survival (% ± SD)[a]
rad7	0	100
	60	0.441 ± 0.053
rad7 sII	0	100
	60	0.107 ± 0.017
rad7rad26	0	100
	60	0.0223 ± 0.0024
rad7rad26sII	0	100
	60	0.0082 ± 0.0011

[a] Average percent survival values were determined from triplicate experiments

Table 2. Comparison of the UV sensitivities of *rad7* and *rad7rad26* mutants in the absence or presence of an *sII* deletion

RAD26 gene product, a protein which may have a role in linking repair to ongoing transcription. The UV sensitivity of both *rad7rad26sII* and *rad16rad26sII* triple mutants was measured and compared to that of the *rad7rad26* and *rad16rad26* double mutants. As can be seen in Fig. 2, the triple mutants manifested modest increases in UV sensitivity, approximately 3 fold, over those of the double mutants. Student's *t* test analysis of data obtained from a typical experiment generated a *P* value of 0.001 for the observed 2.7 fold difference in UV sensitivity between *rad7rad26* and *rad7rad26sII* mutant cells (Table 2). These results suggest that *RAD26* and *SII* may not contribute to NER efficiency in the same manner, for deleting both genes caused a more severe phenotype than when only one of them was deleted. The double mutant *rad26sII* also exhibited a small increase in UV sensitivity compared to the NER proficient wild-type cells; this NER deficiency was detected without the need to eliminate the efficient global genomic repair pathway.

RNA Polymerase II Mutants Defective in Their Response to SII Stimulation Confer UV Sensitivity

Since SII is known to interact with the largest subunit of RNA polymerase II encoded by the *RPO21*(or *RPB1*) gene (Wu et al. 1996), mutations in the Rpo21 polypeptide that affect SII binding might also be expected to confer UV sensitivity. Mutations in *RPO21* that compromised the ability of polymerase II to interact with SII had first been identified in a genetic screen (Archambault et al. 1992a, b). Two of these mutated RNA polymerase II enzymes, represented by alleles *rpo21-18* and *rpo21-24* (Fig. 3), have a 50-fold reduction in binding affinity for SII and are severely compromised in their ability to perform SII-stimulated RNA cleavage and readthrough at intrinsic arrest sites (Wu et al. 1996). As with our analysis of the effects of deleting the *SII* gene, strains carrying these two *rpo21* mutations were crossed to *rad7* or *rad16* null mutants to create double mutants. The analysis of their capacity to survive UV irradiation showed an expected enhanced UV sensitivity over that displayed by *rad7* or *rad16* strains (Fig. 4 and data not shown). Like cells with a deletion of the *SII* gene, the single *rpo21* mutants behaved exactly like wild-type cells and showed no increase in sensitivity to UV.

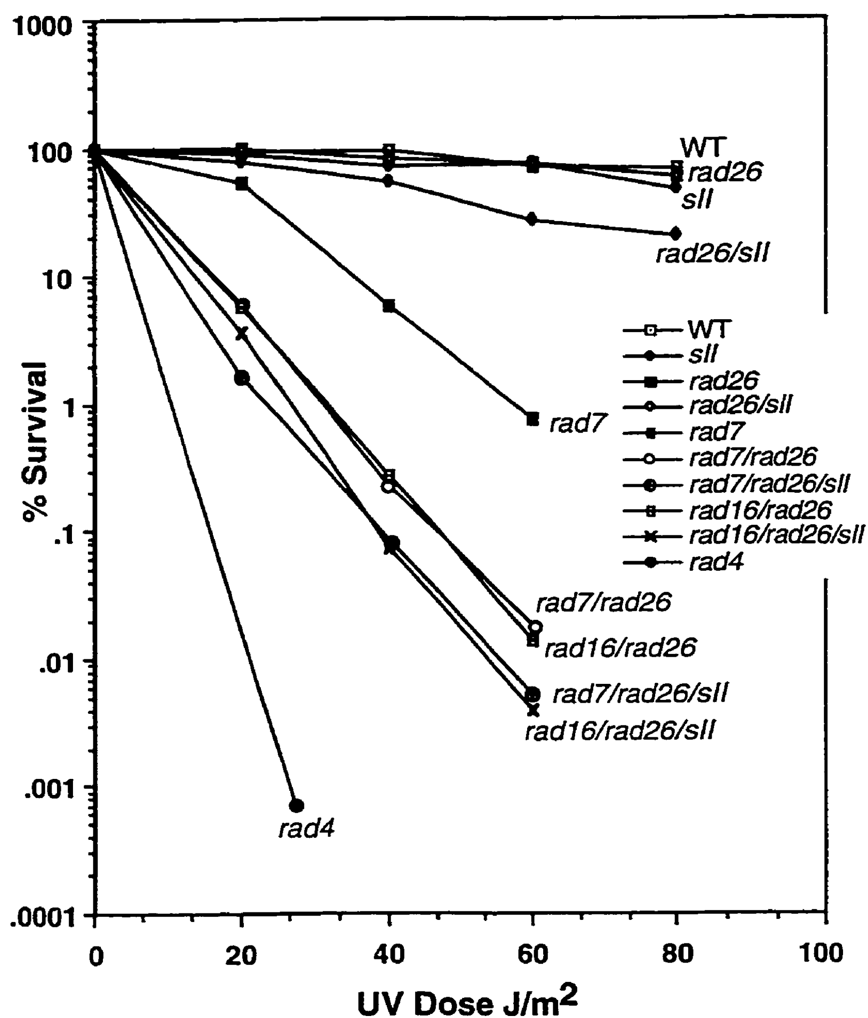

Fig. 2. *SII* and *RAD26* contribute to UV resistance in different genetic pathways. Yeast strains with the *SII* gene deleted in various *rad* backgrounds were irradiated with increasing doses of UV light. Procedures for cell plating and construction of survival curves were as described in Fig. 1.

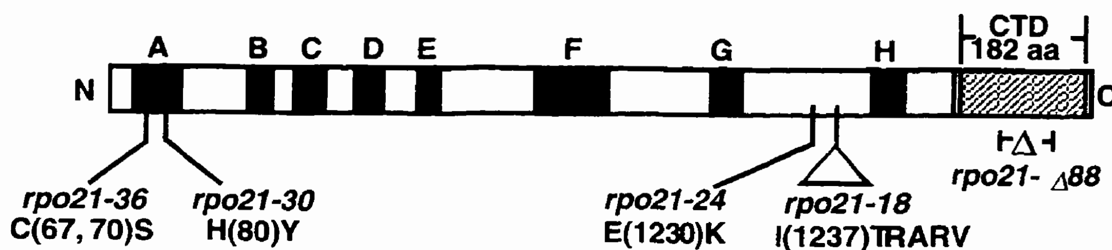

Fig. 3. Schematic diagram of the *RPO21* gene product showing the locations of the mutations used in this study. The black boxes represent the regions that are most conserved in various organisms. The striped box represents the carboxyl-terminal domain (CTD) unique to the largest subunit of eukaryotic RNA polymerase II.

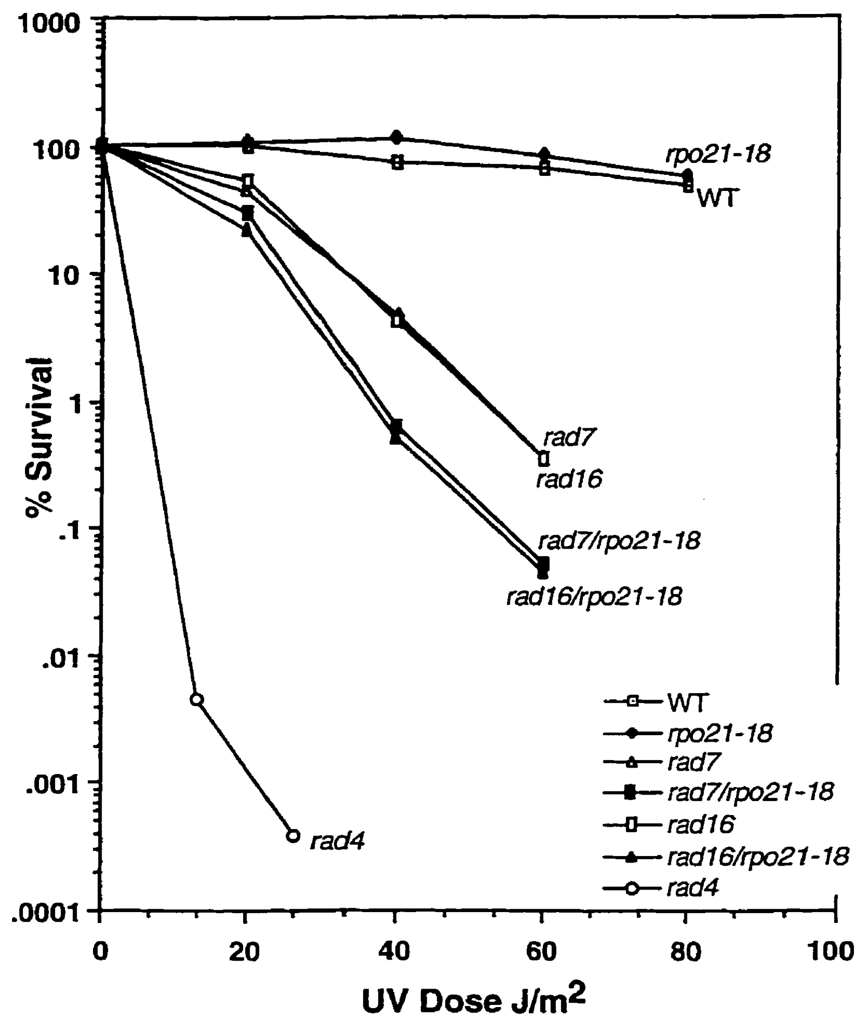

Fig. 4. An RNA Polymerase II mutant with reduced affinity for SII increases UV sensitivity in the absence of global repair. Various yeast strains (see Table 1) harboring the Rpo21-18 mutant polymerase were UV irradiated and plated as described in Fig. 1. Colonies were counted to assess survival rate at various UV doses.

In addition to the mutations in the Rpo21 polypeptide, other RNA polymerase II mutants have also been shown to be defective in transcription elongation (Awrey et al. 1997; Powell et al. 1996). One such mutant polymerase lacks the Rpb9 polypeptide, one of the twelve subunits that make up the core RNA polymerase II enzyme, and is defective in SII-stimulated readthrough *in vitro* (Awrey et al. 1997). To investigate if yeast cells expressing this mutant RNA polymerase II had enhanced UV sensitivity, double and triple mutant strains carrying an *rpb9* deletion were constructed. As with the *rpo21-18* mutation described above (Fig. 4), enhanced UV sensitivity was observed for the *rpb9* cells, but once again only in a *rad7* background (Fig. 5). Furthermore, the *rad7rbp9sII* triple mutant displayed a level of UV sensitivity similar to that of the *rad7rpb9* double mutant (Fig. 5). This finding is consistent with the fact that RNA polymerase II lacking its Rpb9 subunit is unable to readthrough arrest sites once it is stalled, even in the presence of SII (Awrey et al. 1997). *RPB9* and *SII* appear to function in the same genetic pathway and disrupting two genes contributing to this shared function does not create a more severe phenotype.

Other *rpo21* Mutations Not Affecting SII Function Also Confer UV Sensitivity

To determine if the kinds of mutations in RNA polymerase II which enhance UV sensitivity are restricted to those with a compromised ability to respond to SII-mediated stimulation of readthrough at arrest sites, we also evaluated RNA polymerase II mutations which map to domains within the RPO21 polypeptide other than its apparent SII-binding domain. Three such mutant strains were chosen, two with mutations in the putative zinc binding region near the N terminus of the Rpo21 polypeptide, *rpo21-30* and *rpo21-36*, and a third with an 88 amino acid deletion from within its carboxyl-terminal repeating heptapeptide domain (CTD) (see Fig. 2). This CTD mutant has only 13 rather than 26 of the YSPTSPS carboxyl-terminal repeats (Allison et al. 1988). Once again, strains carrying each of these mutant forms of RNA polymerase II were crossed to a *rad7* strain, in anticipation that the global repair pathway might mask any effects of the polymerase II mutations. As can be seen in Fig. 6, single *rpo21* mutants showed no heightened

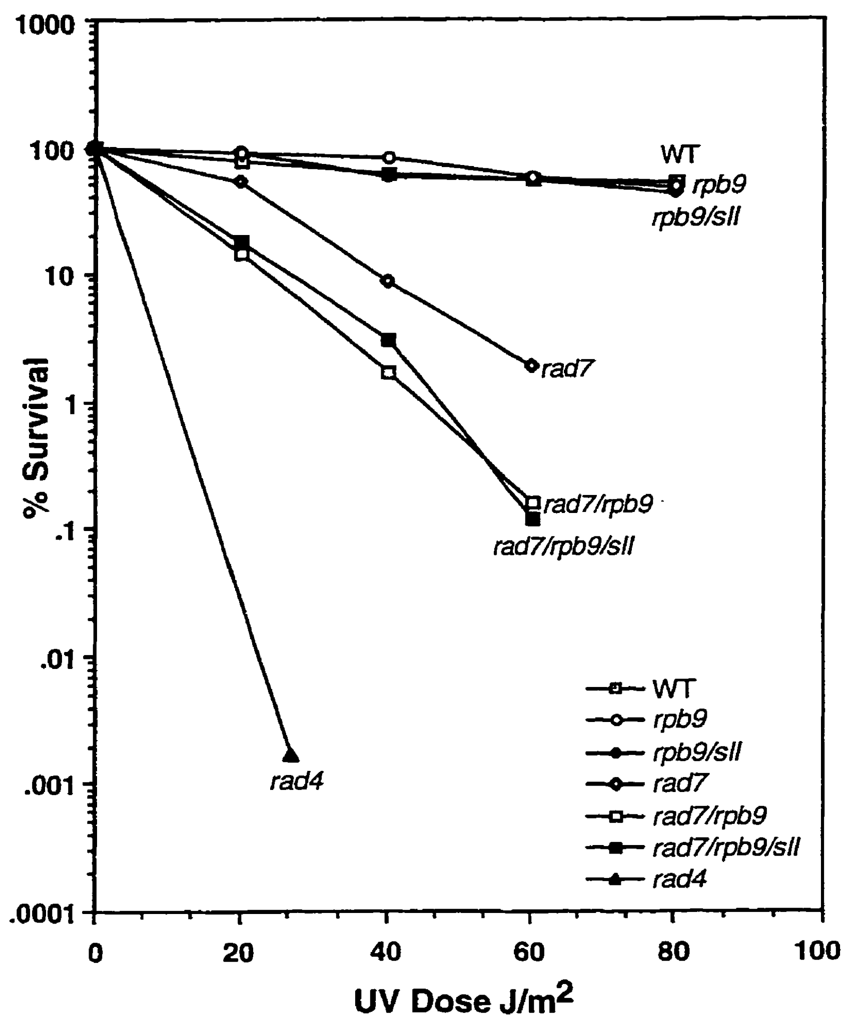

Fig. 5. RNA Polymerase II lacking the Rpb9 subunit can also increase UV sensitivity. Various yeast strains (see Table 1) harboring a mutant RNA polymerase lacking the Rpb9 subunit and defective in response to SII stimulation were UV irradiated and plated as described in Fig. 1. Colonies were counted to assess survival rate at various UV doses.

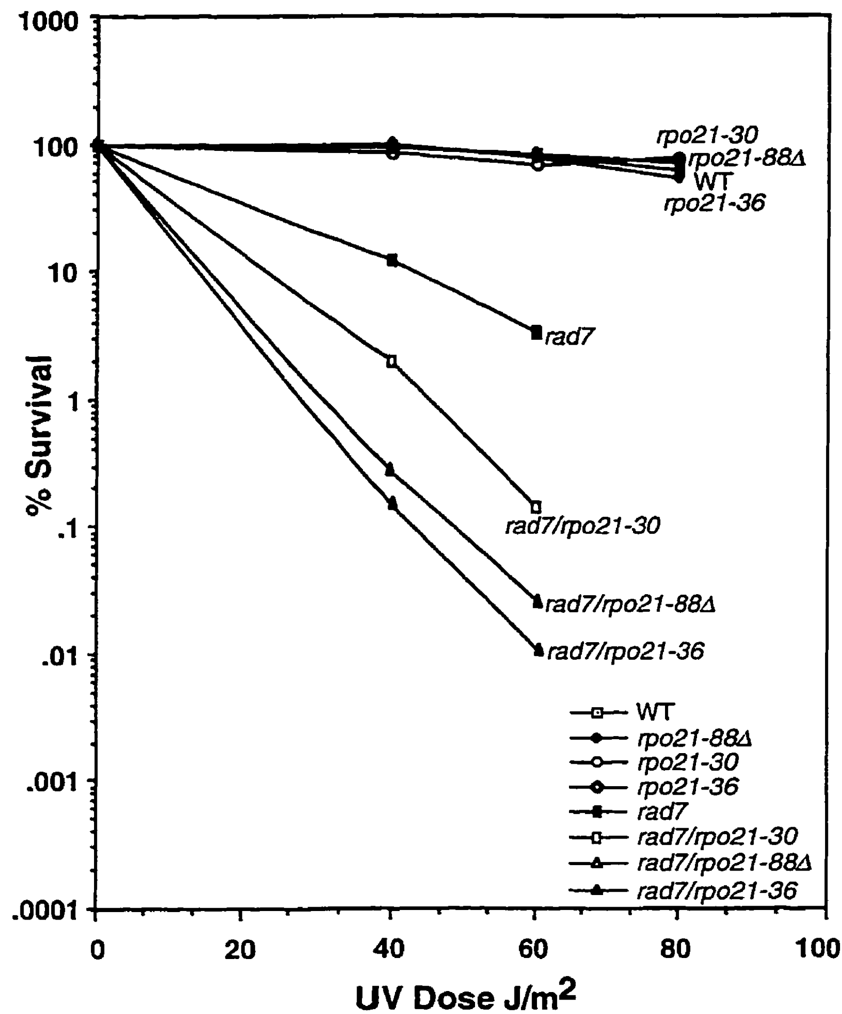

Fig. 6. Other mutations in *RPO21* not affecting RNA polymerase II-SII interaction can also confer UV sensitivity. Yeast cells with *rpo21* mutations outside the SII binding site were subjected to increasing doses of UV radiation. Cells were plated and incubated as described in Fig. 1. Percentage survival rates were measured and plotted as shown.

sensitivity to UV light as compared to the isogenic wild-type cells. However, in the absence of global repair i.e. in the presence of a *rad7* mutation, each of these three RNA polymerase II mutations led to enhanced UV sensitivity compared to that seen with deletion of *RAD7* alone (Fig. 6). The effects of the internal CTD deletion and the *rpo21-36* allele were comparable and somewhat more marked than that of the *rpo21-30* mutation (Fig. 6). These results suggest that RNA polymerase II mutants with defective ability to respond to SII are only one class of mutants that can enhance sensitivity to UV. A spectrum of different mutations spanning the entire *RPO21* gene have similar effects on the survival of yeast cells after UV irradiation provided that the global repair pathway is crippled.

Underproduction of the Largest Subunit of RNA Polymerase II Can Increase UV Sensitivity

In view of the above observations showing that a variety of mutations in the RNA polymerase II enzyme itself can have similar effects in terms of sensitizing yeast to the lethal effects of UV irradiation, we reasoned that an intrinsic physical defect in one of the subunits might not be the only route to this sort of enhanced UV sensitivity. Specifically, a decreased steady-state level of the RNA polymerase II complex itself might be sufficient to enhance UV killing. To test this, we adopted a system described by Archambault et al. (1996) in which the endogenous amount of RNA polymerase II can be manipulated by controlling the expression of the *RPO21* gene product. In this system the *RPO21* open reading frame is fused to the *LEU2* promoter, which can be repressed by the addition of leucine to the culture medium (Archambault et al. 1996). By appropriate crossings, yeast strains with or without the *RAD16* deletion and with the chromosomal *RPO21* locus replaced by an episomal *pLEU2-RPO21* construct (Archambault et al. 1996) were obtained. After UV irradiation, the *pLEU2-RPO21*-containing yeast cells were plated on minimal plates with or without addition of leucine in order to modulate *RPO21* expression and hence the endogenous concentration of active RNA polymerase II complex. The intracellular concentration of RNA polymerase II did not have any effect on the UV survival of cells if the global repair pathway was intact (Fig. 7). On the other hand,

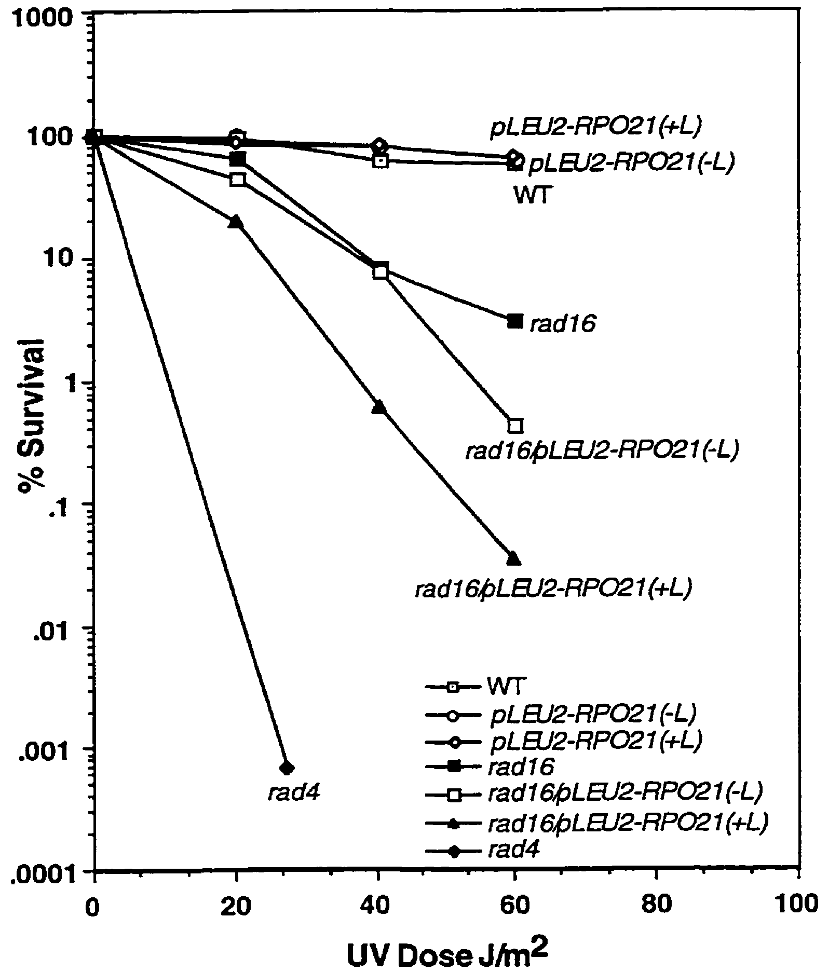

Fig. 7. Decreased steady-state levels of RNA polymerase II lead to increased UV sensitivity in the absence of global genome repair. Yeast cells expressing *RPO21* under the control of the *LEU2* promoter in the presence or absence of a *rad16* null mutation were irradiated and then plated on solid minimal medium with (+Leu) or without (-Leu) addition of leucine.

when the *RAD16* gene was deleted, the *pLEU2-RPO21* cells that had been grown under repressing conditions on leucine-containing plates displayed a marked increase in sensitivity to UV irradiation compared to *rad16* cells (a 100-fold increase at 60 J/m^2). The *rad16* cells expressing *RPO21* from the *LEU2* promoter under derepressed conditions (minus leucine) only showed a difference in UV sensitivity over that of *rad16* cells at the highest UV dose used (60 J/m^2). This finding may be due to a slight decrease of cellular Rpo21 polypeptide content when this polypeptide is expressed from the *LEU2* promoter under the derepressed (minus leucine) conditions compared to the content of Rpo21 in cells expressing this gene from its natural promoter (Archambault et al. 1996). The effect of this small decrease in Rpo21 levels may only have become significant at higher UV doses. The marked enhancement in UV sensitivity seen in *rad16/pLEU2-RPO21* cells under repressed conditions, however, clearly demonstrated that enhanced UV sensitivity in a *rad16* strain can be brought about by simply lowering the steady-state level of RNA polymerase II.

DISCUSSION

We initially wanted to determine whether the RNA polymerase II elongation factor SII (TFIIS) played a role in DNA repair *in vivo*. Several years ago, Hanawalt (1994) had suggested that SII might be required for TCR because it had the unique property of enabling RNA polymerase II to transcribe through natural arrest sites. Since a UV-induced lesion in DNA represents an absolute block to elongation by RNA polymerase II (Donahue et al. 1994), it is conceivable that, once the lesion is removed by the TCR subpathway of NER, SII might be required to reactivate the arrested polymerase. Here we show that deletion of the *SII* gene by itself did not contribute to any heightened sensitivity to UV irradiation, whereas deletion of the *SII* gene did enhance UV sensitivity when the *RAD7*- and *RAD16*-dependent global genomic repair pathway was also compromised. This observation is reminiscent of the effect of deletion of *RAD26*. An effect on UV sensitivity of a *rad26* deletion was observed only in association with a *rad7* or *rad16* null mutation (Verhage et al. 1996 and see Fig. 1). In contrast, mutations in the human homolog of *RAD26*, the *CSB* gene, do, by themselves, confer UV sensitivity in human cells (Troelstra et al. 1992; Nance and Berry 1992). A simple explanation for the lack of UV sensitivity in the single *rad26* or *sII* mutants is that the UV-induced lesions are more rapidly repaired in *S. cerevisiae* cells than in human cells by the very efficient pathway of global genome repair. The increase in UV sensitivity we observed in our experiments with cells lacking SII was small, about 3-fold, but this difference was statistically significant and was reproduced in multiple experiments (Table 2). In this respect, it is perhaps curious that Verhage et al. (1997), who were also investigating the potential role of *SII* in TCR, did not detect this increased UV sensitivity in their *rad7sII* and *rad26sII* mutants. These apparently contradictory results may be due to differences in the UV irradiation protocols, minor strain differences and/or the growth conditions of the yeast cells. Most importantly, however, Verhage et al. (1997) clearly established that *SII* was not required for strand-specific repair of the *RPB2* gene in either the *rad7* or *rad16* background. In contrast, deletion of the *RAD26* gene did eliminate strand-specific repair (van Gool et al. 1994; Verhage et al.

1996). This *sII* phenotype, a modest increase in UV sensitivity in the presence of mutant *rad7* or *rad16* with an apparent lack of any defect in TCR, is similar to that reported for yeast cells deleted for the *RAD28* gene, the yeast homolog of the *CSA* gene (Bhatia et al. 1996). However, unlike the *rad28* deletion, deletion of the *SII* gene synergizes with the effects of deleting the *RAD26* gene, conferring an increase in UV sensitivity over that seen in either *rad7(or rad16)rad26* or *rad7(or rad16)sII* mutants (Fig. 2 and Table 2). Thus, it would appear that *SII* contributes to UV resistance in a different manner than does *RAD26* or *RAD28.*

Consistent with this phenotype caused by deleting the *SII* gene, mutations in the largest subunit of RNA polymerase II which compromise its ability to respond to stimulation of elongation by SII, also confer sensitivity to UV irradiation in the *rad7* or *rad16* background. Surprisingly, however, we found that this phenotype of enhanced sensitivity to UV light can also be conferred by other quite different RNA polymerase II mutations not known to affect the function of SII. Indeed, even a simple reduction of the intracellular levels of RNA polymerase II itself had the same effect. Taken together, these observations argue that the increased UV sensitivity may be the consequence of impaired transcription rather than any specific defect in the TCR pathway of repair. This conclusion is also consistent with the lack of correlation seen between UV sensitivity and strand-specific repair seen in either *rad7sII* or *rad7rad28* cells (Verhage et al. 1997; Bhatia et al. 1996 and Table 3)

We suggest that the phenotype of enhanced UV killing is related to the inability by RNA polymerase II to perform optimal transcription of certain genes required to deal with the effects of UV-induced damage. Candidates for the genes involved in conferring UV resistance are *RAD* genes involved in the actual repair. Indeed, transcription of genes such as *RAD2* (Robinson et al. 1986) and *RAD7* (Jones et al. 1990), *RAD16* (Bang et al. 1995) and *RAD23* (Madura et al. 1990) are known to be induced upon exposure to UV. It is possible that in the absence of the highly efficient global repair pathway, cellular viability is compromised by a weakened transcriptional response to DNA damage. For example in the recent yeast genome wide expression study of Holstege et al (1998), *RAD2* mRNA was shown to be transcribed infrequently and present in cells at a very low level. Shifting *Rpb1-1* cells

Mutations/Deletions	UV sensitivity	TCR
Human *RAD26 (CSB)*	**increase** (Troelstra et al, 92)	**decrease** (van Hoffen et al, 93)
Yeast *RAD26*	**increase** **(only if *rad7/16* deleted)** (Verhage et al, 96; van Gool et al, 94)	**decrease** (Verhage et al, 96; van Gool et al, 94)
Human *RAD28 (CSA)*	**increase** (Henning et al, 95)	**decrease** (van Hoffen et al, 93)
Yeast *RAD28*	**increase** **(only if *rad7/16* deleted)** (Bhatia et al, 96)	**no defect** (Bhatia et al, 96)
SII (TFIIS)	**increase** **(only if *rad7/16* deleted)** (this study)	**no defect** (Verhage et al, 97)
RPO21	**increase** **(only if *rad7/16* deleted)** (this study)	**not determined**

Table 3. UV Sensitivity and Transcription-Coupled Repair

to the nonpermissive temperature and thus immediately halting all polymerase II transcription had a particularly marked effect on the *RAD2* transcript concentration, reducing it more than 10 fold.

As has been pointed out by Archambault et al. (1996), transcription of genes controlled by limiting regulatory factors are particularly sensitive to mutations which compromise transcription efficiency. It is therefore interesting to note that mRNA levels of *UME6* which encodes a transcriptional regulator involved in the damage-induced expression of several DNA repair genes including *RAD2* (Sweet et al. 1997), also dropped significantly (6-fold) when *rpb1-1* cells were shifted to the nonpermissive temperature (Holstege et al. 1998). Another yeast gene particularly sensitive to the effect of impaired transcription is *INO1*. The *INO1* gene is under the control of an *INO2*-dependent loop (Ashburner and Lopes 1995a, b). A 4 to 6 fold reduction of *INO1* transcription, brought about by underproducing the *Rpo21* polypeptide (Archambault et al. 1996) or by mutations that affect components of the transcription machinery including CTD truncation or other mutations in the *RPO21 (RPB1)* and *RPB2* genes (Scafe et al. 1990a-c), is sufficient to cause inositol auxotrophy. The set of pleiotropic phenotypes caused by a wide variety of mutations in the RNA polymerase II transcription machinery may therefore include not only this inositol auxotrophy and, as previously noted (Archambault et al. 1996), the temperature sensitivity and slow growth phenotypes, but also the UV sensitivity which we now report.

Our findings may have relevance for understanding the molecular defect underlying Cockayne syndrome, a genetic disease caused by mutations in the *CSA* or *CSB* genes, which are, respectively, the homologs of the *S. cerevisiae RAD28* and *RAD26* genes. Our results support the suggestion of others (Friedberg 1996) that the primary defect leading to developmental abnormalities in CS could be a defect in transcription rather than TCR. Several recent reports have shown that the CSA and CSB proteins may have a more direct role in transcription than serving only to link efficient NER to ongoing transcription by RNA polymerase as the *mfd* gene product appears to do in *E. coli*. Both *in vivo* and *in vitro* experiments have provided evidence that CS cells support a reduced level of transcription directed by RNA

polymerase II (Balajee et al. 1997; Dianov et al. 1997) and Selby and Sancar (1997b) showed that transcription elongation *in vitro* can be stimulated by the CSB protein. In light of these findings, it can be envisaged that the lack of functional CS proteins may alter the expression of one or more important genes whose efficient transcription is more dependent on CS proteins than are others. Indeed just such a deficiency in transcription of the human *OGG1* gene in CSB cells has been recently reported (Dianov et al. 1999). Underexpression of this and other genes during post-natal growth may result in the pathological features associated with CS. Our study in yeast cells implies that mutations in additional components of the RNA polymerase II transcription machinery other than the dual function repair/transcription factor TFIIH (Lehmann 1998) can confer on yeast an increase in sensitivity to UV irradiation which is also a hallmark of CS. Thus, CS may represent a *bona fide* "transcription syndrome" with the characteristic clinical features including UV sensitivity as well as the lack of TCR being secondary to a primary transcription defect. Identification of the important effector genes underexpressed in CS or in the mutant yeast strains used in this study may provide further insight into the pathology of this syndrome.

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CHAPTER IV

Assessing the Role of Replication Protein A in Base Excision Repair in Yeast and Human Cell Extracts

This chapter represents preliminary work still in progress

I did all of the experiments in this Chapter with some assistance from I. Pasic

SUMMARY

The base excision repair (BER) pathway is responsible for removing lesions in DNA that, in general, have not caused major distortions of the double helix. Two examples of lesions repaired by BER are the uracil residues which arise from deamination of cytosine and thymine glycols which are formed as a consequence of oxidative reactions. DNA glycosylases participate in the first step of BER, the recognition and excision of damaged bases. Human uracil DNA glycosylase (UDG) was recently reported to interact with the 34 kDa subunit of RPA, raising the possibility that RPA may be involved in BER. To investigate the role, if any, of RPA in BER reactions in both yeast and human cells, *in vitro* BER assays were developed using yeast and HeLa cell extracts. These assays employ plasmid DNAs containing uracil or thymine glycols as substrates and quantitate incorporation of ^{32}P-labelled dNTP into DNA during the repair DNA synthesis step which follows base excision. Extracts made from *rad7* or *rad4* yeast strains known to be defective in nucleotide excision repair supported efficient synthesis of labelled DNA on damaged but not undamaged substrate DNAs; these findings argue that NER does not contribute significantly to the observed repair synthesis. Yeast extracts made from *rfa2* mutants with various C-terminal deletions can now be tested for activity in these assays of BER. These truncations span the region of the Rpa2 polypeptide which, in human RPA2, is responsible for the interaction with UDG. To assess the role of RPA in human BER, extracts from HeLa cells have been fractionated chromatographically to deplete endogenous RPA. This RPA-depleted extract can now be tested for BER activity in the presence or absence of added purified RPA.

INTRODUCTION

The genetic material is constantly subject to damage caused by environmental insults, including UV light and chemical mutagens, and by toxic metabolic side products such as reactive oxygen species. For lesions, including those with alkylated bases or uracils in DNA, that do not cause major distortions of the helix, the principal pathway of repair is the base excision repair (BER) system (Lindahl, 1997; Krokan et al, 1997; Seeberg et al, 1995). As its name implies, the first step in BER involves the removal of the damaged base by a class of enzymes known as DNA glycosylases. These enzymes catalyze the hydrolysis of the N-glycosyl bond linking the altered base to deoxyribose. The resulting abasic or apurinic/apyrimidinic (AP) site is then processed by an AP endonuclease which cleaves 5' to the AP site leaving a 3'-hydroxyl and a deoxyribose 5'-phosphate moiety (dRp). In mammalian cells, the major AP endonuclease is called HAP1/APE/BAP1 (Robson and Hickson, 1991; Demple et al, 1991) and has also been isolated as the nuclear redox protein Ref-1 (Rothwell et al, 1997). *S. cerevisiae* also has one major AP endonuclease encoded by the *APN1* gene, deletion of which causes hypersensitivity of yeast cells to oxidative and alkylating agents (Ramotar et al, 1991). Following cleavage at the AP site, two alternative pathways differing in the size of the repair patch of DNA synthesis may be utilized for completing the process of BER in eukaryotic cells. Short-patch repair results in incorporation of a single nucleotide residue during DNA repair synthesis, whereas long-patch BER generates a repair patch of 2-7 nucleotides.

Short-patch BER has been reconstituted with human uracil-DNA glycosylase (UDG), AP endonuclease (HAP1), DNA polymerase β (Polβ), XRCC1 and either DNA ligase III or DNA ligase I (Kubota et al, 1996). In addition to its polymerization activity, DNA polymerase β also has an intrinsic dRpase activity which can remove the dRp residue at the incised AP site (Matsumoto and Kim, 1995). That DNA ligase III is probably the enzyme utilized *in vivo* is supported by the observation that its stability is modulated by the interaction with XRCC1, a scaffold protein that interacts

with both ligase III and DNA polymerase β (Taylor et al, 1998; Cappelli et al, 1997; Caldecott et al, 1995; Caldecott et al, 1994).

The existence of a second long-patch mode of BER was first demonstrated by studies in yeast and mammalian cell extracts showing that a circular plasmid molecule containing multiple AP sites was efficiently repaired by BER with a resulting patch size for the newly synthesized DNA of approximately 7 nucleotides (Wang et al, 1992; Frosina et al, 1994). *In vitro* reconstitution of these long-patch BER reactions showed that the initial reactions can use the same DNA glycosylase(s) and AP endonuclease as the short-patch pathway, but that PCNA is required in the repair synthesis step (Klungland and Lindahl, 1997). These observations confirmed earlier findings that, in a *Xenopus laevis* oocyte system, BER occurred not only via a DNA polymerase β-dependent pathway but also by a PCNA-dependent pathway. Since PCNA acts as a processivity factor for both DNA polymerase δ and ε (Kelman, 1997), these two polymerases may have a role in this mode of repair. Indeed, the requirement for either polymerases δ or ε in BER has been demonstrated both in the yeast and *Xenopus* oocyte systems (Blank et al, 1994; Wang et al, 1993; Matsumoto et al, 1994). Another factor that is essential for the long-patch mode of BER is the flap structure-specific nuclease DNase IV/FEN-1, required for the cleavage of the displaced dRp-containing parental strand of DNA (Klungland and Lindahl, 1997). Interestingly, the activity of DNase IV/FEN-1 is also stimulated by both PCNA (Wu et al, 1996; Li et al 1995) and RPA (Biswas et al, 1997).

In vivo, other factors may modulate the efficiency of BER. The possibility that replication protein A (RPA) may also have a role in the long patch mode of BER is particularly intriguing as it is known to stimulate the activities of DNA polymerases δ and ε (Podust and Hubscher, 1993; Tsurimoto and Stillman, 1989; Lee et al, 1991) and FEN-1, the same activities that are also stimulated by PCNA. A role of RPA in BER has also been suggested by the observation that the C-terminus of the 34 kDa subunit of RPA (RPA2) interacts with uracil DNA glycosylase, the enzyme responsible for removing uracil from DNA (Nagelhus et al, 1997). Uracil can spontaneously arise as a result of deamination of cytosine; if not removed, the

mispairing of uracil with guanosine can cause transition mutations (Mosbaugh and Bennett, 1994). To address the role, if any, of RPA in BER, I have set up assay systems capable of performing BER in mammalian and yeast extracts.

EXPERIMENTAL PROCEDURES

Preparation of Yeast and HeLa Whole Cell Extracts

The *S. cerevisiae* strains used in this study were *rad* mutant strains, MGSC131 (*rad4Δ::URA3*), MGSC104 (*rad7Δ::LEU2*) and their isogenic parental strain W303-1b (Verhage et al, 1994, 1996) (kindly provided by Dr. J. Brouwer, Leiden University). The procedure for the preparation of BER-proficient yeast extract was identical to that previously used for studying nucleotide excision repair (He et al, 1996). A step-by-step description of this protocol has been published (Wong et al, 1998 and Appendix I of this thesis). Human cell-free extracts were prepared from HeLa cells according to published protocols (Sopta et al, 1985). Protein concentrations were determined by the Bio-Rad colorimetric assay using bovine serum albumin as standard. Extracts containing equivalent protein content were used in BER assays.

Fractionation of HeLa Whole Cell Extracts

Whole cell extract (200 mg, 10 ml) was fractionated on a 100 ml phosphocellulose (Whatman P11) column as previously described (Shivji et al, 1992). The column was equilibrated in Buffer A (25 mM HEPES-KOH, pH7.8, 1 mM EDTA, 0.01% NP40, 10% glycerol, 1 mM dithiothreitol, 0.1 mM phenymethylsulfonyl fluoride) containing 0.1 M KCl. Fractions (3-5 ml) were collected at a flow rate of 90 ml/hr. The column was washed with Buffer A until no protein came out as measured by Bio-Rad protein assay. After collection of flow-through fractions, bound protein was eluted with Buffer A containing 1.0 M KCl. The peak fractions from the flow-through (CFI) and the high-salt elution fractions (CFII) were pooled, dialyzed, concentrated by polyethylene glycol and stored in 100 μl aliquots at -70°C.

Expression and Purification of Recombinant Proteins

Recombinant human replication protein A was purified from *E. coli* cells essentially as described previously (Henricksen et al, 1994) with the following

modifications. Lysate from 4 L of induced culture was fractionated on Affi-Gel Blue (100 ml) and hydroxylapatite (10 ml) columns. After dialysis, peak fractions from the hydroxylapatite column were applied to a single-stranded DNA agarose (Pharmacia) column. After loading, the column was washed with 2 column volumes each of buffer containing 0.5 M and 0.75 M NaCl. Elution was carried out using a buffer containing 1.5 M NaCl. Peak fractions were pooled and dialyzed against several changes of ACB (Formosa et al, 1991) containing 0.1 M NaCl.

To purify human PCNA, the coding sequence was amplified by PCR reactions and subcloned into a pET19b (Novagen) vector which expressed the protein as an N-terminal fusion with 10 histidine residues (His_{10}-hPCNA). Purification by one-step nickel-chelate (Qiagen) affinity chromatography was done according to the manufacturer's instructions. Cells from a litre culture expressing recombinant PCNA was harvested and resuspended in 5 ml of Binding Buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH7.9) containing protease inhibitors. After sonication, the clarified lysate was incubated with 0.5 ml of nickel-chelate resin for 1 hr in a batch-binding fashion. The resin was then washed once with Binding Buffer (10 ml) and twice with 10 ml of Wash Buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH7.9). Washed resin was packed in a Bio-Rad Poly-Prep chromatography column and bound protein was eluted with 3 ml of Elute Buffer (0.5M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH7.9). Peak protein fractions were pooled and dialyzed against ACB overnight.

Preparation of DNA Substrates

DNA from plasmids pUC18 (2.7 kb) and pGEM-3Zf(+) (3.2 kb) was isolated by alkaline lysis and CsCl-ethidium bromide equilibrium centrifugation. pGEM-3Zf(+) was used as the undamaged control whereas the smaller pUC18 was used to prepare damaged DNA. UV-irradiated pUC18 was obtained by irradiating the DNA (50 μg/ml) in a thin layer using a Stratalinker UV crosslinker (Stratagene) at a dose of 450 J/m^2. To prepare osmium tetroxide-damaged DNA, pUC18 plasmid (100 μg) was mixed with osmium tetroxide (Sigma) at a concentration of 300 μg/ml in 300 μl of

STE buffer (100 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. After an incubation at 70°C for 90 min, the plasmid DNA was purified by centrifugation at 28, 000 rpm for 17 hr at 4°C on a linear 5 to 20% sucrose gradient, a step which removed nicked plasmid DNA (Wong et al, 1998; Wang et al, 1992). To prepare uracil-containing pUC18 DNA, the plasmid was propagated in the *E. coli* dut^- ung^- strain CJ236 (Kunkel et al, 1987). Uracil-containing plasmid DNA was isolated as above.

BER Synthesis Assay

Standard reactions were set up essentially as described previously (He et al, 1996). Reaction mixtures (50 µl) contained 300 ng of damaged pUC18 and 300 ng of control pGEM-3Zf(+) DNA, 45 mM HEPES-KOH (pH 7.8), 70 mM KCl, 7.4 mM $MgCl_2$, 0.9 mM DTT, 0.4 mM EDTA, 20 µM each of dGTP, dATP, TTP, 8 µM dCTP, 2 µCi of [α-^{32}P]dCTP (3000 Ci/mmol), 2 mM ATP, 40 mM disodium phosphocreatine, 2.5 µg creatine kinase, 3.4% glycerol, 360 µg/ml of bovine serum albumin, and yeast or HeLa extracts as indicated. Reactions were incubated at 28°C for 2 hr. Plasmid DNA was purified from the reaction mixtures, linearized by digestion with *Hin*dIII, analyzed on a 1% agarose gel, and autoradiographed.

RESULTS

Base Excision Repair Synthesis in Yeast Extracts

Previously, we have developed a yeast cell-free system that supports efficient nucleotide excision repair (He et al, 1996, see Chapter II and Appendix I of this thesis). Using this system, we showed that yeast Rpa, like its human counterpart, is involved in nucleotide excision repair. As we wished to determine the role of Rpa in BER, the same *in vitro* system was also tested for its ability to support BER. Since the freezing and grinding of yeast cells in liquid nitrogen during extract preparation minimizes proteolytic degradation, the integrity of the factors that normally perform BER activity is also likely to be preserved.

We first examined the capacity of our yeast extracts to carry out BER on a UV-irradiated pUC18 plasmid. BER was monitored by the incorporation of radiolabelled nucleotides into damaged-plasmid DNA during repair DNA synthesis. An untreated plasmid was also included in each reaction to monitor repair-independent nucleotide incorporation. As shown in Fig. 1A, radioincorporation as a result of repair synthesis was detected on plasmid pUC18 DNA irradiated with UV light. Unirradiated control plasmid showed only very low levels of background incorporation. To exclude the possibility that the repair synthesis observed was only the result of nucleotide excision repair, we also performed the repair reaction in extracts derived from NER-deficient *rad* strains. Extracts prepared from mutant strains with deletions of the *RAD4* or *RAD7* gene, both of which are known to be required for yeast NER (Prakash et al, 1993) (and which we previously showed were required for *in vitro* NER in these same extracts (He et al, 1996)) still supported substantial levels of radioactive dCTP incorporation into the UV-irradiated plasmid (Fig. 1A). However, noticeably lower levels of radioincorporation were observed in the *rad4* and *rad7* extracts as compared to the wild-type extract, indicating that some of this DNA synthesis was the result of NER. This result is in keeping with the fact that UV irradiation can produce several types of photoproducts that are substrates by for both base and nucleotide excision repair (Boorstein et al, 1989; Breimer and

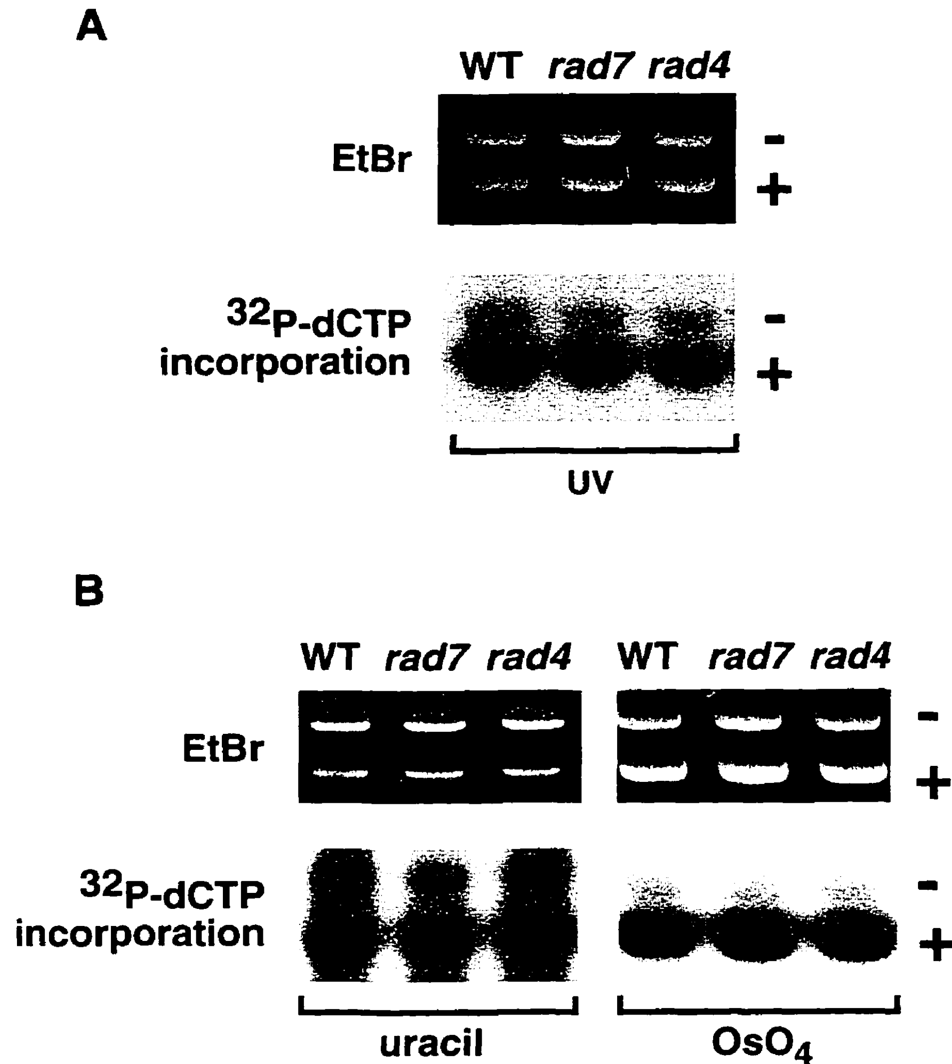

Figure 1. ***In vitro*** **BER in yeast extracts.** The BER activity in yeast cell extracts was assessed by detecting DNA repair synthesis. The incorporation of radioactively labelled deoxycytidine triphosphate into control or damaged DNA isolated from repair reaction mixtures was detected by autoradiography after agarose gel electrophoresis. In the upper panels the DNAs were visualized by ethidium bromide staining; the lower panels show the autoradiograms of the gels. (A) BER activity in isogenic wild-type or *rad* mutant extracts using UV-irradiated DNA as substrate (B) BER activity in wild-type or *rad* mutant extracts using either uracil-containing DNA or osmium tetroxide-treated DNA as substrate.

Lindahl 1984). Since the BER pathway involves the use of various glycosylases, each of which recognizes a specific type of damage, DNA substrates with other types of damage should also be processed by our extracts if they can indeed support BER. Thymine glycol in DNA is known to be a substrate for thymine glycol-DNA glycosylase (Demple and Harrison, 1994). To determine if our yeast extract can carry out BER on thymine glycol-containing DNA, we incubated the extract with pUC18 DNA treated with osmium tetroxide, an agent that promotes oxidation of thymine into thymine glycol. As shown in Figure 1B, similar repair synthesis was observed with both wild-type and the *rad* mutant extracts. Since thymine glycols are not substrates for the *rad4*- or *rad7*-dependent NER pathway, these mutations were not expected to compromise BER. In addition, a DNA substrate containing uracil was prepared and assayed for BER activity. As was the case with the substrate containing thymine glycol, uracil-containing DNA was utilized efficiently in this repair synthesis assay and there was a significant amount of radioincorporation. Again, extracts derived from NER-deficient *rad4* and *rad7* yeast strains also supported repair synthesis of uracil-containing DNA as well as wild-type extracts (see Fig. 1B). This efficient recognition of uracil-containing DNA in our assay was important for subsequent experiments, since our objective of examining the role of RPA in BER, was derived, in part, from the finding that human RPA has been shown to bind to human uracil DNA glycosylase (UDG).

Base Excision Repair Synthesis in HeLa Extracts

As mentioned above, the published finding of an interaction between human RPA and UDG provided the initial impetus for this work. In order to test for any role of RPA in BER in a human system, I also chose to set up a cell-free BER system using HeLa whole cell extracts. Incubation of either osmium tetroxide-treated or uracil-containing DNA with increasing amounts of a HeLa cell extract resulted in increasing levels of radioincorporation (Fig. 2). We concluded from these experiments that both HeLa and yeast extracts prepared as described here can support efficient BER.

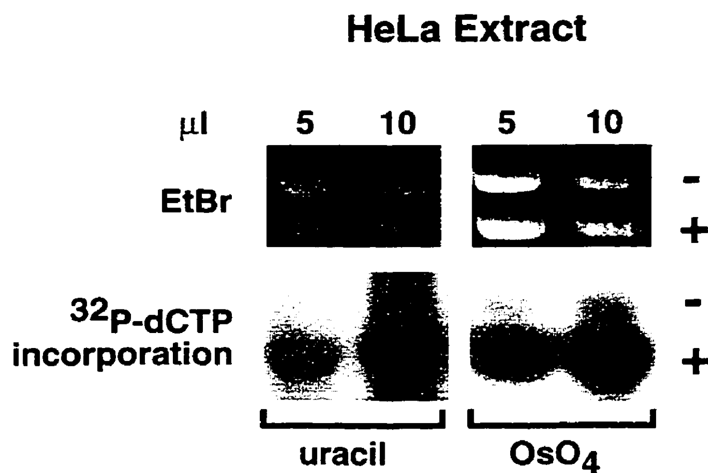

Figure 2. ***In vitro*** **BER in HeLa whole cell extracts.** The BER activity of HeLa whole cell extracts was assessed by detecting DNA repair synthesis, as described in Fig. 1. In the upper panels the DNAs were visualized by ethidium bromide staining; the lower panels show the autoradiograms of the gels. BER activity was measured with 5 and 10 µl of HeLa extracts using either a uracil-containing DNA or osmium tetroxide-treated DNA as substrate.

Purification of human RPA and PCNA

Since reconstitution of BER activity in the RPA-depleted extracts by addition of recombinant RPA is a critical control, the availability of purified human RPA is essential to our approach. To produce RPA in sufficient quantity and purity, I utilized a vector that allows simultaneous expression of all three subunits of human RPA in *E. coli*. This vector has previously been used to produce a high yield of active human RPA (Henricksen et al, 1994; He et al, 1995). As human PCNA is also depleted by phosphocellulose, the expression and purification of human PCNA was also required. Human PCNA cDNA was subcloned into a vector that expresses the protein as a fusion with 10 N-terminal histidine residues. This allowed one-step purification of the tagged human PCNA using nickel-chelate affinity chromatography. As shown in Fig. 3, both human RPA and PCNA were purified in sufficient quantity and to a high degree of purity.

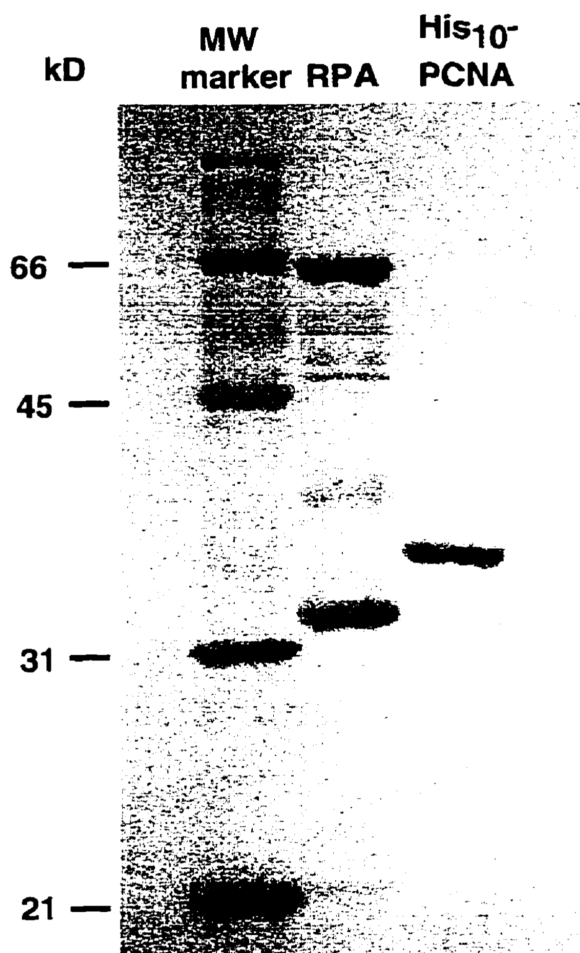

Figure 3. Purified human RPA and PCNA

An SDS-polyacrylamide gel showing Coomassie blue-stained human RPA and histidine-tagged PCNA (2 μg of each). Molecular size markers (2 μg; Bio-Rad) are shown on the left.

DISCUSSION

We have modified a protocol for preparing yeast cell extracts for an assay of *in vitro* NER activity, to make what appears to be a BER-proficient yeast extract. Our success with the NER protocol may have been dependent upon the freezing and grinding of yeast cells under liquid nitrogen, steps which may have preserved cellular activities and minimized protease activity. The high protein concentration achieved by ammonium sulfate precipitation may also have been important. Since these procedures appear to maintain the functional integrity of cellular components, we reasoned that the activity of proteins involved in BER may also be preserved by this protocol. To specifically measure BER activity, we utilized DNA with the different types of damage known to be substrates for DNA glycosylases of the BER pathway. Our yeast extracts preferentially incorporate radiolabelled nucleotides into DNA containing uracil or into DNA damaged either by osmium tetroxide or UV, which produce thymine glycol or pyrimidine dimers and other photoproducts, respectively. With all three types of substrate, efficient BER synthesis was observed even with extracts made from mutant strains such as *rad7* and *rad4*, previously shown to be completely defective *in vitro* in yeast NER. Taken together, our results strongly suggest that the repair synthesis seen in the yeast extracts resulted from BER rather than NER activity. To substantiate our claim that repair synthesis in our assay truly reflects BER, it will be necessary to perform the assay in extracts derived from a yeast strain with a deletion of a gene essential for BER. The gene encoding the major AP endonuclease in yeast (*APN1*) seems to be a good candidate, since its deletion should cripple all steps subsequent to the step carried out by glycosylase. Such an experiment has already been performed by Zhigang Wang et al. (1992) using an analogous system; however, no effect of the *APN1* deletion was seen on BER activity in their assay system, suggesting that a second AP endonuclease may exist in yeast and compensate for the *APN1* deletion. Indeed, a second yeast AP endonuclease gene, named *APN2*, has recently been reported (Johnson et al, 1998). Deletion of both genes may therefore be required.

Although a yeast extract that supports BER has been reported, it requires the preparation of spheroplasts and nuclei (Wang et al, 1992). Not only is our whole cell extract system simple to make, it does not require the preparation of yeast nuclei and appears to support the complete BER reaction. As our yeast transcription extracts also permit efficient transcription by RNA polymerase II, it may be possible, by using a DNA substrate containing an RNA polymerase II promoter, to employ this system to investigate aspects of BER coupled to transcription. Interestingly, recent reports showed that repair of thymine glycols *in vivo* occurs at a faster rate on the transcribed strand than in the untranscribed strand of active genes (Leadon and Cooper, 1993; Cooper et al, 1997). BER, like NER, appears to have a subpathway coupled to ongoing transcription.

We intend to use this yeast system to examine the role of yeast Rpa in BER. Although the original observation that RPA2 interacts with UDG was made with human proteins, the region of UDG interacting with human RPA2 shares some homology with the equivalent region of the yeast Rpa2 polypeptide (Nagelhus et al, 1997). Yeast Rpa2 may therefore also interact in a similar way with yeast UDG. The strategy we will adopt involves making yeast extracts from strains carrying mutations in the *RFA2* gene (Philipova et al, 1996). These mutants contain a series of truncations from the C-terminus of yeast Rpa2 that span the region in yeast Rpa2 analogous to that portion of human RPA2 shown to interact with human UDG. If the same interactions also occur between yeast Rpa2 and yeast UDG, and if they are important for BER, then some of the mutant extracts will be expected to perform BER less efficiently than the wild-type extract. Other future experiments will also include protein-protein interaction studies employing affinity chromatography to assess whether yeast Rpa can interact with yeast UDG, as is the case in the human system.

The approach we will be using to test for a role of human RPA in BER was first used to dissect the protein requirements for SV40 DNA replication (Wold et al, 1989; Prelich et al, 1987) and later for NER (Shivji et al, 1992). This strategy is based on the elution profile of human RPA and PCNA, which flow through phosphocellulose columns while other NER proteins are retained and elute at high

salt. We reason that this strategy will provide a means to effectively remove endogenous RPA and PCNA from HeLa whole cell extracts, although we also made the assumption that other BER proteins will be retained. If our assumption is correct, this fractionated extract should allow us to assess the effect of RPA depletion on BER activity. This approach is worth pursuing since NER and BER share the use of many similar proteins, including DNA polymerases and ligases, in the repair synthesis steps. We would expect that adding back human RPA and PCNA (the latter required only for long-patch BER) should confirm their requirements for BER. To this end, human RPA and PCNA have been expressed and purified. In the event that this chromatographic depletion strategy using phosphocellulose fails, alternative approaches could also be used. The effect of addition of anti-RPA antibodies can also be assessed. These same antibodies could also be used to immunodeplete endogenous RPA from HeLa extracts.

Although human RPA could directly bind to uracil DNA glycosylase (UDG), this interaction did not result in increased glycosylase activity *in vitro* (Nagelhus et al, 1997). This result suggests that RPA does not function to enhance the recognition of uracil. However, RPA may link the early steps of BER to the subsequent steps of DNA repair synthesis by contacting the damage recognition molecule UDG. A similar coordinating role of RPA has previously been suggested to occur during the steps of nucleotide excision repair, as RPA also interacts with the NER recognition protein XPA (He et al, 1995). In addition, it is conceivable that RPA may have a role in the late steps of repair synthesis. Indeed, recent studies using a reconstituted *in vitro* system showed that RPA can stimulate long-patch BER by enhancing strand displacement synthesis (DeMott et al, 1998).

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APPENDIX I

Nucleotide Excision Repair in *Saccharomyces cerevisiae* Whole Cell Extracts

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1. INTRODUCTION

Nucleotide excision repair (NER) is a particularly versatile pathway of DNA repair capable of removing a broad spectrum of DNA lesions in both prokaryotes and eukaryotes *(1-3)*. NER involves steps of damage recognition, incision and excision of the lesion and its flanking DNA, and repair DNA synthesis to fill in the resulting single-stranded gap. Here we describe the techniques used to prepare extracts from *S. cerevisiae* cells capable of performing NER reactions and the details of this *in vitro* NER assay. Studies of DNA repair in *S. cerevisiae* have the advantage of being amenable to powerful genetic analyses within a completely sequenced yeast genome. In fact, most of the earlier work on NER in yeast relied on the genetic analyses of *rad* mutants. On the other hand, the potential for biochemical analysis of NER in yeast has not yet been fully realized owing in part to the lack of a simple *in vitro* repair system. This is in contrast to the situation in human cells in which the *in vitro* system developed by Wood and his colleagues *(4)* has proven instrumental in dissecting the human pathway of NER.

So far, two cell-free repair systems employing *S. cerevisiae* extracts have been described, both by Wang *et al.* from the laboratory of E. Friedberg *(5, 6)*. The first described system utilizes a nuclear extract supplemented with whole cell extract made from a yeast strain that overexpresses the Rad2 protein *(5)*. The second system is simpler and makes use of yeast whole cell extracts. However, this protocol requires the preparation of spheroplasts and same day processing after the harvesting step *(6)*. Here, we describe a simple *in vitro* system using whole cell extract prepared from frozen *S. cerevisiae* cells *(7)*. Some unique features of our system include ease of preparation and general applicability for use with practically any yeast strain. The harvested yeast can be stored frozen at -70°C until the day of extract preparation. Moreover, this extract preparation can be used as the starting material for further biochemical manipulations, such as antibody-depletion experiments to remove a particular protein component *(7)*.

This procedure for the preparation of NER-proficient yeast extract is a modification of protocols originally used for studies of *in vitro* transcription by

RNA polymerases *(8)*. More recently, this same extract has also been shown to support chromatin assembly*(9)*. The critical steps in our own protocol appear to be the freezing and grinding of yeast cells under liquid nitrogen to preserve cellular activities and minimize protease activity, as well as the high protein concentration achieved by ammonium sulfate precipitation. Further characterization of the activities of this extract is certainly worth pursuing since it may provide a system for *in vitro* studies of additional pathways of DNA repair.

2. MATERIALS

2.1. Equipment

1. Cryogenic and leather gloves.
2. Stainless steel spatula: cool with dry ice before use.
3. Matched porcelain mortar and pestle: e.g. Coors #60319 (275 ml, 115 X 70 mm) and #60320. Store at -20°C the day before use.
4. Cryoflask for storing liquid nitrogen.
5. 50 ml sterile, disposable polypropylene tubes.
6. 4 L Erlenmeyer flasks.
7. Beakers: 1 litre, 500 ml, and 250 ml
8. Syringe: 20 ml. Cooled in -20°C freezer before use.
9. Pasteur pipets.
10. Blotting paper: 0.33 mm (Grade 238, VWR Scientific) or equivalent.
11. Dialysis tubing: 12,000-14,000 Mr cutoff.
12. 1.5 ml microcentrifuge tubes.

2.2. Solutions and Reagents

1. Water: High-quality deionized water such as that provided from a Milli-Q (Millipore) system should be used in all procedures and for making up all solutions. Buffers should be made using autoclaved water or stock solutions.

2. Growth Media: YPD (also known as YEPD, 1% yeast extract, 2% Bacto-Peptone, 2% glucose) (*see* Note 1).
3. Yeast Extraction Buffer: 0.2 *M* Tris-acetate (pH 7.5), 0.39 *M* $(NH_4)_2SO_4$, 10 m*M* $MgSO_4$, 20% (v/v) glycerol, 1 m*M* EDTA. Store at 4°C. Add DTT to 1 m*M* and protease inhibitors before use.
4. Protease inhibitor stocks (*see* Notes 2 and 3): 100 m*M* phenymethylsulfonyl fluoride (PMSF) (Sigma) in absolute ethanol (100X), 1 *M* benzamidine hydrochloride (Sigma) in water (1000X), 3.5 mg/ml pepstatin A (Calbiochem) in DMSO (1000X), 1 mg/ml leupeptin (Calbiochem) in water (1000X), 0.35 mg/ml bestatin (Boehringer Mannheim) in water (1000X), and 2 mg/ml aprotinin (Sigma) in water (200X). Store all stocks at -20°C.
5. Solid ammonium sulfate: Analytical grade.
6. Dialysis buffer: 20 m*M* HEPES-KOH (pH 7.5), 20 % (v/v) glycerol, 10 m*M* $MgSO_4$, 10 m*M* EGTA, 5 m*M* dithiothreitol, and 1 m*M* PMSF. Cool in cold room (4-10°C) before use.
7. Plasmid DNA: pUC18 (2.7 kilobase pairs) and pGEM-3Zf(+) (3.2 kilobase pairs). Purify by CsCl centrifugation according to established protocols *(10)* or by any other procedures such as with a QIAGEN DNA purification kit that yield similarly high quality DNA.
8. *N*-acetoxy-2-acetylaminofluorene (National Cancer Institute Chemical Carcinogen Repository, catalog# AM0030): 1 m*M* in ethanol, store at -20°C.
9. dNTP stocks and ATP: Ultrapure set (Pharmacia): Supplied as 100 m*M* solutions, pH 7.5.
10. 4X Repair Buffer A: 180 m*M* HEPES-KOH (pH 7.8), 280 m*M* KCl, 29.6 m*M* $MgCl_2$, 3.6 m*M* DTT, 1.6 m*M* EDTA, and 13.6 % glycerol. Store at -20°C.
11. 4X Repair Buffer B: 8 m*M* ATP, 80 μ*M* dGTP, 80 μ*M* dATP, 80 μ*M* dTTP, 32 μ*M* dCTP. Store at -20°C.
12. 10 mg/ml bovine serum albumin (Sigma, Fraction V): Dissolve in water and store in aliquots at -20°C.

13. Disodium phosphocreatine (Sigma): 1*M* stock in water. Store at -20°C.
14. Creatine phosphokinase (Sigma): 2.5 mg/ml in 50 m*M* HEPES-KOH (pH 7.6), 20 m*M* Mg acetate, and 50% glycerol. Store at -20°C.
15. [α-^{32}P]dCTP (New England Nuclear): Specific activity of 3000 Ci/mmol.
16. 10% SDS: 10% (w/v) sodium dodecyl sulfate in water. Store at room temperature.
17. Proteinase K (Boehringer Mannheim): Make a 20 mg/ml stock in water. Store in 50 μl aliquots at -20°C.
18. Ribonuclease A (Sigma): 10 mg/ml in water. Boil for 10 min to inactivate DNase. Store in aliquots at -20°C.
19. Phenol/chloroform: Buffered-saturated phenol (Gibco-BRL):chloroform:isoamyl alcohol (25:24:1). Phases are allowed to separate after vigorous shaking. Wrap in foil and store at room temperature.
20. 7.5*M* ammonium acetate.
21. Ethanol: Absolute (100%) and 70%.
22. Restriction enzyme HindIII (Gibco-BRL): Store 10X buffer and enzyme in small aliquots at -20°C to ensure optimal activity.
23. 1X TBE: 89 m*M* Tris-base, 89 m*M* boric acid, and 2 m*M* EDTA, pH 8.0.
24. Agarose: Molecular biology grade.
25. 5% and 20% Sucrose: Dissolve ultrapure grade sucrose in 10 m*M* Tris-HCl (pH 7.5), 1m*M* EDTA, and 0.5 *M* NaCl. Prepare fresh and cool to 4°C.
26. Ethidium bromide stock: Dissolve 10 mg/ml in water. Wrap in foil and store at room temperature.
27. TE buffer: 10 m*M* Tris-HCl (pH 8.0), and 1 m*M* EDTA. Prepare with autoclaved stock solutions.

3. METHODS

3.1. Yeast Growth and Harvesting

1. Prepare a starter culture by innoculating the yeast strain into 10 ml of growth media (YPD) in a 50 ml sterile disposable tube (*see* Note 1). Grow until saturation at 27°C with shaking (250 rpm).
2. Innoculate 2 L YPD in a 4 L Erlenmeyer flask with the 10 ml starter culture. Incubate at 27°C with shaking overnight (24 h) (*see* Note 4).
3. Chill the flask in ice water. Transfer culture to 1 L centrifuge bottle (weigh empty bottle, *see* Step 8). Cultures should be kept cold during the entire harvesting procedure.
4. Harvest cells by centrifugation at approximately 4500 X g for 5 min at 4°C. For a Beckman J6-HC centrifuge fitted with a JS-4.2 rotor, the speed is 4000 rpm.
5. Discard supernatant. Add any remaining culture and recentrifuge.
6. Wash cells by thoroughly resuspending in 100 ml of ice-cold water. Pellet cells by centrifugation as in step 4. Discard supernatant.
7. Resuspend cells in 100 ml of cold Yeast Extraction Buffer with DTT and protease inhibitors (*see* Notes 2 and 3). Centrifuge as above.
8. Drain supernatant. Remove residual supernatant with a pipet. The wet weight of cells can be determined at this step if the yield per L of culture is of interest (*see* Note 5).
9. With the help of a spatula, scoop the yeast paste into a cooled 20 ml syringe with the plunger removed. Do not fit the syringe with a needle.
10. Extrude the yeast cells directly into a small plastic container (e.g. a 250 ml beaker) filled with liquid nitrogen (N_2). Keep filling container with liquid nitrogen so that yeast "noodles" are always completely submerged under N_2.
11. Break yeast noodles into pieces with the help of a spatula or a pestle. This helps to keep volume down and allows easier handling in future steps.
12. The frozen yeast can now be transferred to a -70°C freezer for long term storage or until the day of extract preparation. The container should be covered with

aluminum foil to allow liquid nitrogen to evaporate. Make sure container is not tightly capped to avoid pressure buildup and explosion.

3.2. Preparation of Whole Cell Extract

1. Transfer the container with yeast (from Step 12 of 3.1) from freezer into a bucket of dry ice.
2. Weigh out the desired quantity of frozen yeast cells into a small beaker (pre-cooled on dry ice) (*see* Note 6). Transfer onto dry ice immediately after weighing. All following steps are carried out in a cold room.
3. Take the cooled mortar and pestle to a cold room. Fill to three-quarter full with N_2 which will evaporate away relatively quickly. Fill mortar with N_2 again. (Caution: Liquid nitrogen is hazardous both for its extremely low temperature as well as the risk of asphyxiation if used in a small, unventilated room).
4. Pour the weighed yeast noodles into the mortar.
5. Start crushing the frozen yeast under N_2 by stomping the noodle fragments gently against the bottom of the mortar with a pestle. Pressing the yeast against the side and bottom also helps. The goal is to reduce the noodles into even smaller chunks or pellets.
6. Replenish N_2 if it has boiled off.
7. Begin the grinding step that will break the cells by driving the pestle in a circumferential path around the mortar, applying pressure against the side wall.
8. Every now and then, refill the mortar with N_2. Wait until most of the N_2 has boiled off before initiating grinding. The optimal amount of N_2 is such that the yeast powder is just barely resuspended in it to form a slurry, with no obvious layer of excess N_2 above the yeast.
9. If some yeast powder sticks to the wall of the mortar, use the pestle to scrape it off to the bottom below the N_2. Grind until the yeast shows a powdery, smooth consistency (*see* Note 7).
10. After the grinding step, pour more N_2 into the mortar washing all the powder to the bottom with the aid of the pestle.

11. Pour the ground yeast/liquid nitrogen suspension into a 1 L plastic beaker previously cooled with N_2. Scrape any remaining yeast powder into the beaker with a spatula.
12. The beaker with the ground yeast can now be covered with aluminum foil and transferred to a -70°C freezer for storage. Alternatively, if extract preparation is to be done right after the grinding step, let N_2 boil off completely from the beaker in a cold room.
13. Add Yeast Extraction Buffer (1 ml per 1 g of yeast) supplemented with DTT and protease inhibitors. It is not necessary to pre-cool the buffer (*see* Note 8).
14. Allow the yeast to thaw slowly in the cold room. It may take 15-20 min to obtain a fluid suspension. Disperse any clumps of powder by pipetting up and down.
15. Transfer the thawed extract to a centrifuge tube on ice. Centifuge at 120,000 X g for 2 h at 4°C. For a Beckman type 70 Ti rotor, the speed would be 33,000 rpm.
16. After centrifugation, recover the clear portion of the supernatant using a pasteur pipet (*see* Note 9). Transfer the material to a 250 ml beaker.
17. Measure the volume of the supernatant collected. Weigh out 337 mg solid ammonium sulfate per ml of lysate.
18. Add the solid ammonium sulfate in small portions over the course of 1 h with gentle continuous stirring by a small magnetic stir bar.
19. Stir the suspension for another 30 min after all the ammonium sulfate is added (*see* Note 10).
20. Recover the precipitated protein by centrifugation at 40,000 X g (approximately 20,000 rpm in a 70 Ti rotor) for 15 min at 4°C.
21. Remove the supernatant. Resuspend the pellet in a small volume (approximately 50 μl per g of yeast) of dialysis buffer containing protease inhibitors (*see* 4 of 2.2).
22. Transfer the resuspended material to dialysis tubing. Dialyze overnight (12-16 h) against 1 L of dialysis buffer.
23. After dialysis, recover the dialysate to a microcentrifuge tube and centrifuge for 1-2 min to pellet any precipitated protein (*see* Note 11).

24. Recover the clarified supernatant. Freeze the sample in small aliquots in dry ice and store at -70°C (*see* Note 12).

3.3. Preparation of Damaged DNA Substrates

The procedures for preparing DNA repair substrates are essentially the same as described *(6, 11)* and are outlined below in detail. Plasmid pGEM-3Zf(+) is used as the undamaged control whereas the smaller pUC18 is used to prepare AAAF-damaged DNA.

1. Incubate 100 μg of pUC18 at 30°C for 3 h in 1 ml of TE buffer containing 3 μ*M* *N*-acetoxy-2-acetylaminofluorene (*see* Note 13). (Caution: AAAF is a potent carcinogen. Dispose of contaminated equipment according to safety guidelines).
2. Layer the DNA sample onto a linear 5-20% sucrose gradient in 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, and 0.5 *M* NaCl.
3. Centrifuge at 28,000 rpm in a Beckman SW41 rotor for 17 h at 4°C.
4. Collect fractions (each 0.5-1 ml) from the bottom of the centrifuge tube.
5. Check 3 μl of each fraction by electrophoresis on a 1% agarose gel cast in 1X TBE containing 0.5 μg/ml ethidium bromide.
6. Identify the fractions containing closed circular, supercoiled DNA.
7. Pool these fractions and recover DNA by ethanol precipitation.
8. Dissolve DNA in TE buffer so that DNA concentration is at least 100 ng/μl. Store at -20°C.

3.4. In vitro DNA Repair Synthesis

Nucleotide excision repair in the yeast cell extract is monitored by the incorporation of radiolabelled nucleotides into AAAF-modified plasmid DNA during repair DNA synthesis. An untreated plasmid is also included in each reaction to monitor damage-independent nucleotide incorporation. The assay is essentially an adaptation of the protocol developed for human cell extracts *(4)*.

1. Quantitate protein concentration in extract (Section 3.2) by Bradford assay (Bio-Rad Protein Assay) using BSA as standard (*see* Note 14).

2. To a 1.5 ml microcentrifuge tube, add 12.5 µl of 4X Repair Buffer A, 12.5 µl of 4X Repair Buffer B, 1.8 µl of 10 mg/ml bovine serum albumin, 2 µl of disodium phosphocreatine, 1 µl of creatine phosphokinase, 300 ng each of AAAF-treated pUC18 and control pGEM-3Zf(+) DNA, and 2 µCi of [α-^{32}P]dCTP (*see* Note 15).
3. Add yeast extract containing 250 µg protein (typically 6-8 µl) and water to a final total volume of 50 µl. Mix gently (*see* Note 16).
4. Incubate at 28°C for 2 h.
5. Stop reaction by adding 1 µl of 1*M* EDTA. Add 0.5 µl of ribonuclease A, mix and incubate at 37°C for 10 min.
6. Add 2.5 µl of 10% SDS and 0.5 µl of proteinase K. Mix and incubate at 37°C for 30 min.
7. Extract with equal volume of phenol/chloroform. Centrifuge for 10 min at room temperature in a microfuge.
8. Carefully transfer upper aqueous phase to a new microcentrifuge tube. Repeat the extraction procedure one more time.
9. Add ammonium acetate to 2.5 *M* (1/2 volume of 7.5 *M* stock) and precipitate the DNA with 2 volume of absolute ethanol at -70°C for a minimum of 10 min.
10. Recover DNA by centrifugation in a microfuge at top speed for 10 min. Remove supernatant.
11. Add 100 µl 70% ethanol. Vortex and centrifuge as above.
12. Allow residual ethanol to evaporate by leaving the tube open for at least 10 min.
13. Digest DNA overnight with HindIII (20 U) in 20 µl final volume.
14. Add gel loading buffer and subject the sample to electrophoresis on a 1% agarose gel plus ethidium bromide.
15. Take photograph of the gel under near-UV transillumination.
16. Transfer gel to blotting paper and vacuum dry at 80°C for 1 h.
17. Expose dried gel to X-ray film to obtain high-quality autoradiogram. Quantitation can be done by phosphoimaging analyses (*see* Note 17).
18. The result of a typical repair assay is shown in Fig. 1 (*see* Note 18).

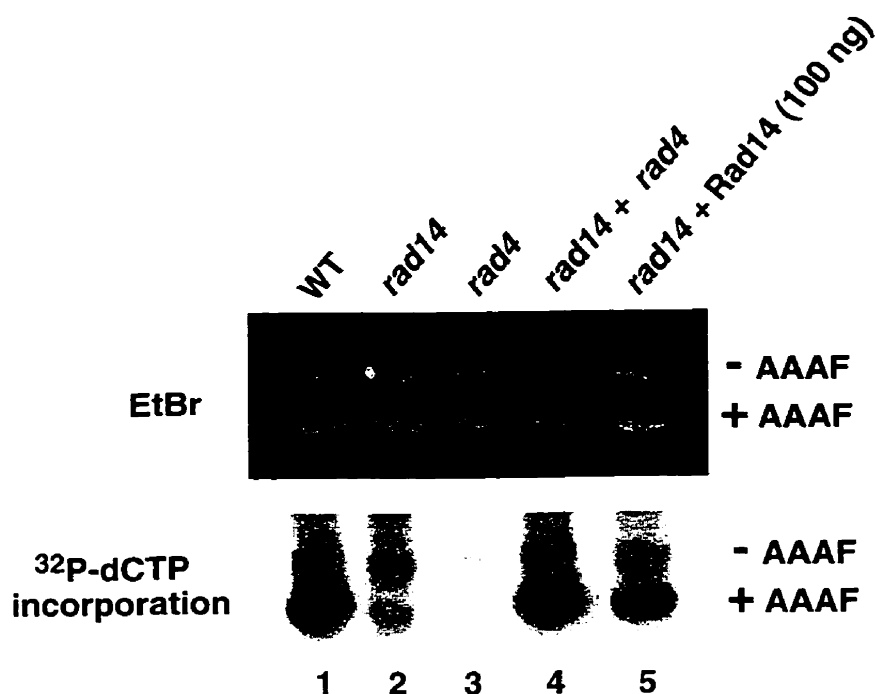

Fig. 1. Nucleotide excision repair in yeast whole cell extracts.
The repair activity was assayed by radioisotope incorporation into control or AAAF-treated DNA during repair DNA synthesis. The upper panel shows the DNA as visualized by ethidium bromide staining, the lower panel shows the autoradiogram of the gel. Extracts prepared from *rad14* and *rad4* deletion strains exhibited only background levels of radiolabel incorporation (lanes 1-3). Mixing equal amounts of mutant extracts complemented the defects in excision repair (lane 4). Adding purified Rad14 protein also restored repair activity to the *rad14* extract (lane 5). See Notes 17 and 18.

4. NOTES

1. Yeast grown in rich medium such as YPD gives the highest yield. Usually 5 g per L can be obtained for a common laboratory strain such as W303. However, if minimal medium such as synthetic complete (SC) medium must be used, a lower yield should be expected.
2. Other protease inhibitor cocktails can also be used (or supplemented) if it is felt necessary. PMSF is highly unstable in aqueous solution of pH > 7 and should be added to the buffer just before use. Consult a Boehringer Mannheim or other supplier catalogue for a description of the specificities of these and other alternative protease inhibitors.
3. We have tried both protease-deficient and wild-type yeast strains (using identical conditions as described here) and they appear to yield similarly active extracts. The protocol described herein probably works for most, if not all strains (*see* Ref. 7 and Note 8).
4. Growth of yeast can be monitored by measuring O.D. at 600 nm. We routinely carry out the harvest when the culture is grown to saturation. (O.D. of 2-4 for W303). Exponential growth is not required to obtain an extract proficient in repair. If cells are cultured in SC medium, it will take longer (2 days at least) to get to saturation if the amount of starter to culture volume is kept at the same ratio i.e. 10 ml to 2 L.
5. Getting a rough idea of the yield of a particular yeast strain in terms of gram per L of media may be helpful in future experiments. This allows one to calculate the minimum volume of cells needed to be grown to give, say, 10 g of yeast.
6. As little as 2 g of yeast can be processed. However, we recommend grinding 10 g of yeast as this is the maximum amount the mortar can comfortably hold. This amount of starting material will eventually yield about 0.8-1 ml of concentrated extract.
7. At the initial phase of the grinding step, the consistency of the yeast can be described as "grainy" and "chunky" as the material is essentially small pellets of frozen yeast. As grinding proceeds, the material turns from "grainy" or "sandy"

into a evenly smooth powder which makes grinding more difficult. The powder will also tend to stick to the pestle and the side of the mortar. We estimate that roughly 300-400 circumferential passes around the mortar is sufficient to break 10 g of yeast. Total time required for just the grinding step is around 20-30 min with some rest periods.

8. The extraction buffer (room temperature) will immediately be frozen on contact with the frozen powder. That cells remain frozen as they are broken and are thawed directly into a buffer containing protease inhibitors are likely the two important features that contribute to the success of this protocol (see Notes 2 and 3).
9. Avoid the gummy material near the pellet of cellular debris at the bottom of the centrifuge tube. Also, avoid the white, loose layer of lipid frequently seen floating on the surface. As a result, up to 1/3 of the supernatant may not be retrievable. This ultracentrifugation step also helps remove yeast DNA.
10. Stirring should be regulated to keep frothing to a minimum. Frothing may promote denaturation and oxidation of proteins. This step of ammonium sulfate precipitation serves to concentrate protein and remove residual yeast DNA (*see* Note 9). However, some yeast proteins will not be precipitated and hence the extract preparation is, after this step, not strictly speaking a whole cell extract.
11. The amount of precipitation appears to vary for unknown reasons. However, the precipitation that occurs during dialysis does not seem to affect activity and may only reduce the final protein concentration.
12. Extracts prepared according to this protocol appear to lose some repair activity after multiple freezings and thawings. Therefore, aliquoting the extract into small fractions is highly recommended.
13. *N*-acetoxy-2-acetylaminofluorene forms DNA adducts known to be corrected only by nucleotide excision repair. Ultraviolet-irradiated plasmid DNA, sometimes used as substrate for NER, is also known to be acted on by base excision repair *(11)*.
14. The protein concentration of a typical extract preparation is usually around 25-35 μg/μl.

15. Optimal repair activity requires disodium phosphocreatine and phosphocreatine kinase which constitute an ATP-regenerating system. The repair activity is also dependent on ATP and Mg^{2+} *(7)*.
16. The extent of damage-dependent repair synthesis increased with the amount of extract protein added *(7)*. An amount of extract corresponding to 250 μg of protein usually produces good signals above the background level of radioisotope incorporation into the control plasmid.
17. The amount of radioactivity in each band can be quantitated by phosphoimaging analysis and comparison to known radioactivity standards which are simultaneously exposed to the same phosphoimaging screen. Our assay routinely supports incorporation of 130-190 fmol of dCMP into the AAAF-treated DNA with background incorporation of 20-40 fmol into the untreated control DNA. This is comparable to that reported by Wang *et al. (5)* and is nearly as active as the human cell extracts described by Wood *et al. (4)*.
18. Note that incorporation of labelled nucleotides into AAAF-treated plasmid is dependent on *RAD* genes such as *RAD14* and *RAD4* (Fig. 1, lanes 2 and 3). A negative control using a *rad* mutant extract should always be included in every experiment. We also recommend performing a complementation experiment by mixing two appropriate mutant extracts (e.g. *rad4* and *rad14*) in order to confirm that the incorporation does represent *bona fide* excison repair activity (Fig. 1, lane 4).

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