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The *RAD3* gene of *Saccharomyces cerevisiae* encodes a DNA-dependent ATPase

(RAD3 protein/DNA repair/yeast)

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ABSTRACT **The *RAD3* gene of the yeast *Saccharomyces cerevisiae* is required for excision repair of damaged DNA and for cell viability. A protein of ≈89 kDa was purified to near homogeneity from yeast strains harboring multicopy plasmids that overproduce RAD3 protein; this protein corresponds closely to the expected size of the RAD3 protein and cross-reacts with the antiserum raised against a truncated RAD3 protein produced in *Escherichia coli*. The purified RAD3 protein shows a single-stranded DNA-dependent ATPase activity that catalyzes hydrolysis of ATP to ADP and P_i. The ATPase activity was coincident with the RAD3 protein during purification and is inhibited by anti-RAD3 antibodies, indicating that the *RAD3* gene encodes this activity.**

In the yeast *Saccharomyces cerevisiae*, many genes are involved in excision repair of DNA damaged by UV light, crosslinking agents, and by chemicals that form bulky adducts. Rather surprisingly, most of these genes affect incision of damaged DNA. The *rad1, rad2, rad3, rad4, rad10*, and *mms19* mutants are highly defective in incision (1–3), whereas *rad7, rad14, rad16*, and *rad23* mutants are less so (1, 3, 4). Several of these genes have been cloned, and their nucleotide sequences were determined in our laboratory and by others (5–9). Our aim now is to overproduce, purify, and characterize the proteins encoded by these genes and to examine the incision mechanism *in vitro*.

RAD3 differs from the other genes involved in incision in that its genomic deletions are recessive lethals (10, 11), whereas deletion mutations of the *RAD1, RAD2, RAD7, RAD10*, and *RAD23* genes do not affect viability. *RAD3* thus functions in excision repair and plays an essential role in the maintenance of cell viability. The nucleotide sequence of *RAD3* predicts a polypeptide of 778 amino acids with a M_r of 89,779 (7, 9). A consensus sequence present in a number of enzymes that bind and hydrolyze ATP also occurs in the RAD3 protein (7).

To elucidate the action mechanism of the RAD3 protein, we have constructed RAD3-overproducing plasmids and purified RAD3 protein from an *S. cerevisiae* strain carrying one such plasmid. In the present communication, we show that *RAD3*-encoded protein is a single-stranded DNA-dependent ATPase that hydrolyzes ATP to ADP and P_i.

MATERIALS AND METHODS

Construction of the RAD3-Overproducing Plasmid. The *RAD3* gene was fused to the highly expressed constitutive yeast alcohol dehydrogenase I (*ADC1*) promoter in the vector pSCW231, which contains the *ADC1* promoter and the *CYC1* terminator as well as the yeast 2-μm plasmid origin of replication for autonomous replication and the *TRP1* gene for selection in yeast. The resulting RAD3-overproducing plasmid, pSCW367, contains the *RAD3* gene from position −60 to ≈700 nucleotides downstream of the *RAD3* TGA termination codon (7).

Yeast Strains and Cell Transformation. The haploid strain CMY135 (*MATα ade2-1 his3-Δ200 lys2-801 trp1-Δ ura3-52 RAD⁺*) was transformed with the plasmid pSCW231 or pSCW367 to Trp^+ by the method of Ito *et al.* (12). The resulting plasmid-bearing strains were cultured in synthetic medium lacking tryptophan; at titers of 1.2×10^7 to 1.5×10^7 per ml, the cells were collected by centrifugation and stored at −70°C.

Buffers. CBB (cell breakage buffer) is 50 mM Tris·HCl (pH 7.5) containing 600 mM KCl, 10% sucrose, 10 mM EDTA, 10 mM 2-mercaptoethanol, and the following protease inhibitors: phenylmethylsulfonyl fluoride and benzamidine hydrochloride at 1 mM, and aprotinin, chymostatin, leupeptin, and pepstatin A at 5 μg/ml. Buffer A is 20 mM KH_2PO_4 (pH 7.4) containing 10% glycerol, 10 mM 2-mercaptoethanol, 0.5 mM EDTA, and 1 mM each of phenylmethylsulfonyl fluoride and benzamidine hydrochloride. Buffer B is 15 mM Tris·HCl (pH 7.5) containing 10% glycerol, 1 mM dithiothreitol, and 0.5 mM EDTA.

Purification of the RAD3 Protein. All the purification steps were done at 0–4°C. For large-scale purification of RAD3 protein (Table 1), 200 grams (wet weight) of frozen yeast cells were thawed on ice and suspended in 500 ml of CBB. The cell suspension was passed through a French press (SLM Aminco, Urbana, IL) twice at $\approx 117 \times 10^6$ Pa. The resulting extract was centrifuged at 110,000 × *g* for 1.5 hr, and solid $(NH_4)_2SO_4$ was added slowly to the clear supernatant to 55% saturation (0.35 g/ml of extract). The protein pellet obtained after centrifugation (20,000 × *g* for 30 min) was dissolved in 40 ml of 50 mM KCl in buffer A that also contained chymostatin and pepstatin A at 20 μg/ml. The protein solution was clarified by centrifugation at 110,000 × *g* for 15 min before being diluted with 50 mM KCl in buffer A to give a conductivity equivalent to that of 150 mM KCl in water. The diluted fraction was layered onto a column of DEAE-Sephacel (Pharmacia; 5 × 25.5 cm) equilibrated with 180 mM KCl in buffer A. The column was washed with 2.5 vol of 180 mM KCl in buffer A and eluted with 270 mM KCl in buffer A. The fractions containing the protein peak were identified using the Coomassie blue dye binding assay of Bradford (13), and then pooled; the protein was precipitated by the addition of solid $(NH_4)_2SO_4$ to 75% saturation (0.52 g per ml of protein solution). The precipitate was harvested by centrifugation (20,000 × *g* for 30 min), dissolved in 20 ml of 80 mM NaCl in buffer B containing phenylmethylsulfonyl fluoride and benzamidine hydrochloride at 1 mM and chymostatin and pepstatin A at 20 μg/ml. The sample was dialyzed, with one

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change against a total of 4 liters of 80 mM NaCl in buffer B for 12 hr. After being diluted to 100 ml with 80 mM NaCl in buffer B, the protein solution was layered onto a column of single-stranded DNA agarose (Bethesda Research Laboratories; 2.6 × 12 cm) equilibrated with the same buffer. The column was washed with 8 vol of 80 mM NaCl in buffer B and eluted with 400 mM NaCl in buffer B. The fractions containing protein were identified, pooled, and assayed for ATPase activity. RAD3 protein ATPase was not assayed prior to this step because of contaminating ATPase activities. The protein pool was loaded directly onto a column of Bio-Gel HTP hydroxylapatite (Bio-Rad; 1.5 × 5.7 cm) equilibrated with buffer C (100 mM KCl in buffer A). After being washed with 10 vol of buffer C, the column was developed with a 200-ml linear gradient of 0–100 mM KH_2PO_4 in buffer C. The fractions containing the RAD3 protein (eluting between 70–80 mM KH_2PO_4) were identified by $NaDodSO_4$/PAGE and by ATPase assays. The protein pool obtained from the Bio-Gel HTP hydroxylapatite column was diluted with buffer A until the conductivity was equivalent to that of 120 mM KCl in water.

The pooled enzyme fractions were passed through a column of Bio-Rex 70 ion exchange resin (Bio-Rad; 1 × 6.4 cm) equilibrated with 90 mM KCl in buffer A, and the column was washed with 5 ml of the same buffer. The flow-through fraction and the wash were combined and applied onto a column of DEAE-Sephacel (1 × 2.6 cm) equilibrated with 150 mM KCl in buffer A. The column was washed with 15 ml of 150 mM KCl in buffer A before being developed with a 40-ml gradient of 150–270 mM KCl in buffer A. The fractions (0.8 ml) were examined for their content of RAD3 protein by $NaDodSO_4$/PAGE and by ATPase assays. The purity of the RAD protein, which elutes at about 230 mM KCl, was greater than 90%. To concentrate the active fractions for various assays, the enzyme pool was diluted with one vol of 20% glycerol in buffer D (buffer A without phenylmethylsulfonyl fluoride and benzamidine hydrochloride) and applied onto a 0.6-ml column of DEAE-Sephacel equilibrated with 100 mM KCl in buffer D. The enzyme was subsequently eluted with buffer D containing 300 mM KCl and 10% glycerol and stored at −70°C.

ATPase Assays. The standard assay mixture (10 μl) contained 20 mM $KC_2H_3O_2$ (pH 5.6), 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg of bovine serum albumin per ml, 25 μg of M13mp8 single-stranded DNA per ml, 0.25 mM [2,8-^{3}H]ATP (specific activity, 0.2 Ci/mmol; 1 Ci = 37 GBq), and 50–100 ng of RAD3 protein. Under these conditions, ATP hydrolysis is linear with time for at least 60 min and is proportional to the amount of RAD3 protein. After 20–40 min at 30°C, the reaction was terminated by the addition of 2 μl of 100 mM EDTA and 10 mM each of ATP, ADP, and AMP used as markers. A small aliquot (1–2 μl) of the reaction mixture was applied onto polyethyleneimine-cellulose strips (Brinkmann), which were then developed in 1 M LiCl as described by Randerath and Randerath (14). The ATP, ADP, and AMP regions, located by illumination with a short-wave UV lamp, were excised from the cellulose strips, and their radioactivities were determined by liquid scintillation counting. The formation of P_i was verified by using [γ-^{32}P]ATP instead of [2,8-^{3}H]ATP in the standard reaction mix. The $^{32}P_i$-labeled spot was identified by autoradiography following chromatography on polyethyleneimine-cellulose strips. RAD3 protein ATPase generates only ADP and P_i as the reaction products.

Purification of a Truncated RAD3 Protein from *Escherichia coli* and Production of Anti-RAD3 Antiserum. A portion (≈80%) of the *RAD3* open reading frame along with 35 nucleotides of the 5′ leader region of the gene was inserted as an *Hae* III–*Eco*RI fragment, representing the *RAD3* gene from position −35 to position +1918 (7) into the plasmid pUC19, which had been digested with *Sma* I and *Eco*RI. The resulting fusion plasmid, pSP119, was introduced into *E. coli* strain JM103 by transformation. The truncated RAD3 protein (≈78 kDa) obtained after induction with isopropylthio-β-D-galactoside (IPTG; Boehringer Mannheim) was purified by $NaDodSO_4$/PAGE and used as an immunogen for antiserum production in rabbits.

Other Procedures. PAGE was carried out on a Studier type

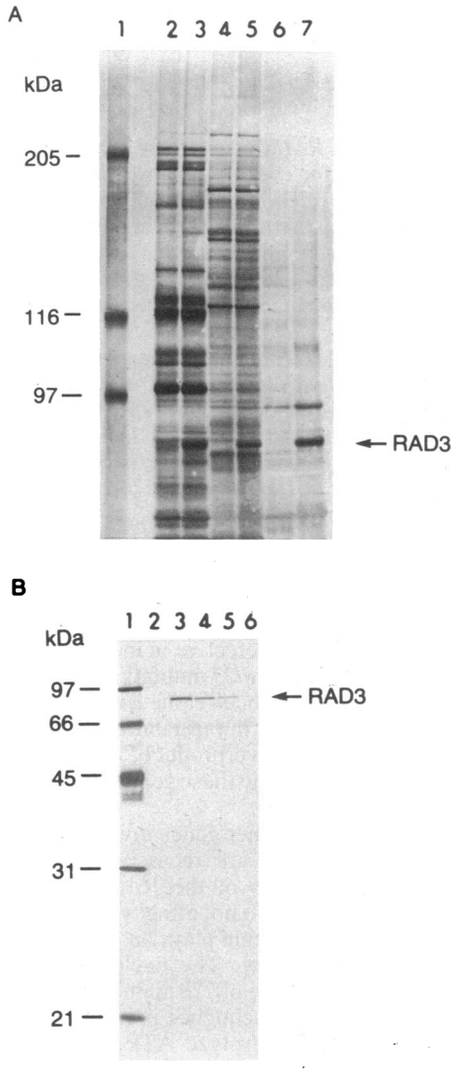

FIG. 1. $NaDodSO_4$/PAGE of different column pools and purified RAD3 protein. (*A*) Aliquots of column pools were analyzed on a 9% $NaDodSO_4$/polyacrylamide gel according to Laemmli (15). The samples were subjected to gel electrophoresis for 12 hr at 18 mA, and the protein bands were revealed using the silver staining method of Wray *et al.* (16). Due to the long time of electrophoresis, proteins with molecular mass <70 kDa migrated beyond the gel. (Lane 1) Molecular mass markers; (lanes 2, 4, and 6) crude cell extract, DNA agarose pool, and Bio-Gel HTP pool from the control yeast strain (CMY135 with plasmid pSCW231), respectively. (Lanes 3, 5, and 7) Equivalent fractions from the RAD3-overproducing strain (CMY135 with plasmid pSCW367). (*B*) The enzyme pool from RAD3-overproducing cells obtained from the second DEAE-Sephacel column was concentrated on a 0.6-ml column of DEAE-Sephacel as described. One μl of the fractions (0.4 ml) from this concentration step was examined on a 10% $NaDodSO_4$/polyacrylamide gel. Staining in the 66-kDa region and below is an artifact inherent in the silver staining technique and also occurred to the same extent in lanes containing only the sample buffer. (Lane 1) Molecular mass markers (350 ng each). Lanes 2–6 correspond to fractions 6–10 from the concentration procedure.

Table 1. Purification of the RAD3 protein from *Saccharomyces cerevisiae*

Step	Volume, ml	Protein, mg	ATPase units*	Specific activity, ATPase units/mg of protein
I Crude extract	500	2700	†	
II First DEAE-Sephacel	230	170	†	
III DNA agarose	80	28	3240	116
IV Bio-Gel HTP	27	4	2800	700
V Bio-Rex 70	42	1.2	2900	2,416
VI Second DEAE-Sephacel	8.5	0.11	1620	14,727

*Reaction mixtures containing 75 μM of [^{3}H]ATP and 2 μl of the various column pools were incubated at 30°C for 1 hr as described. One unit of activity corresponds to the hydrolysis of 1 nmol of ATP under the stated conditions.

†RAD3 protein ATPase could not be assayed due to large quantities of background ATPase activities.

slab gel apparatus using the discontinuous system of Laemmli (15). After electrophoresis the gels were silver-stained as described by Wray *et al.* (16). For the determination of protein concentrations, the Coomassie blue dye binding method of Bradford (13) was used. Pyrimidine dimers were introduced into different kinds of DNA by using a UV dose of 900 J/m^2.

RESULTS

Purification of the RAD3 Protein. The RAD3 protein was purified as described from the Rad$^+$ yeast strain CMY135 carrying the RAD3-overproducing plasmid, pSCW367. At each stage of purification, the RAD3 protein was visualized by electrophoresis in polyacrylamide gels containing 0.1% $NaDodSO_4$ (Fig. 1); in later steps, ATPase activity could be monitored as well (Table 1). A distinct protein band of approximately 89 kDa was seen in crude extracts from the strain CMY135[pSCW367] (Fig. 1*A*, lane 3) but was not seen in control extracts from strain CMY135[pSCW231] (Fig. 1*A*, lane 2). Subsequent purification steps were done identically for extracts from the overproducing yeast strain and from control cells. During purification, the 89-kDa protein band was seen only in extracts from cells containing the RAD3-overproducing plasmid (Fig. 1*A*, compare lane 4 with 5 and lane 6 with 7). After the final chromatographic step (Table 1), the RAD3 protein was >90% pure (Fig. 1*B*).

The RAD3 Protein Has ATPase Activity. Purified RAD3 protein obtained after step VI (Table 1) was seen to catalyze the hydrolysis of ATP to ADP and P_i in the presence of single-stranded DNA; no ^{3}H-labeled AMP was detected as a product of ATP hydrolysis. Coelution of DNA-dependent ATPase activity with the RAD3 protein was examined in fractions from Bio-Gel HTP hydroxylapatite columns (step IV, Table 1) and from the second DEAE-Sephacel column (step VI, Table 1). Fig. 2 shows the pattern of proteins in $NaDodSO_4$/PAGE in fractions from Bio-Gel HTP hydroxylapatite columns. In samples from the RAD3-overproducing yeast strain, a prominent protein band of 89 kDa appears in fractions 16, 17, 18, and 19 (Fig. 2*A*), whereas no such band is visible in the samples from the control yeast strain (Fig. 2*B*). In the samples from the RAD3-overproducing yeast strain, the DNA-dependent ATPase activity elutes as a single peak (Fig. 2*C*), and the ATPase activity level in different fractions closely parallels the amount of RAD3 protein in these fractions (Fig. 2*A*). The small ATPase activity peak in the control Rad$^+$ strain (Fig. 2*C*) may be coded for by the yeast chromosomal *RAD3* gene. In the last step of purification, the second DEAE-Sephacel column (Table 1), single-stranded DNA-dependent ATPase activity again coeluted with the RAD3 protein (results not shown).

Inhibition of RAD3 ATPase by Anti-RAD3 Antibodies. To establish that the *RAD3* gene encodes the 89-kDa ATPase rather than a positive regulatory protein that enhances the production of this 89-kDa protein, we examined whether the ATPase activity is inhibited by anti-RAD3 antibodies. As described, antibodies were raised against a truncated RAD3 protein produced in *E. coli*. In immunologic blots, only the

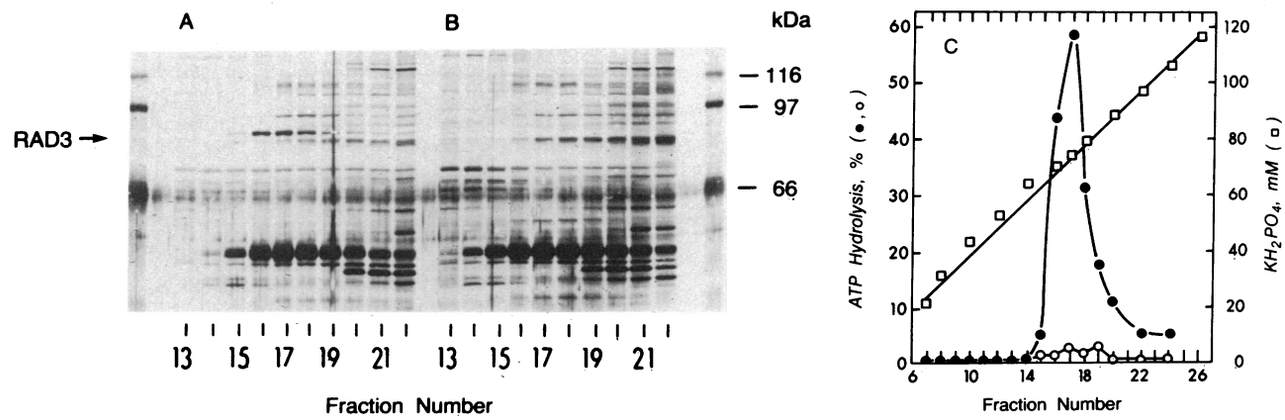

FIG. 2. Coelution of ATPase activity with RAD3 protein on Bio-Gel HTP hydroxylapatite columns. Thirty grams each of RAD3-overproducing cells (strain CMY135 with plasmid pSCW367) and control cells (CMY135 with plasmid pSCW231) were disrupted using a French press, and the extracts were processed on DEAE-Sephacel (2.6 × 13 cm) and DNA agarose (1.5 × 4.5 cm) as described. The protein pools obtained from DNA agarose was further fractionated on Bio-Gel HTP (1 × 2 cm) using a 20-ml KH_2PO_4 gradient from 0–100 mM in buffer C (this buffer contained an additional 20 mM KH_2PO_4). $NaDodSO_4$/PAGE of fractions 13–22 from Bio-Gel HTP columns originating from extracts of RAD3-overproducing cells (*A*) and control cells (*B*). (*C*) Two microliters of the fractions from the Bio-Gel HTP columns was examined for RAD3 ATPase activity in reaction mixtures containing 75 μM [^{3}H]ATP and other components as specified. The incubation time for this experiment was 1 hr. ●, ATPase activity profile of fractions from the RAD3-overproducing cells; ○, ATPase activity in fractions from the control cells.

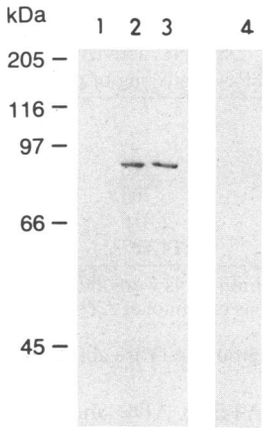

FIG. 3. Immunoblot of protein pools from the DNA agarose step and purified RAD3 protein. A nitrocellulose blot of $NaDodSO_4$/polyacrylamide gel was probed with anti-RAD3 antiserum. (Lane 1) DNA agarose protein pool (10 μg of total protein) from the control yeast strain (CMY135 with plasmid pSCW231); (lane 2) DNA agarose protein pool (10 μg of total protein) from the RAD3-overproducing strain (CMY135 with plasmid pSCW367); (lane 3) 75 ng of purified RAD3 protein; (lane 4) 75 ng of purified RAD3 protein probed with preimmune serum. No cross-reaction was observed.

89-kDa protein present in various column fractions from the RAD3-overproducing strain cross-reacts with the anti-RAD3 antiserum (Fig. 3). For inhibition of ATPase activity, anti-RAD3 antibodies were added to the standard assay mix containing 100 ng of RAD3 protein. The addition of 20 μg and 40 μg of anti-RAD3 IgG resulted in inhibition of ATPase activity by 22% and 35%, respectively. No inhibition of RAD3 ATPase activity was observed with control IgG isolated from preimmune serum. The modest level of inhibition of ATPase activity by anti-RAD3 antibodies may be due to the fact that $NaDodSO_4$-denatured and truncated RAD3 protein was used as the immunogen.

Characterization of RAD3 ATPase. As shown in Table 2, RAD3 ATPase activity requires single-stranded DNA. All of the characterization of RAD3 ATPase was done under

Table 2. DNA effectors for RAD3 protein ATPase activity

Polynucleotide	ATPase activity, %
Control (no DNA)	0
Single-stranded DNA	
M13mp8	100*
M13mp8, UV-irradiated	92
Poly(dA)	106
dT_{10}	50
Duplex DNA	
M13um21†	0
M13um21, UV-irradiated†	0
Circular pBR322‡	7
Circular pBR322, UV-irradiated‡	6
Linear pBR322§	2
Linear pBR322, UV-irradiated§	4

Reaction mixtures containing 250 ng of the various polynucleotides and 75 ng of RAD3 protein were incubated at 30°C for 40 min as described.

*100% ATPase activity corresponds to the hydrolysis of 26.3% of input ATP.

†M13um21 DNA was purchased from International Biotechnologies (New Haven, CT).

‡Circular pBR322 DNA contained about 60% supercoiled and 40% relaxed forms.

§pBR322 DNA was linearized at its unique *Eco*RI site.

Table 3. Effect of nucleotides on RAD3 protein ATPase activity

Reaction mixture	NTP	ATPase activity, %
1	Complete reaction mix	100*
2	1 + ATP[γS]	36
3	1 + ADP	57
4	1 + GTP	94
5	1 + CTP	63
6	1 + UTP	110
7	1 + dATP	19
8	1 + dGTP	106
9	1 + dCTP	128
10	1 + dTTP	94

The complete reaction mixture contained 0.25 mM [^{3}H]ATP, 50 ng of RAD3 protein, and other components as specified. The various nucleotides were added to the complete reaction mixture to a final concentration of 1.5 mM; incubation time for this experiment was 20 min.

*100% ATPase activity corresponds to the hydrolysis of 9.8% of input ATP.

conditions where ATP hydrolysis was linear with time and proportional to RAD3 protein concentration. Poly(dA) is as effective as single-stranded M13 DNA, but oligonucleic acid dT_{10} is only 50% as effective. UV irradiation of M13 single-stranded DNA does not stimulate ATPase activity. Little or no ATPase activity is observed with unirradiated or UV-irradiated duplex DNA or in the absence of DNA. The optimal pH for RAD3 ATPase is near 5.6. The ATPase activity requires Mg^{2+}, but Mg^{2+} can be replaced by Mn^{2+}; however, ATPase activity is lost when Mg^{2+} is replaced by Ca^{2+} or Zn^{2+}. The optimal Mg^{2+} concentration is from 2 to 10 mM. At 2 mM and 10 mM Mn^{2+}, ATPase activity is 67% and 20%, respectively, of that observed with equivalent concentrations of Mg^{2+}. KCl stimulates the ATPase activity maximally at 100–150 mM, and slight inhibition (10–20%) is seen at 210 mM concentration of the salt.

The effect of additions of rNTPs and dNTPs on ATPase activity is shown in Table 3. To the complete reaction mix containing 0.25 mM [^{3}H]ATP, 1.5 mM concentrations of other nucleotides were added. ATP hydrolysis was decreased by ≈80% by the addition of dATP, and ≈40–60% by the addition of ATP[γS], ADP, and CTP. Addition of GTP, UTP, dGTP, dCTP, and dTTP had no inhibitory effect on ATPase activity. Using [γ-^{32}P]dATP, we observed that RAD3 protein also hydrolyzes dATP in a single-stranded DNA-dependent manner (data not shown). The pH optimum for dATP hydrolysis is the same as for ATP hydrolysis. Extrapolation from a double-reciprocal plot of ADP formation versus ATP concentration yields an apparent K_m for ATP of 67 μM.

DISCUSSION

In this study, we purified the *RAD3*-encoded protein from a yeast strain carrying RAD3-overproducing plasmids. The molecular mass of the purified protein, as determined by $NaDodSO_4$/PAGE, is ≈89 kDa, which is in good agreement with the 89.779 kDa predicted from the *RAD3* coding sequence. The 89-kDa protein cross-reacts with an antiserum raised against a truncated RAD3 protein produced in *E. coli*. Furthermore, a change of the lysine 48 codon to methionine constructed by *in vitro* mutagenesis in the *RAD3* gene results in an alteration of the elution profile of the 89-kDa mutant protein on Bio-Gel HTP hydroxylapatite, as verified by immunoblotting (P.S., unpublished data). The single-stranded DNA-dependent ATPase activity coelutes with the 89-kDa protein and is inhibited by anti-RAD3 antibodies. From these observations, we conclude that the *RAD3* gene encodes the single-stranded DNA-dependent ATPase.

The ATPase activity of the RAD3 protein catalyzes the hydrolysis of ATP to ADP and P_i in the presence of single-stranded DNA. Little or no ATPase activity is seen with double-stranded DNA or without DNA, and UV-irradiation of DNA has no effect on this activity. The RAD3 ATPase requires Mg^{2+}, but this can be partially replaced by Mn^{2+}. The ATPase has a pH optimum of 5.6. RAD3 ATPase activity at physiological pH *in vivo* might be enhanced through interaction with other protein cofactors. The RAD3 ATPase differs in size and properties from the three other DNA-dependent ATPases, I, II, and III, described in yeast (17, 18).

The ATP binding and hydrolysis by the RAD3 protein could play a role in excision repair and in maintaining cell viability in several ways. ATP could act as an allosteric effector by binding to RAD3 protein and producing a conformational change that allows the assembly of RAD3 monomers or allows binding with other proteins in the repair and possibly the replication complex. ATP binding could stimulate the DNA binding activity of RAD3 protein. ATP-bound UvrA protein of *E. coli* has a higher affinity for damaged DNA than UvrA protein alone (19). DnaB and Rep proteins required for replication of *E. coli* and phage chromosomes, respectively, form a binary complex with ATP, which shows a strong affinity for single-stranded DNA (20, 21). ATP hydrolysis may be linked to dissociation of RAD3 protein from DNA as seen for other DNA-dependent ATPases (20–22). Alternatively, the energy from ATP hydrolysis may be used for translocation of RAD3 protein or the associated protein complex on DNA and for unwinding of duplex DNA.

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