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RAD3 protein of *Saccharomyces cerevisiae* is a DNA helicase

(DNA unwinding/DNA-dependent ATPase/DNA replication/DNA repair)

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ABSTRACT **The *Saccharomyces cerevisiae RAD3* gene, which is required for cell viability and excision repair of damaged DNA, encodes an 89-kDa protein that has a single-stranded DNA-dependent ATPase activity. We now show that the RAD3 protein also possesses a helicase activity that unwinds duplex regions in DNA substrates constructed by annealing DNA fragments of 71–851 nucleotides to circular, single-stranded M13 DNA. The DNA helicase activity is dependent on the hydrolysis of ATP, has a pH optimum of ≈5.6, and is inhibited by antibodies raised against a truncated RAD3 protein produced in *Escherichia coli*. The RAD3 helicase translocates along single-stranded DNA in the 5′→3′ direction. The direction of RAD3 helicase movement is consistent with the possibility that it unwinds DNA duplexes in advance of the replication fork during DNA replication.**

The *RAD3* gene of *Saccharomyces cerevisiae* is required for excision repair of DNA damaged by ultraviolet light and by other agents such as those that form bulky adducts or cross-links in DNA. Some of the *rad3* mutants show defects in incision of damaged DNA (1–3), and others appear to be defective in a post-incision step(s) (4). Genomic deletions of *RAD3* are recessive lethal, indicating an essential role for *RAD3* in cell viability (5, 6).

The nucleotide sequence of *RAD3* predicts an encoded protein of 778 amino acids and M_r 89,779 (7, 8). A consensus amino acid sequence present in many enzymes that bind and hydrolyze ATP also occurs in the RAD3 protein (7). We have described (9) purification of RAD3 protein from yeast cells harboring a RAD3-overexpressing plasmid in which the *RAD3* gene was fused to the highly expressed constitutive yeast alcohol dehydrogenase (*ADC1*) promoter. The purified RAD3 protein exhibits a single-stranded DNA-dependent ATPase activity (9).

Since the *RAD3* gene is required for cell viability (5, 6) and since the *rem1* mutation of *RAD3* increases spontaneous mutation and spontaneous mitotic recombination (10, 11), a phenotype characteristic of DNA replication mutants (12, 13), RAD3 protein might be involved in DNA replication. The RAD3 protein ATPase activity could supply the energy for unwinding the parental DNA duplex in advance of the replication fork. All DNA helicases from *Escherichia coli* and its phages are single-stranded DNA-dependent nucleoside 5′-triphosphatases (14). Utilizing the energy from nucleoside triphosphate hydrolysis, these enzymes move unidirectionally along single-stranded DNA and separate duplexed DNA strands (15–22). In this paper, we show that RAD3 protein is also a DNA helicase that unwinds duplex DNA in the presence of ATP and Mg^{2+}. Our studies indicate that RAD3 helicase unwinds duplex DNA in a 5′→3′ direction with respect to the strand to which it binds. These results suggest the possibility that, during DNA replication, RAD3 helicase moves along the template strand for lagging-strand synthesis and unwinds duplex DNA ahead of the replication fork. In its role in DNA repair, RAD3 with DNA polymerase may initiate DNA strand-displacement synthesis from a nick introduced at or near the DNA lesion by the excision–repair enzyme complex.

MATERIALS AND METHODS

Purification of the RAD3 Protein. The haploid yeast strain DH225 containing a RAD3-overproducing plasmid pSCW-367, in which the *RAD3* open reading frame had been fused to the yeast *ADC1* promoter (9), was used in the present studies. This strain has better growth properties than strain CMY135[pSCW367] used in ref. 9. The following modifications of the purification procedure (9) were adopted: 37% rather than 50% ammonium sulfate was used to precipitate RAD3 protein from the 100,000 × *g* supernatant, and 140 mM NaCl rather than 80 mM NaCl was used for washing the DNA agarose columns. These modifications not only reduce the quantity of proteins handled, thus making smaller columns and shorter processing times possible, but also result in a preparation of greater purity.

Helicase Substrates. The DNA substrate used in helicase assays is a ^{32}P-labeled partial duplex of circular M13mp7 single-stranded DNA and one of a number of complementary fragments of different lengths (ref. 20; Fig. 1*A*). The substrate for determining the directionality of the DNA unwinding reaction was constructed from a 341 bp partial duplex that possesses a *Cla* I restriction site in the duplex region. The partial duplex was cleaved with *Cla* I, and all 3′-hydroxyl termini were labeled by the addition of two nucleotides using the Klenow fragment of *E. coli* DNA polymerase I, dGTP, and [α-^{32}P]dCTP. The resulting linear product contains a ^{32}P-labeled duplex region of 143 bp at one end, another such region of 202 bp at the other end, and a single-stranded central region ≈7000 bases long (ref. 20; Fig. 1*B*).

Helicase Assay. The helicase assay measures the displacement of a ^{32}P-labeled DNA fragment from a partial duplex of M13 single-stranded DNA and the annealed labeled fragment (Fig. 1*A*). The standard reaction mixture (10 μl) was assembled at room temperature and contained 20 mM KOAc (pH 5.6), 5 mM $MgCl_2$, 1 mM dithiothreitol, bovine serum albumin (70 μg/ml), 2 μM DNA substrate, 50–100 ng of RAD3 protein, and 1 mM ATP, added last to initiate unwinding of the substrate. After 5–15 min at 30°C, the reaction was terminated by the addition of 5 μl of 50 mM EDTA, 40% (vol/vol) glycerol, 1% $NaDodSO_4$, and 0.1% bromophenol blue. Reaction mixtures were analyzed by 8% $NaDodSO_4$/PAGE at 12 V/cm for 4 hr in 80 mM Tris borate, pH 8.0/2 mM EDTA. The gels were dried, and the ^{32}P-labeled products of the DNA unwinding reaction were revealed by autoradiography at −70°C in film cassettes fitted with DuPont Cronex

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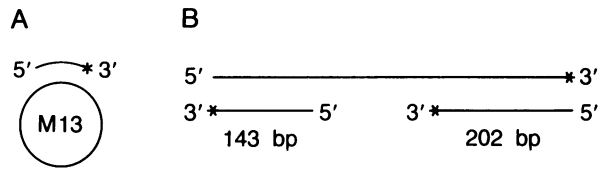

FIG. 1. Helicase substrates used in this study. The substrate in *A* is a partial duplex of M13mp7 single-stranded DNA and one of a number of complementary fragments of various lengths (20). The linear substrate in *B*, which contains terminal duplex regions of 143 base pairs (bp) and of 202 bp and a long central single-stranded region of ≈7000 nucleotides, was constructed (20). As explained in the *Results*, unwinding of duplex DNA in the 3′→5′ direction or 5′→3′ direction with respect to the bound single-stranded DNA will result in the displacement of the 143-base fragment or 202-base fragment, respectively. Asterisk, position of ^{32}P label.

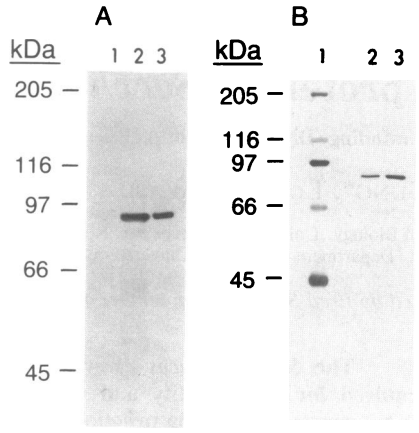

FIG. 2. Immunoblot analysis and NaDodSO$_4$/PAGE of yeast cell extracts and purified RAD3 protein. (*A*) Nitrocellulose blot of a 9% NaDodSO$_4$/polyacrylamide gel was probed with anti-RAD3 antibodies and developed by using the indirect peroxidase procedure (26). Lane 1 contains crude extract (20 μg of protein) from wild-type yeast cells harboring the vector pSCW231 that lacks the *RAD3* gene (9). The low level of RAD3 protein encoded by the chromosomal *RAD3* gene was not detectable under the experimental conditions used. Lane 2 contains crude extract (20 μg of protein) from yeast cells harboring the RAD3-overproducing plasmid pSCW367. Lane 3 contains 50 ng of purified RAD3 protein. In separate experiments, no cross-reaction was observed between RAD3 protein and preimmune sera (9). (*B*) Samples of purified RAD3 protein were electrophoresed on a 9% NaDodSO$_4$/polyacrylamide gel at 20 mA for 4 hr according to Laemmli (24). The gel was stained with silver nitrate as described by Wray *et al.* (25). Lanes: 1, molecular weight markers (100 ng, each); 2 and 3, 50 ng and 100 ng of purified RAD3 protein, respectively.

Lightning Plus intensifying screens. The intensity of images of the radioactive bands was quantitated by densitometry.

Miscellaneous. Transformation of yeast, isolation of a 78-kDa truncated RAD3 protein from *E. coli* JM103 harboring plasmid pSP119, and the production of antisera in New Zealand White rabbits using the truncated polypeptide as immunogen have been reported (9). To test the authenticity of the truncated RAD3 protein, a deletion spanning the *Bgl* II site in the *RAD3* gene at position +942 and the *Bam*HI site in the polylinker region of pSP119 was constructed. The deletion results in the synthesis of a smaller polypeptide (M_r, 40,000) as predicted (data not shown), confirming that the 78-kDa *E. coli* protein used for the production of antisera is encoded by the partial *RAD3* gene in pSP119.

ATPase assays were carried out as described (9). For the determination of protein concentrations, the Coomassie blue dye-binding method of Bradford (23) was employed. NaDodSO$_4$/PAGE was performed according to Laemmli (24). After electrophoresis, the proteins in gels were either revealed by silver staining (25) or transferred to nitrocellulose sheets. The protein blots were probed with anti-RAD3 antisera and developed by using the indirect peroxidase procedure (26) with horseradish peroxidase-conjugated goat anti-rabbit IgG and horseradish peroxidase color reagent purchased from Bio-Rad.

RESULTS

RAD3 Protein Purification. We have reported (9) the purification of an 89-kDa protein possessing single-stranded DNA-dependent ATPase activity from yeast cells harboring the RAD3 protein-overproducing plasmid pSCW367. Column fractions originating from wild-type yeast cells harboring the *ADC1* vector lacking the *RAD3* gene have much reduced levels of the 89-kDa polypeptide and the ATPase activity. Immunoblot analysis, using antisera raised against a truncated RAD3 protein produced in *E. coli*, confirmed that the 89-kDa yeast protein is encoded by the *RAD3* gene (ref. 9; Fig. 2*A*). The purified RAD3 protein is nearly homogeneous as judged by NaDodSO$_4$/PAGE (Fig. 2*B*).

RAD3 Protein Exhibits a DNA Unwinding Activity. The helicase activity of the RAD3 protein was assayed by measuring the displacement of a 3′-end-labeled 71-nucleotide DNA fragment annealed to circular M13mp7 single-stranded DNA (Fig. 1*A*). The displaced fragment was separated from the residual hybrid on 8% polyacrylamide gels, and the degree of unwinding was determined by densitometry following autoradiography of the dried gels. As shown in Fig. 3*A*, the purified RAD3 protein catalyzes the unwinding of the 71-bp helicase substrate in a reaction mixture that contains ATP and Mg^{2+}. The RAD3 helicase activity is highly pH dependent and has an optimal pH of ≈5.6 (Fig. 3*B*). Fifty percent reduction in activity occurred at pH 6.2, and little unwinding was observed above pH 6.6. This pH optimum is also shared by the RAD3 ATPase activity (Fig. 3*B*), suggesting that DNA unwinding by RAD3 protein is coupled to the hydrolysis of ATP.

Inhibition of RAD3 Helicase Activity by Anti-RAD3 Antibody. The RAD3 ATPase activity can be partially inhibited by total IgG isolated from antisera raised against a truncated RAD3 protein produced in *E. coli* (9). To determine if the helicase activity is affected by the IgG preparation, various quantities of the latter were included in the standard helicase assay mixture containing the 71-bp partial duplex and 100 ng of RAD3 protein. Maximal inhibition of 37% was observed at 20 μg of anti-RAD3 IgG. In the control experiment, 20 μg of IgG purified from preimmune sera showed no inhibition of RAD3 helicase activity. The anti-RAD3 antibodies used were raised against a truncated *E. coli* RAD3 protein that had been denatured with NaDodSO$_4$ (9). Hence, our antibody preparation, while useful for immunoblot analyses where denatured proteins are studied, may not possess sufficient reactivity toward the native yeast RAD3 protein to cause complete inhibition of the ATPase and helicase activities.

Characteristics of the RAD3 Protein Helicase Activity. The RAD3 protein helicase activity is strictly dependent on the presence of ATP or dATP; adenosine 5′-[γ-thio]triphosphate or other NTPs or dNTPs do not support unwinding to any significant degree (Table 1). Maximal unwinding of the 71-bp partial duplex was observed at 1 mM of ATP or dATP, and higher concentrations of the nucleotides are inhibitory (data not shown). The requirement for a divalent cation can be satisfied by Mg^{2+} or Mn^{2+}, but Ca^{2+} and Zn^{2+} are ineffective. The optimal concentration of Mg^{2+} and Mn^{2+} for unwinding is 5–10 mM and 2 mM, respectively. At 2 mM Mn^{2+}, the unwinding activity is 70% of that at 5 mM Mg^{2+} (data not shown). The RAD3 helicase activity exhibits linear kinetics for at least 10 min (Fig. 4*A*). Fig. 4*B* shows a nonlinear relationship between the extent of unwinding and RAD3

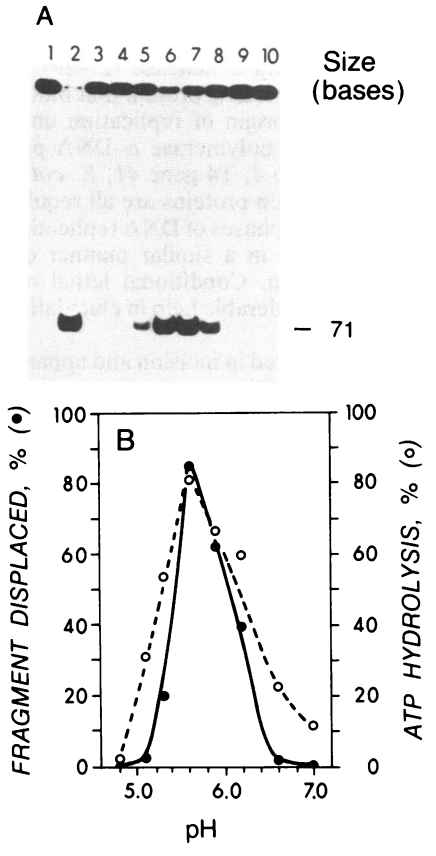

FIG. 3. Effect of pH on the RAD3 helicase and ATPase activities. The RAD3 helicase and ATPase activities were examined at pH 4.8, 5.1, 5.3, 5.6, and 5.9 by using 20 mM KOAc and at pH 6.2, 6.6, and 7.0 by using 20 mM imidazole hydrochloride. In the helicase assays, 50 ng of RAD3 protein was incubated with the 71-bp partial duplex at 30°C for 10 min. ATPase reaction mixtures contained 100 ng of RAD3 protein and 0.25 mM [2,8-^{3}H]ATP and were incubated at 30°C for 20 min. (*A*) Autoradiogram showing the displaced fragment resulting from unwinding of the 71-bp partially duplex helicase substrate at the pH values tested. Lanes: 1, no RAD3 protein; 2, heat-denatured DNA substrate; 3–10, unwinding at pH 4.8, 5.1, 5.3, 5.6, 5.9, 6.2, 6.6, and 7.0, respectively. (*B*) Graphical representation of the RAD3 helicase and ATPase activities as a function of pH.

protein concentration at lower concentrations. Since the overall shape of the curve is not sigmoidal, whether the nonlinearity arises from cooperativity or oligomerization of the RAD3 protein at higher concentrations remains to be established.

Direction of Unwinding by RAD3 Helicase. The various helicases examined to date have been shown to translocate unidirectionally along single-stranded DNA and unwind duplex DNA from a specific direction (15–22). To determine if RAD3 helicase also unwinds DNA with a specific polarity, we used a linear substrate that contains terminal duplex regions of 143 bp and 202 bp and a long central single-stranded region of ≈7000 nucleotides (Fig. 1*B*). If RAD3 protein binds the central single-stranded DNA and moves in the 5′→3′ direction, displacing the annealed fragment from its 3′ terminus, then the 202-nucleotide fragment should be released. If the 143-nucleotide fragment is released, then RAD3 moves in the 3′→5′ direction (Fig. 1*B*). As shown in Fig. 5*A*, the RAD3 helicase activity catalyzes exclusively the unwinding of the 202-bp duplex region, indicating that RAD3 helicase unwinds DNA in the 5′→3′ direction with respect to the single-stranded DNA on which it is bound. Incubation of the same DNA substrate with the UvrD protein of *E. coli*, a 3′→5′ helicase (20), results in the displacement of the 143-base fragment as expected (data not shown).

RAD3 helicase also unwinds the 343-bp partial duplex (Fig. 5*B*) used in the construction of the linear substrate for determining the direction of RAD3 helicase movement (Fig. 1*B*), and unwinding of an 851-bp substrate can be demonstrated (data not shown). These observations indicate that RAD3 helicase is capable of unwinding long stretches of duplex DNA.

Table 1. Nucleotide requirement for RAD3 helicase activity

Nucleotide	% fragment displaced
ATP	70.0
CTP	1.6
GTP	1.6
UTP	1.1
ADP	1.8
ATP[S]	0.0
dATP	53.0
dCTP	1.8
dGTP	2.0
dTTP	1.5

Helicase reaction mixtures contained 50 ng of RAD3 protein and 1 mM of the various nucleotides and were incubated at 30°C for 7 min. ATP[S], adenosine 5′-[γ-thio]triphosphate.

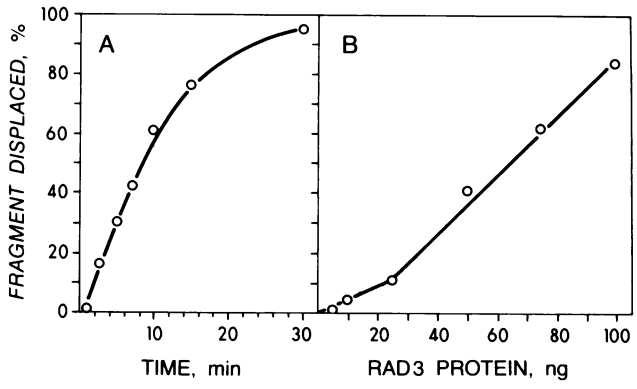

FIG. 4. RAD3 helicase activity as a function of time and protein concentration. (*A*) Scaled up helicase assay mixture containing 500 ng of RAD3 protein in a final volume of 100 μl was incubated at 30°C. At the times indicated, a 10-μl aliquot was withdrawn from the reaction mixture, mixed with 5 μl of stop buffer, and assayed for displacement of the fragment from the 71-bp helicase substrate. (*B*) Helicase assay mixtures containing 5–100 ng of RAD3 protein were incubated at 30°C for 7 min.

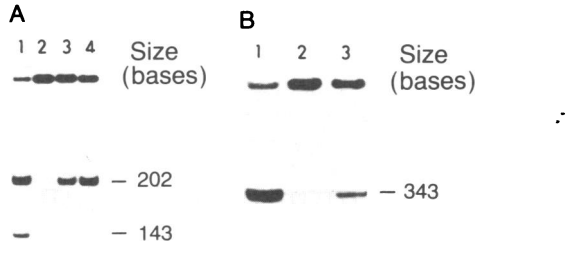

FIG. 5. Direction of unwinding by RAD3 helicase. (*A*) To determine the direction of the RAD3 helicase activity, RAD3 protein was incubated with the linear substrate described in Fig. 1*B* at 30°C for 15 min. Only the 202-base fragment was displaced (lanes 3 and 4), indicating that RAD3 protein moves on the single-stranded DNA in the 5′→3′ direction. Lanes: 1, heat-denatured DNA substrate; 2, no RAD3 protein; 3 and 4, 50 ng and 100 ng of RAD3 protein, respectively. (*B*) RAD3 protein also unwinds the 343-bp partial duplex used for the construction of the linear substrate used in *A*. The incubation time for this experiment was 15 min. Lanes: 1, heat-denatured DNA substrate; 2, no RAD3 protein; 3, 100 ng of RAD3 protein.

DISCUSSION

We have shown that RAD3 protein, a single-stranded DNA-dependent ATPase (9), also possesses a helicase activity. This activity was found to unwind DNA duplexes 71–851 bp long. Like other helicases, RAD3 helicase requires a hydrolyzable nucleoside triphosphate, which can be satisfied by ATP or dATP. With adenosine 5′-[γ-thio]triphosphate, an inhibitor of RAD3 ATPase activity (9), no helicase activity was observed. The pH optimum for RAD3 ATPase and helicase activities is ≈5.6. *In vivo* activities of RAD3 protein at physiological pH may be enhanced by interaction with other proteins. For instance, phage T4 gene *41* helicase activity is elevated by its interaction with other T4 replication proteins (15), and *E. coli* DnaB helicase is stimulated by a combination of single-strand DNA binding protein and primase (22).

All helicases move unidirectionally on single-stranded DNA and unwind duplex DNA with a specific polarity. Presumably, ATP hydrolysis is required for translocation, for unwinding, or for both. RAD3 helicase also exhibits a unidirectional movement, translocating on single-stranded DNA in the 5′→3′ direction. Phage T7 gene *4*, phage T4 gene *41*, and *E. coli* DnaB proteins possess helicase activities that, like RAD3 helicase, move along single-stranded DNA in the 5′→3′ direction and unwind duplex DNA (15, 19, 22). These phage and *E. coli* proteins are multifunctional and play several roles in DNA replication. Each one apparently binds and moves on the lagging-strand template and unwinds duplex DNA in advance of the replication fork. In addition, these proteins interact with other replication proteins and are involved in the synthesis of RNA primers on the lagging strand. T7 gene *4* protein is also a primase (19), T4 gene *41* helicase and gene *61* primase work together for primer synthesis (15), and *E. coli* DnaB helicase has been suggested to interact with the DnaG primase (22). In eukaryotes, the multifunctional simian virus 40 tumor antigen, required for transformation of eukaryotic cells and for viral DNA replication, is an ATPase and a helicase (27–29). It is also a sequence specific DNA binding protein that binds sequences at the simian virus 40 origin of replication and physically interacts with the DNA polymerase α–DNA primase complex (27). Thus, T7 gene *4*, T4 gene *41*, *E. coli* DnaB, and simian virus tumor antigen proteins are all required for both initiation and elongation phases of DNA replication. Whether RAD3 protein also acts in a similar manner during DNA replication is not known. Conditional lethal mutations of *RAD3* should be of considerable help in elucidating its *in vivo* role.

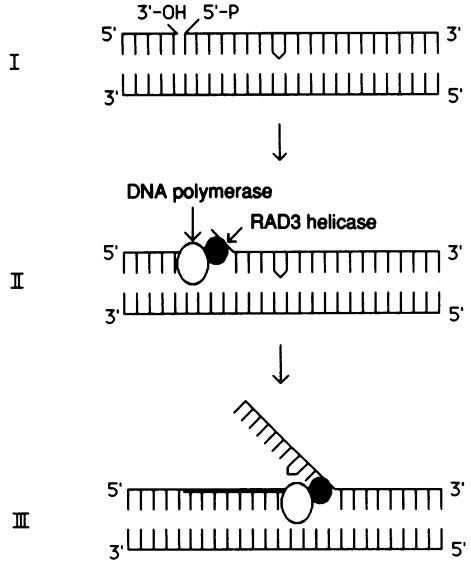

FIG. 6. A model showing RAD3 helicase action during excision repair of pyrimidine dimers. (*I*) Damaged DNA strand is presumed to be incised on the 5′ side of the pyrimidine dimer (▯). (*II*) RAD3 protein binds the single-stranded region, formed by the action of a DNA polymerase and accessory proteins, and translocates in the 5′→3′ direction. Alternatively, RAD3 protein could initiate unwinding at the nick. (*III*) RAD3 helicase further displaces the bound strand from the template, facilitating DNA synthesis, indicated by the heavy line. Following the dissociation of RAD3 helicase, the displaced fragment could be degraded exonucleolytically, or it could be released if a second nick is made by the incision enzyme on the 3′ side of the pyrimidine dimer as in *E. coli* (36, 37).

RAD3 protein is involved in incision and apparently also in post-incision steps of excision repair. The *rad3-1* and *rad3-2* mutants are totally defective in incision (1–3), indicating that RAD3 protein may be a component of the incision enzyme complex. We have observed that *rad3* mutants in which lysine-48 in the putative ATP-binding consensus sequence (7) has been changed, incise UV-damaged DNA to a limited extent but are defective in the removal of pyrimidine dimers (4). This suggests that, like the *E. coli* UvrD protein, RAD3 protein may be involved in dissociation of the incision enzyme complex from DNA (30, 31) and in repair synthesis (20). RAD3 helicase, in conjunction with yeast DNA polymerase, may catalyze strand-displacement repair synthesis at the site of incision in DNA. Most DNA polymerases, such as human DNA polymerase α, T4 DNA polymerase, and T7 DNA polymerase, are unable to initiate synthesis from nicks (32–34). Addition of T4 gene *41* and T7 gene *4* proteins greatly stimulates strand-displacement synthesis by the respective DNA polymerases at single-strand interruptions in duplex DNA (15, 35). A model for RAD3 helicase action during excision repair is shown in Fig. 6. In this scheme RAD3 protein binds the strand containing the damage and moves along it in the 5′→3′ direction, separating it from the template strand. The gap thus created can then be filled in by a DNA polymerase.

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