Mutation of lysine-48 to arginine in the yeast RAD3 protein abolishes its ATPase and DNA helicase activities but not the ability to bind ATP

Patrick Sung¹, David Higgins^{1,3},
Louise Prakash² and Satya Prakash¹

¹Department of Biology, University of Rochester, Rochester, NY 14627 and ²Department of Biophysics, University of Rochester School of Medicine, Rochester, NY 14642, USA

³Present address: NCI-Frederick Cancer Research Facility, Frederick, MD 21701, USA

Communicated by D. Bootsma

The *RAD3* gene of *Saccharomyces cerevisiae* is required for excision repair of DNA damaged by UV radiation and is also essential for cell viability. The ~89 kd protein encoded by *RAD3* possesses single-stranded DNA dependent ATPase and DNA helicase activities. The sequence Gly-X-Gly-Lys-Thr, believed to be involved in the interaction with purine nucleotides in proteins that bind and hydrolyze the nucleotides, is present in the *RAD3* primary structure between amino acids 45 and 49. We report here that the point mutation of Lys-48 to arginine abolishes the *RAD3* ATPase and DNA helicase activities but not the ability to bind ATP. These observations highlight the involvement of this lysine residue in the hydrolysis of ATP and indicate that the positive charge on arginine can replace that of the lysine residue in the binding of ATP but not in its hydrolysis. The *rad3 Arg-48* mutant is apparently defective in a step subsequent to incision at the damage site in DNA; it can incise UV damaged DNA, but does not remove pyrimidine dimers. The role of the ATPase and DNA helicase activities of the *RAD3* protein in its DNA repair and viability functions is discussed.

Key words: ATP binding/ATP hydrolysis/DNA helicase/*RAD3* protein/*S.cerevisiae*

Introduction

In the yeast *Saccharomyces cerevisiae*, excision repair of DNA damaged by UV radiation and by agents that form bulky lesions is an intricate process requiring at least ten genes. Of these, the *RAD1*, *RAD2*, *RAD3*, *RAD4* and *RAD10* genes are essential for incision of damaged DNA (Reynolds and Friedberg, 1981; Wilcox and Prakash, 1981; Miller *et al.*, 1982a), and mutations in these five genes render cells extremely susceptible to killing by UV radiation and other agents. The remainder of the known excision repair genes, *RAD7*, *RAD14*, *RAD16*, *RAD23* and *MMS19*, affect the proficiency of damage excision (Miller *et al.*, 1982a,b), and strains carrying mutations of these genes display moderate UV sensitivity. Several of these genes have been isolated and their nucleotide sequences determined in our laboratory and by others (Naumovski and Friedberg, 1985; Reynolds *et al.*, 1985a,b; Madura and Prakash, 1986; Perozzi and Prakash, 1986; Reynolds *et al.* 1987). *RAD3* is unique among

the cloned excision repair genes in that it is also essential for cell viability (Higgins *et al.*, 1983; Naumovski and Friedberg, 1983).

RAD3 protein contains, near its amino terminus, a glycine rich segment which includes the sequence Gly-X-Gly-Lys-Thr (Reynolds *et al.*, 1985a), features that are characteristic of the 'type A' consensus sequence present in various proteins that bind and hydrolyze ATP (Walker *et al.*, 1982) including the *Escherichia coli* DNA replication, recombination and repair proteins DnaA, DnaB, RecA, RecB, UvrA and UvrD (Table I). In our laboratory, *RAD3* protein has been purified to apparent homogeneity from yeast strains genetically tailored to overproduce the protein and shown to possess ssDNA dependent ATPase (Sung *et al.*, 1987a) and DNA helicase (Sung *et al.*, 1987b) activities. *RAD3* helicase translocates on the bound DNA strand in the 5' to 3' direction and is capable of unwinding a duplex region 851 bp in length (Sung *et al.*, 1987b).

In the sequence Gly-X-Gly-Lys-Thr, present in the *RAD3* protein between amino acids 45 and 49, Gly-47 and Lys-48 represent invariant residues in the consensus sequence (Table I). To examine the functional significance of the invariant lysine and the positive charge of this lysine in ATP binding, ATP hydrolysis and in DNA helicase activity, we mutated Lys-48 in the *RAD3* protein to the similarly charged arginine. The protein encoded by the *rad3 Arg-48* mutant gene was overproduced in yeast and purified to apparent homogeneity, and the biochemical properties of the purified *rad3* mutant protein were compared to those of *RAD3* wild-type protein. We show that *rad3 Arg-48* protein retains the ability to bind ATP but lacks ATPase and DNA helicase activities. We find that yeast strains bearing a genomic *rad3 Arg-48* allele are viable but are as sensitive to UV radiation as the well characterized *rad3-1* and *rad3-2* mutants. Interestingly, however, unlike the latter mutants, which are defective in the incision of damaged DNA, the *rad3 Arg-48* mutant can carry out incision but does not remove pyrimidine dimers from DNA, apparently due to a defect in a post-incision step. The role of the invariant lysine in ATP binding and hydrolysis, and the interplay between the biochemical activities and biological functions of *RAD3* protein are the subjects of this paper.

Results

Overproduction and purification of *RAD3* and *rad3 Arg-48* proteins

Lysine-48 in *RAD3* protein was changed to arginine by oligonucleotide-directed mutagenesis (see Materials and methods for details). To overproduce *RAD3* and *rad3 Arg-48* proteins in yeast for purification, ORFs of the *RAD3* and *rad3 Arg-48* genes were fused to the promoter of the highly expressed constitutive yeast alcohol dehydrogenase I (*ADCI*) gene as described in Sung *et al.* (1987a) and in Materials and methods. The overproduction of *RAD3* and

Table I. Homology between nucleotide binding sequence of RAD3 protein and several *E.coli* ATPases

Protein	Residues	Sequence	Reference
RAD3	35–55	<u>G</u> <u>G</u> N S I <u>L</u> E M P S <u>G</u> T <u>G</u> K T V S L L S L	1
UvrA	633–653	<u>G</u> L F T C I T <u>G</u> V S <u>G</u> S <u>G</u> K S T L I N D T	2
UvrD	22–42	R S N L L V L A <u>G</u> A <u>G</u> S <u>G</u> K T R V L V H R	3
RecA	59–79	<u>G</u> R I V E I Y <u>G</u> P E S S <u>G</u> K T T L T L Q V	4
RecB	16–36	Q <u>G</u> E R L I E A S A <u>G</u> T <u>G</u> K T F T I A A L	5
DnaA	165–185	Y N P L F L Y <u>G</u> <u>G</u> T <u>G</u> L <u>G</u> K T H L L H A V	6
DnaB	223–243	S D L I I V A A R P S M <u>G</u> K T T F A M N L	7

Identical or similar residues shared by all proteins are boxed. The glycine residues are underlined. References: (1) Reynolds *et al.* (1985a); (2) Husain *et al.* (1986); (3) Finch and Emmerson (1984); (4) Sancar *et al.* (1980); (5) Finch *et al.* (1986); (6) Hansen *et al.* (1982); (7) Nakayama *et al.* (1984).

rad3 Arg-48 proteins in yeast strains harboring the *ADCI::RAD3* and *ADCI::rad3 Arg-48* chimeric plasmids was verified by immunoblot analysis of cell extracts of the strains (Figure 1A) using anti-RAD3 antibodies raised against a truncated RAD3 protein produced in *E.coli* (Sung *et al.*, 1987a). RAD3 and rad3 Arg-48 proteins were purified to apparent homogeneity (Figure 1B) by a procedure modified from that used previously (Sung *et al.*, 1987b; see Materials and methods).

The rad3 Arg-48 mutation does not affect binding to single-stranded DNA

The RAD3 protein hydrolyzes ATP to ADP and P_i only in the presence of a ssDNA cofactor (Sung *et al.*, 1987a). To establish that the change of Lys-48 to arginine in RAD3 does not alter the ssDNA binding properties, the ability of the rad3 Arg-48 mutant protein to bind ssDNA was compared to that of RAD3 wild-type protein using a filter-binding assay (see Materials and methods). It is clear from the data presented in Table II that rad3 Arg-48 protein binds single-stranded M13 DNA with the same affinity as RAD3 protein.

The rad3 Arg-48 mutation abolishes the ATPase activity

The RAD3 ATPase activity exhibits a pH optimum of 5.6 (Sung *et al.*, 1987a). To determine whether rad3 Arg-48 protein possesses ATPase activity, 100 ng of the purified protein was added to reaction mixtures (10 μ l) assembled in buffers of pH 5.1–7.5. As shown in Figure 2, whereas 80% of the input ATP was hydrolyzed by an equivalent quantity of RAD3 protein after 20 min at pH 5.6, no ATP hydrolysis was observed with rad3 Arg-48 protein at any of the pH values tested. The same result was obtained when the amount of rad3 Arg-48 protein in reaction mixtures was increased 5-fold to 500 ng (not shown). Since the RAD3 ATPase activity is totally inactivated by the conservative change of Lys-48 to the similarly charged arginine, it can be concluded that the invariant lysine in the Walker 'type A' consensus sequence plays an essential role in ATP hydrolysis.

rad3 Arg-48 protein lacks DNA helicase activity

RAD3 protein unwinds duplex DNA of 71–851 bp in the presence of ATP (Sung *et al.*, 1987b). DNA unwinding by RAD3 protein has the same pH optimum (pH 5.6) as the RAD3 ATPase activity and does not occur when ATP is replaced by a nonhydrolyzable nucleotide (Sung *et al.*, 1987b), suggesting that the unwinding process is coupled

Fig. 1. Immunoblot analysis of extracts from yeast cells overproducing the wild-type RAD3 and the mutant rad3 Arg-48 proteins and SDS-PAGE of the purified proteins. (A) The nitrocellulose blot of a 9% SDS-polyacrylamide gel was probed with anti-RAD3 antibodies and developed by the indirect peroxidase method as described previously (Sung *et al.*, 1987a,b). **Lane 1**, extract (30 μ g protein) from yeast cells harboring the vector pSCW231 (Sung *et al.*, 1987a) which contains the yeast *ADCI* promoter and lacks any *RAD3* sequence. **Lane 2**, extract (30 μ g protein) from cells harboring the *ADCI::RAD3* plasmid pSCW367 that overproduces RAD3 protein (Sung *et al.*, 1987a). **Lane 3**, extract (30 μ g protein) from cells harboring the *ADCI::rad3 Arg-48* plasmid pPS5 that overproduces rad3 Arg-48 protein. (B) 200 ng of purified RAD3 protein (**lane 2**) and rad3 Arg-48 (**lane 3**) protein were electrophoresed on a 9% SDS-polyacrylamide gel at 1 mA/cm for 4 h according to Laemmli (1970). The protein bands in the gel were visualized by the silver-staining procedure of Wray *et al.* (1981). **Lane 1** contains mol. wt markers (150 ng each). Staining in the 66 kd region and below is an artefact inherent in the silver staining technique.

to and requires the hydrolysis of ATP. Since the change of Lys-48 to arginine abolishes the ATPase activity of RAD3 protein, the mutation is likely to affect the DNA helicase activity. To examine this, purified rad3 Arg-48 protein was incubated with a circular ssDNA substrate that contains a 71 bp duplex region. We used this substrate, rather than one

Table II. Binding of single-stranded DNA by RAD3 and rad3 Arg-48 proteins

Amount of protein (ng)	% DNA bound	
	RAD3	rad3 Arg-48
75	44	46
150	61	62

Reaction mixtures containing 75 ng or 150 ng of RAD3 wild-type protein or rad3 Arg-48 mutant protein and 100 ng of ^3H -labeled single-stranded M13 DNA (2345 c.p.m.) were incubated at 30°C for 20 min, and the amount of DNA bound by the proteins was determined as described in Materials and methods.

Fig. 2. The RAD3 ATPase activity is inactivated by the mutation of Lys-48 to arginine. ATP hydrolysis was examined over a broad range of pH. Reaction mixtures containing 100 ng of RAD3 protein (●) or rad3 Arg-48 protein (○) were incubated at 30°C for 20 min as described in Materials and methods.

with a longer duplex region, to ensure the detection of any residual DNA unwinding activity that may be possessed by rad3 Arg-48 protein. As shown in Figure 3, rad3 Arg-48 protein does not have the ability of the RAD3 protein to denature the 71 bp duplex region in the helicase substrate. Increasing the amount of rad3 Arg-48 protein in the reaction mixture from 100 ng to 500 ng did not result in any detectable unwinding of the DNA substrate (not shown). Since the rad3 mutant protein binds ATP (see below), the lack of helicase activity clearly demonstrates that ATP binding alone is insufficient to realize the unwinding of DNA.

rad3 Arg-48 protein retains the ability to bind ATP

Results from previous studies suggest that the positive charge on the invariant lysine residue in the sequence Gly-X-Gly-Lys-Thr contributes to the binding of purine nucleotides by interacting with the α and/or β phosphoryl group(s) of the nucleotides (Jurnak, 1985; la Cour *et al.*, 1985; Fry *et al.*, 1986). Since Arg-48 in the rad3 mutant protein also possesses a positive charge, we investigated if the mutant protein

Fig. 3. rad3 Arg-48 protein does not possess DNA helicase activity. DNA helicase activity was examined over a broad range of pH. Reaction mixtures containing RAD3 protein or rad3 Arg-48 protein and, as helicase substrate, a partial duplex of circular single-stranded M13mp7 DNA and a ^{32}P -labeled 71 base complementary fragment, were incubated at 30°C for 5 min. The displacement of the 71 base fragment from the single-stranded circle was determined by PAGE and autoradiography as described in Materials and methods. Lane 1, no RAD3 protein; lane 2, heat-denatured (95°C for 2 min) helicase substrate; lanes 3–10, reaction mixtures containing rad3 Arg-48 mutant protein at pH 5.1, 5.3, 5.6, 5.9, 6.2, 6.6, 7.0 and 7.5 respectively. lanes 11 and 12, reaction mixtures containing wild-type RAD3 protein at pH 5.6 and 5.9.

retains the ability to bind ATP. ATP-binding by RAD3 and rad3 Arg-48 proteins was measured using a gel filtration method adapted from that described by Penefsky (1977). The Sephadex matrix used in this method of determining protein–ligand complex formation, unlike nitrocellulose in filter binding, does not interact with the protein of interest, thereby minimizing the possibility of protein unfolding and the loss of the bound ligand. Figure 4 shows that ATP binding by RAD3 protein has the same pH optimum (pH 5.6) as the RAD3 ATPase and helicase activities, and that from pH 5.3 to 7.0, rad3 Arg-48 mutant protein has ~40% of the wild-type RAD3 ATP-binding activity. Thus, it is clear that ATP binding does not have an absolute requirement for the invariant lysine.

The rad3 Arg-48 mutation impairs a post-incision step in excision repair

The rad3 Arg-48 mutant is highly UV sensitive, its UV sensitivity being identical to the incision defective rad3-2 mutant (Higgins *et al.*, 1983). We examined the incision capacity of the rad3 Arg-48 mutation by coupling it with the *ts cdc9-2* mutation, defective in DNA ligase, and analyzing DNA from these cells after UV irradiation on alkaline sucrose gradients. In the *RAD*⁺ *cdc9* strain, single-strand breaks are observed in DNA from UV irradiated cells incubated at 36°C for 1 h, the restrictive temperature for *cdc9*, but not in unirradiated cells held at 36°C for 1 h (Figure 5A; Wilcox and Prakash, 1981). The accumulation of single-strand breaks in DNA from UV irradiated *RAD*⁺ *cdc9* cells held at the restrictive temperature must result from

Fig. 4. *rad3* Arg-48 protein retains the ability to bind ATP. ATP binding was examined over a broad range of pH. Reaction mixtures containing RAD3 protein (●) or *rad3* Arg-48 protein (○) and [3 H]ATP were incubated at 25°C. Protein bound [3 H]ATP was separated from free [3 H]ATP by filtration of the reaction mixtures through Sephadex G50 columns. ATP-binding activity was determined by liquid scintillation counting of the filtrates from the Sephadex columns as described in Materials and methods.

the inability to seal the nick remaining after repair synthesis subsequent to damage excision. These breaks are undetectable in a *RAD⁺ CDC⁺* strain presumably because the incision nicks are repaired rapidly in a ligase-proficient cell. To test the incision proficiency of the *rad3* Arg-48 mutant, the *rad3* Arg-48 mutation was coupled to the *cdc9-2* mutation and DNA from the double mutant examined after UV treatment. As shown in Figure 5B, single-strand breaks accumulate in *rad3* Arg-48 *cdc9* cells irradiated with UV light and incubated for 1 h at the restrictive temperature of 36°C. Thus, the *rad3* Arg-48 mutant retains the capacity to incise UV irradiated DNA; however, the extent of incision in the *rad3* Arg-48 *cdc9* mutant is reduced compared to that in the *RAD⁺ cdc9* strain (Figure 5).

Since incisions are made in the *rad3* Arg-48 *cdc9* mutant, we examined the removal of pyrimidine dimers in this strain by assaying for sites in DNA sensitive to the *Micrococcus luteus* pyrimidine dimer DNA glycosylase. However, no loss of glycosylase-sensitive sites occurred in DNA from UV irradiated *rad3* Arg-48 *cdc9* cells even after 4 h of post UV incubation in growth medium at the permissive temperature of 25°C (Figure 6), indicating that the *rad3* Arg-48 mutant is defective in the removal of pyrimidine dimers. In the *RAD⁺ cdc9* strain H280 treated similarly, all pyrimidine dimers were removed within 1 h (results not shown). Thus,

even though incision breaks are formed in the *rad3* Arg-48 mutant, no removal of pyrimidine dimers occurs in this strain. These observations indicate that the *rad3* Arg-48 mutation affects a post-incision step of excision repair. In *rad3* Arg-48 cells, in the absence of a proper post-incision event, such as the dissociation of the incision enzyme complex from the damage site in DNA or strand displacement synthesis following incision (Sung *et al.*, 1987b), joining of incision nicks by DNA ligase would result in defective pyrimidine dimer removal.

Discussion

In proteins that have a requirement for a purine nucleotide, different segments of the protein, by virtue of their ability to interact with the adenine-ribose or guanine-ribose and the phosphoryl moieties of the nucleotide, collectively define the binding site for the nucleotide (Jurnak, 1985; la Cour *et al.*, 1985; Fry *et al.*, 1986; Knight and McEntee, 1986). It has been shown in the *E. coli* RecA protein and rabbit muscle adenylate kinase that the adenine-ribose moiety of the ATP ligand is located inside a hydrophobic pocket constituted within residues 257–280 of RecA protein (Knight and McEntee, 1986) and the first 40 or so residues of adenylate kinase (Hamada *et al.*, 1979; Fry *et al.*, 1986). Except for their content of clusters of hydrophobic residues, the sequences in RecA protein and adenylate kinase that give rise to this adenosine-binding motif do not share any noticeable amino acid homology (Knight and McEntee, 1986). While surveying a number of known ATPases for common structural features, Walker *et al.* (1982) identified two highly conserved segments which they called the 'type A' and 'type B' sequences. The 'type B' sequence, whose distinguishing feature is the presence of 4–5 consecutive hydrophobic residues followed by an aspartate, has been shown by NMR techniques to flank the triphosphate chain of MgATP in rabbit muscle adenylate kinase (Fry *et al.*, 1986). It was suggested that the aspartate may interact with Mg^{2+} on MgATP and the hydrophobic residues of this sequence may serve to exclude water from the ATP reaction centre (Fry *et al.*, 1986). The 'type A' sequence being focused upon in this paper is rich in glycine and contains the distinguishing configuration Gly-X-Gly-Lys-Thr (Table I). The studies of Fry *et al.* (1986) with adenylate kinase indicate that ionic interaction between lysine-21, which corresponds to the invariant lysine residue in the conserved sequence, and the α phosphoryl group of the bound nucleotide (MgATP) is highly probable as they are only 4 ± 2 Å apart. A similar conclusion was drawn from examining X-ray diffraction data obtained with *E. coli* elongation factor Tu crystallized in the presence of GDP (Jurnak, 1985; La Cour *et al.*, 1985). Replacement of the invariant lysine (lysine-16) in the human Ha-ras encoded protein with asparagine causes a 100-fold reduction in the affinity of the protein for GDP and GTP (Sigal *et al.*, 1986).

Our finding that *rad3* Arg-48 protein lacks the ability to hydrolyze ATP firmly establishes the involvement of the invariant lysine in the 'type A' sequence in defining the nucleotide binding and hydrolysis reaction centre. In particular, we have demonstrated that in nucleotide binding, arginine is able to replace the lysine residue most probably because it also carries a positive charge. In nucleotide hydrolysis, however, the lysine residue is specifically

Fig. 5. Alkaline sucrose sedimentation of DNAs from the *RAD*⁺ *cdc9-2* and *rad3 Arg-48 cdc9-2* mutant strains. (A) DNA from the *RAD*⁺ *cdc9-2* yeast strain H280, irradiated with 14 J/m² of UV light. DNA from unirradiated cells incubated for 1 h at 36°C (—); DNA from UV irradiated cells incubated for 1 h at 36°C after UV (---). (B) DNA from the *rad3 Arg-48 cdc9-2* yeast strain DH293-1 irradiated with 14 J/m² of UV light; DNA from unirradiated cells incubated for 1 h at 25°C (—) or 36°C (—); DNA from UV irradiated cells incubated for 1 h at 25°C after UV (·····) or at 36°C after UV (---). Incubations were in the dark in media. Spheroplasts were layered onto a lysing solution on top of a 15–30% alkaline sucrose gradient and centrifuged at 4°C at 17 000 r.p.m. for 17 h and 18 min in a Beckman SW41 rotor.

required, as even the similarly charged arginine fails to substitute for the residue. The change of the invariant lysine in the 'type A' sequence to arginine may prove to be a generally useful method for uncoupling the functions of nucleotide binding and nucleotide hydrolysis in enzymes that interact with purine nucleotides.

Yeast strains carrying the *rad3 Arg-48* allele are viable but are as susceptible to killing by UV radiation as the *rad3-1* and *rad3-2* mutants, indicating that the mutation of Lys-48 to arginine results in the inactivation of the excision repair pathway. However, we find that unlike the incision defective *rad3-1* and *rad3-2* mutants (Wilcox and Prakash, 1981), the *rad3 Arg-48* mutant can carry out incision of DNA damaged by UV light but is unable to remove pyrimidine dimers from the incised DNA. In *E. coli*, this type of excision repair defect is seen in mutants of the *UvrD* gene (Kuemmerle and Masker, 1980), which encodes a DNA helicase (Kumura and Sekiguchi, 1984) that shows considerable sequence homology to RAD3 protein (Foury and Lahaye, 1987). The phenotypic and biochemical similarities between yeast RAD3 protein and the *E. coli* UvrD protein suggest that in excision repair, the two enzymes may play an evolutionarily conserved role in reactions subsequent to the nicking of damaged DNA. The *E. coli* UvrD protein (helicase II), acting in concert with DNA polymerase I, catalyzes the turnover of the UvrABC repair endonuclease by displacing the

enzyme complex from incised DNA (Caron *et al.*, 1985; Husain *et al.*, 1985). Subsequent to turnover of the incision complex, UvrD helicase may also function in repair synthesis (Matson, 1986). If the post-incision role of RAD3 protein is analogous to that of UvrD protein, then our findings indicate that RAD3 ATPase and helicase activities are needed for the release of the yeast incision nuclease complex from DNA, and possibly also for repair synthesis. It remains to be established whether the enzymatic turnover of the *E. coli* UvrABC nuclease is impaired by mutating the invariant lysine in the 'type A' consensus sequence in UvrD protein (Finch and Emmerson, 1984; and Table I). Even though RAD3 may function in a manner similar to the UvrD protein in post-incision reactions, it should be emphasized that while nicking of damaged DNA in *E. coli* apparently can occur without UvrD protein, the incision defectiveness of the yeast *rad3-1* and *rad3-2* mutants indicates that RAD3 protein also participates in incision, or in a pre-incision step such as damage recognition, or in the assembly of the incision nuclease complex at the damage site in DNA.

The ATPase and helicase activities of RAD3 protein are dispensable in the viability function of the protein, as the *Arg-48* mutation is not lethal. Thus, RAD3 protein must possess an as yet unidentified activity that is essential for the maintenance of cell viability. The bacteriophage T4 gene 41 protein, bacteriophage T7 gene 4 protein, *E. coli* DnaB

Fig. 6. The *rad3* Arg-48 mutant is defective in the removal of pyrimidine dimers. *M.luteus* pyrimidine dimer DNA glycosylase-sensitive sites in DNA of the *rad3* Arg-48 *cdc9-2* yeast strain DH293-1 irradiated with 14 J/m² of UV light. DNA from unirradiated cells (—), DNA from UV irradiated cells with no post-UV incubation (---) and DNA from UV irradiated cells with 4 h post-UV incubation at 25°C in media (·····). DNAs were treated with *M.luteus* pyrimidine dimer DNA glycosylase and then layered onto 1 M NaOH on top of a 15–30% alkaline sucrose gradient. Centrifugation was at 4°C at 28 000 r.p.m. for 15 h and 18 min in a Beckman SW41 rotor.

protein and the SV40 T antigen are multifunctional proteins that possess DNA helicase activity (Venkatesan *et al.*, 1982; Matson and Richardson, 1983; Lebowitz and McMacken, 1986; Stahl *et al.*, 1986; Dean *et al.*, 1987) and the ability to interact with various protein factors involved in the initiation, priming and synthesis phases of DNA replication (Venkatesan *et al.*, 1982; Gough and Lane, 1986; Lebowitz and McMacken, 1986; Nakai and Richardson, 1986; Nakayama *et al.*, 1987; Sekimezu *et al.*, 1987). In addition, the SV40 T antigen binds the viral origin of DNA replication in the initiation of DNA synthesis (Tooze, 1980; Gough and Lane, 1986), and the phage T7 gene 4 protein is also a DNA primase (Tabor and Richardson, 1981). The RAD3 protein may also physically interact with and influence the activity of DNA replication components such as DNA polymerase and primase, or it may be a sequence specific DNA binding protein involved in the initiation of DNA synthesis at the origins of DNA replication. In view of the apparent dispensability of the helicase activity of RAD3 protein in its putative involvement in DNA replication, it will be of interest to determine whether inactivation of the DNA helicase activity of T antigen, gene 4, gene 41 and DnaB

proteins will have an adverse effect on DNA replication *in vivo*. For instance, the DNA helicase activity of DnaB protein may not be essential in DNA replication since *E.coli* contains at least six additional proteins that possess DNA helicase activity (Lee and Mariani, 1987; Wood and Matson, 1987).

Materials and methods

Oligonucleotide-directed mutagenesis

Lys-48, an AAA codon, in the *RAD3* gene was changed to the AGA arginine codon using the oligonucleotide TGT CCA TCT TGC CAG by the two primer method of Zoller and Smith (1984). This oligonucleotide corresponds to nucleotides +136–+150 in the *RAD3* ORF. The presence of the intended mutation in the host plasmid was established by DNA sequencing.

Overproduction and purification of RAD3 and *rad3* Arg-48 proteins

The placement of the *RAD3* ORF downstream of the yeast *ADCI* promoter to yield pSCW367 (2 μ *ADCI::RAD3 TRP1*) has been described (Sung *et al.*, 1987a). The *rad3* Arg-48 mutant gene was fused to the *ADCI* promoter as follows. Subsequent to the attachment of *EcoRI* linkers (phosphorylated 8mer, Pharmacia) to the 2.2 kb *HaeIII*–*HaeIII* fragment from nucleotide position –35 to position +2137 of the *rad3* Arg-48 allele, the DNA fragment was digested with an excess of *EcoRI*. The 1.8 kb fragment from this digestion was purified from a 0.8% agarose gel and used to replace the *EcoRI*–*EcoRI* fragment of the *ADCI::RAD3* construction in pSCW367 to give pPS5 (2 μ *ADCI::rad3* Arg-48 *TRP1*). The plasmids pSCW367 and pPS5 were introduced into the yeast strain DH225Δ (*MATa rad3Δ ura3-52 trp1-289*; Reynolds *et al.*, 1985a) harboring plasmid pSP32 (2 μ , *RAD3*, *URA3*) by cell transformation (Ito *et al.*, 1983). The plasmid pSP32 was subsequently removed from the transformed strains on medium containing 5-fluoroorotic acid (Boeke *et al.*, 1984). Yeast cells harboring plasmids were cultured in complete synthetic medium lacking tryptophan in 10 l batches in fermentors to a density of 3–4 $\times 10^7$ cells/ml. For the purification of the RAD3 and *rad3* Arg-48 proteins, 200 g (wet weight) of yeast cells harboring the appropriate plasmid were disrupted by passage through a French Press, and proteins in cell extracts were subjected to a combination of ammonium sulfate precipitation and chromatographic fractionation on columns of DEAE Sephacel, ssDNA agarose, hydroxyapatite and Bio-Rex 70, as described previously (Sung *et al.*, 1987a,b). Fraction V from this purification scheme, corresponding to the flow-through fraction from the Bio-Rex 70 step, was applied onto a MonoQ column (HR 5/5, Pharmacia). The column was washed with 10 ml of 200 mM KCl in 20 mM KH₂PO₄ (pH 7.4) containing 10% glycerol, 1 mM DTT and 0.5 mM EDTA, and developed with a 35 ml gradient of 200–460 mM KCl in the same buffer. Fractions containing RAD3 protein or *rad3* Arg-48 protein (total 300 μ g), which elute between 370 and 390 mM KCl, were pooled and concentrated to 0.25 ml using a Centricon 30 concentrator (Amicon). Following the addition of 0.2 ml glycerol, the enzyme preparations were stored at –20°C.

DNA binding assay

The nitrocellulose filters (Millipore HAWP 25) used in DNA binding assays were first treated with 0.3 M NaOH for 10 min at 25°C (Moranelli and Lieberman, 1980). Reaction mixtures containing 75–150 ng of RAD3 protein or *rad3* Arg-48 protein and 100 ng of ³H-labelled single-stranded M13 DNA were assembled in buffer R (20 mM potassium MES (pH 5.6), 40 mM KCl, 1 mM ATP, 1 mM DTT and 50 μ g/ml bovine serum albumin (BSA)) and incubated at 30°C for 20 min. Following the addition of 0.35 ml ice-cold buffer W (buffer R that did not contain BSA and ATP), the reaction mixtures were sucked through nitrocellulose filters (flow rate: 3 ml/min) with the aid of a water pump. The filters were washed with 8 ml of ice-cold buffer W (flow rate: 20 ml/min), dissolved in 10 ml of Filtron-X (National Diagnostics) and radioactivities determined by scintillation counting.

ATPase and helicase assays

The buffers used were 20 mM KOAc (pH 5.1, 5.3, 5.6 and 5.9), imidazole–HCl (pH 6.2, 6.6 and 7.0) and Tris–HCl (pH 7.5). ATPase reaction mixtures (10 μ l) contained 100 ng of RAD3 protein or *rad3* Arg-48 protein, 200 ng of single-stranded M13mp8 DNA, 0.5 mM [2-³H]ATP (sp. act. 0.1 Ci/mmol; 1 Ci = 37 GBq), 100 μ g BSA/ml, 6 mM MgCl₂, 1 mM DTT and 60 mM KCl in one of the buffers listed above. After 20 min at 30°C, the reaction was terminated by the addition of 2 μ l of 50 mM EDTA and 20 mM each of ADP and ATP. Nucleotides were separated by TLC on PEI cellulose using 1.2 M LiCl as the developing solvent (Randerath

and Randerath, 1964). The conversion of [^3H]ATP to [^3H]ADP was determined by liquid scintillation counting of the radioactive spots from PEI cellulose (Sung *et al.*, 1987a).

Helicase assay mixtures (10 μl) contained one of the buffers, 100 ng of RAD3 protein or rad3 Arg-48 protein, 4 μM (nucleotides) of a helicase substrate that contains a 71 bp duplex region (Matson, 1986; Sung *et al.*, 1987b), 6 mM MgCl_2 , 60 $\mu\text{g/ml}$ BSA, 1 mM DTT and 1 mM ATP being added last to initiate unwinding of the substrate. DNA unwinding proceeded at 30°C for 5 min and was terminated by the addition of 5 μl of 50 mM EDTA, 40% glycerol, 1% NaDodSO₄ and 0.1% bromophenol blue. The displaced 71 base fragment was separated from the unreacted substrate on 8% polyacrylamide gels, and revealed by autoradiography (Sung *et al.*, 1987b).

ATP binding assay

The gel filtration method used in the determination of ATP binding was based on that described by Penefsky (1977), with modifications to minimize the sticking of RAD3 and rad3 Arg-48 proteins to the Sephadex matrix. The buffers for these experiments were 20 mM KOAc (pH 5.1, 5.3, 5.6 and 5.9) and 20 mM imidazole-HCl (pH 6.2, 6.6 and 7.0). Sephadex G50-80 (Sigma) was swollen at 25°C in distilled water containing 200 mM KCl and de-aerated using a water suction pump. One ml tuberculin syringes (Becton Dickinson & Co., NJ) were plugged with glass wool and filled to the 1 ml mark with the gel filtration matrix. The gel was drained for 1 min and washed at 25°C with 4 ml of one of the buffers listed above containing 6 mM MgCl_2 , 50 $\mu\text{g/ml}$ BSA, 1 mM DTT, 200 mM KCl and 0.025% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Boehringer Mannheim). After being washed with an additional 4 ml of the same buffer at 0°C, the columns were placed in 15 ml polystyrene tubes (Sarstedt), drained at 2°C for 5 min and centrifuged at 100 g for 1.5 min. ATP-binding reaction mixtures (50 μl) contained 2 μg (0.44 μM) of RAD3 protein or rad3 Arg-48 protein, 8.6 μM [^3H]ATP (23 Ci/mmol; 1 Ci = 37 GBq), 120 μg BSA/ml, 6 mM MgCl_2 , 1 mM DTT and 30 mM KCl in one of the buffers listed above, and were incubated at 25°C for 25 min. After adjusting the concentration of CHAPS and KCl to 0.025% and 200 mM by the addition of 2 μl of 0.525% CHAPS/3.57 M KCl, 40 μl of the reaction mixtures were applied onto the Sephadex columns. The columns were centrifuged at 100 g for 1 min and the filtrates (50 μl) collected into 500 μl Eppendorf microfuge tubes. A 20 μl aliquot of the filtrates was mixed with 9 ml Liquiscint (National Diagnostics) and the ^3H content determined by liquid scintillation counting. The recovery of proteins was examined by immunoblotting.

Incision of UV damaged DNA and removal of pyrimidine dimers
The yeast strains were H280 (*MATa ade1 lys2-1 his7 tyr1 gal1 ura1 RAD⁺ cdc9-2 q^o*) and DH293-1 (*MATa ade1 and/or ade2 leu2-3 leu2-112 ura1 ura3-52 rad3 Arg-48 cdc9-2 q^o*). In strain DH293-1, the chromosomal *RAD3* gene was replaced by the *rad3 Arg-48* mutation. The method for assaying incision breaks in DNA was as described in Wilcox and Prakash (1981). Removal of pyrimidine dimers was determined as in Miller *et al.* (1982b).

Acknowledgements

We thank Steven Matson for providing the helicase substrate and Susan Wallace for the gift of ^3H -labeled M13 DNA. The use of the gel filtration method in ATP binding was suggested by David Hinkle. This work was supported by NIH grant CA-35035 and DOE grant DE-FG02-88ER60621.

References

- Boeke, J., La Croute, F. and Fink, G. (1984) *Mol. Gen. Genet.*, **197**, 345–346.
Caron, P.R., Kushner, S.R. and Grossmann, L. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 4925–4929.
Dean, F.B., Bullock, P., Murakami, Y., Wobbe, C.R., Weissbach, L. and Hurwitz, J. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 16–20.
Finch, P.W. and Emmerson, P.T. (1984) *Nucleic Acids Res.*, **12**, 5789–5799.
Finch, P.W., Storey, A., Chapman, K.E., Brown, K., Hickson, I.D. and Emmerson, P.T. (1986) *Nucleic Acids Res.*, **14**, 8573–8582.
Foury, F. and Lahaye, A. (1987) *EMBO J.*, **6**, 1441–1449.
Fry, D.C., Kuby, S.A. and Mildvan, A.S. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 907–911.
Gough, G. and Lane, D. (1986) *Trends Genet.*, **2**, 274–275.
Hamada, M., Palmieri, R.H., Russell, G.A. and Kuby, S.A. (1979) *Arch. Biochem. Biophys.*, **195**, 155–177.

- Hansen, E.B., Hansen, F.G. and Von Meyenburg, K. (1982) *Nucleic Acids Res.*, **10**, 7373–7385.
Higgins, D.R., Prakash, S., Reynolds, P., Polakowska, R., Weber, S. and Prakash, L. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 5680–5684.
Husain, I., Van Houten, B., Thomas, D.C., Abdel-Monem, M. and Sancar, A. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 6774–6778.
Husain, I., Van Houten, B., Thomas, D.C. and Sancar, A. (1986) *J. Biol. Chem.*, **261**, 4895–4901.
Ito, H., Fukuda, Y., Murata, I. and Kimura, A. (1983) *J. Bacteriol.*, **153**, 163–168.
Jurnak, F. (1985) *Science*, **230**, 32–36.
Knight, K.L. and McEntee, K. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9289–9293.
Kuemmerle, N.B. and Masker, W.E. (1980) *J. Bacteriol.*, **142**, 535–546.
Kumura, K. and Sekiguchi, M. (1984) *J. Biol. Chem.*, **259**, 1560–1565.
la Cour, T.F.M., Nyborg, J., Thirup, S. and Clark, B.F.C. (1985) *EMBO J.*, **4**, 2385–2388.
Laemmli, U.K. (1970) *Nature*, **227**, 680–685.
Lebowitz, J.H. and McMacken, R. (1986) *J. Biol. Chem.*, **261**, 4738–4748.
Lee, M.S. and Mariani, K.J. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 8345–8349.
Madura, K. and Prakash, S. (1986) *J. Bacteriol.*, **166**, 914–923.
Matson, S.W. (1986) *J. Biol. Chem.*, **261**, 10169–10175.
Matson, S.W. and Richardson, C.C. (1983) *J. Biol. Chem.*, **258**, 14009–14016.
Miller, R., Prakash, L. and Prakash, S. (1982a) *Mol. Cell. Biol.*, **2**, 939–948.
Miller, R., Prakash, L. and Prakash, S. (1982b) *Mol. Gen. Genet.*, **188**, 235–239.
Moranelli, F. and Lieberman, M.W. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 3201–3205.
Nakai, H. and Richardson, C.C. (1986) *J. Biol. Chem.*, **261**, 15208–15216.
Nakayama, N., Arai, N., Bond, N.W., Kaziro, Y. and Arai, K.I. (1984) *J. Biol. Chem.*, **259**, 97–101.
Nakayama, N., Bond, M.W., Miyajima, A., Kobori, J. and Arai, K. (1987) *J. Biol. Chem.*, **262**, 10475–10480.
Naumovski, L. and Friedberg, E.C. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 4818–4821.
Naumovski, L. and Friedberg, E.C. (1985) *Mol. Cell. Biol.*, **5**, 17–26.
Penefsky, H.S. (1977) *J. Biol. Chem.*, **252**, 2891–2899.
Perozzi, G. and Prakash, S. (1986) *Mol. Cell. Biol.*, **6**, 1497–1507.
Randerath, K. and Randerath, E. (1964) *J. Chromatogr.*, **16**, 111–125.
Reynolds, P., Higgins, D.R., Prakash, L. and Prakash, S. (1985a) *Nucleic Acids Res.*, **13**, 2357–2372.
Reynolds, P., Prakash, L., Dumais, D., Perozzi, G. and Prakash, S. (1985b) *EMBO J.*, **4**, 3549–3552.
Reynolds, P., Prakash, L. and Prakash, S. (1987) *Mol. Cell. Biol.*, **7**, 1012–1020.
Reynolds, R.J. and Friedberg, E.C. (1981) *J. Bacteriol.*, **146**, 692–704.
Sancar, A., Stachelek, C., Konigsberg, W. and Rupp, W.D. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 2611–2615.
Sekimezu, K., Bramhill, D. and Kornberg, A. (1987) *Cell*, **50**, 259–265.
Sigal, I.S., Gibbs, J.B., D'Alonzo, J.S., Temeles, G.L., Wolanski, B., Socher, S.H. and Scolnick, E.M. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 952–956.
Stahl, H., Droge, P. and Knippers, R. (1986) *EMBO J.*, **5**, 1939–1944.
Sung, P., Prakash, L., Weber, S. and Prakash, S. (1987a) *Proc. Natl. Acad. Sci. USA*, **84**, 6045–6049.
Sung, P., Prakash, L., Matson, S.W. and Prakash, S. (1987b) *Proc. Natl. Acad. Sci. USA*, **84**, 8951–8955.
Tabor, S. and Richardson, C.C. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 205–209.
Tooze, J. (ed.) (1980) *Molecular Biology of Tumor Viruses*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Part 2.
Venkatesan, M., Silver, L.L. and Nossal, N.G. (1982) *J. Biol. Chem.*, **257**, 12426–12434.
Walker, J.E., Sarsate, M., Runswick, M.J. and Gay, N.J. (1982) *EMBO J.*, **1**, 945–951.
Wilcox, D.R. and Prakash, L. (1981) *J. Bacteriol.*, **148**, 618–623.
Wood, E.R. and Matson, S.W. (1987) *J. Biol. Chem.*, **262**, 15269–15276.
Wray, W., Boulikas, T., Wray, V.P. and Hancock, R. (1981) *Anal. Biochem.*, **118**, 197–203.
Zoller, M.J. and Smith, M. (1984) *DNA*, **3**, 479–487.

Received on May 26, 1988; revised on June 24, 1988