DNA fragmentation/degradation is an important step for apoptosis. However, in unicellular organisms such as yeast, this process has rarely been investigated. In the current study, we revealed eight apoptotic nuclease candidates in *Saccharomyces cerevisiae*, analogous to the *Caenorhabditis elegans* apoptotic nucleases. One of them is Tat-D. Sequence comparison indicates that Tat-D is conserved across kingdoms, implicating that it is evolutionarily and functionally indispensable. In order to better understand the biochemical and biological functions of Tat-D, we have overexpressed, purified, and characterized the *S. cerevisiae* Tat-D (scTat-D). Our biochemical assays revealed that scTat-D is an endo-/exo-nuclease. It incises the double-stranded DNA without obvious specificity via its endonuclease activity and excises the DNA from the 3′- to 5′-end by its exonuclease activity. The enzyme activities are metal-dependent with Mg\(^{2+}\) as an optimal metal ion and an optimal pH around 5. We have also identified three amino acid residues, His\(^{185}\), Asp\(^{325}\), and Glu\(^{327}\), important for its catalysis. In addition, our study demonstrated that knockout of Tat-D in *S. cerevisiae* increases the TUNEL-positive cells and cell survival in response to hydrogen peroxide treatment, whereas overexpression of Tat-D facilitates cell death. These results suggest a role of Tat-D in yeast apoptosis.

Apoptosis is an important biological process required for maintaining tissue homeostasis, removal of redundant or damaged cells, normal developmental progression, and response to various toxic stimuli (1–6). It is a programmed cell death characterized by cell shrinkage, membrane blebbing, chromatin condensation around the periphery of the nucleus, and DNA fragmentation and degradation (1). Failure of this process could lead to developmental defects, immunological and neurodegenerative disorders, and formation of cancers in mammals and other higher organisms (7–10).

A critical and late step in apoptosis is DNA degradation, which requires participation of endo- and exonucleases. So far, at least three nucleases have been well characterized to play a role in DNA degradation for apoptosis, including DFF40/CAD, Cps-6/endonuclease G, and Nuc I/DNase II (11–18). Of the three nucleases, DFF40/CAD is caspase-dependent, whereas the other two are caspase-independent. More recently, seven additional nucleases have been identified for apoptosis in *Caec- norhabditis elegans*, including Crn-1/FEN-1 and Crn-2/Tat-D (19). Further analysis indicated that Crn-1/FEN-1, a flap endo- 5′ to 3′ exonuclease critical for DNA replication, repair, and recombination (20), degrades DNA through coordination with Cps-6/endonuclease G in *C. elegans* cells (19).

Evidence is accumulating that apoptosis also exists in unicellular organisms, such as in budding yeast *Saccharomyces cerevisiae* (21–23). The apoptotic response of *S. cerevisiae* has been observed in aging cells (24) and mutants with mutations in ATPase CDC48 (25) or anti-silencing protein ASF1 (26). In addition, weak acidic (27, 28), oxidative stress (29), salt stress (30, 31), UV irradiation (32), and mating pheromone treatment (33) can also induce apoptosis in yeast cells. Dying yeast cells under these conditions display several markers that are characteristic of apoptosis. These include the rapid exposure of phosphatidylserine on the outer cell membrane, the margination of chromatin in nuclei, nuclear fragmentation, and the degradation of DNA. Exposure of the cells to the protein translation inhibitor, cycloheximide, prevents these death-associated changes, indicating that the death response requires active protein synthesis (29).

Although yeast has been a good model system for apoptotic studies (34), the process and mechanism of apoptotic DNA fragmentation/degradation in yeast has rarely been investigated. It is unclear which nucleases are involved in yeast apoptosis. To gain knowledge on apoptotic nucleases in yeast cells, we started with a comprehensive homology search based on presently available information on apoptotic nucleases of multicellular organisms, including caspase-dependent DFF40/CAD and caspase-independent nucleases, such as Cps-6/endonuclease G, Nuc-1/DNase IIa, Crn-1/FEN-1, Crn-2/Tat-D, Crn-3/PM/ScI-100, Crn-4/RNase T, Crn-5/Rrp46, Crn-6/DNase IIb, Cyp-13/Cyp E, DNase I, and DNase γ. The search resulted in eight candidate nucleases for yeast apoptotic DNA fragmentation/degradation, which correspond to the above listed except for DFF40/CAD, Nuc-1/DNase IIa, DNase I, and DNase γ.

Among the eight potential apoptotic nucleases in yeast, Tat-D appears most conserved. It exists in organisms across all kingdoms. However, the biochemical and biological functions of Tat-D are still poorly understood. Tat-D was first implicated in *Schizosaccharomyces pombe* to have a role in spindle elongation and chromosome decondensation during progression of late anaphase (35). It was later, however, considered to be an integral membrane component of the Sec-independent protein export complex that is the so-called twin arginine translocation (Tat) system in *Escherichia coli* (36). Further analysis indicated that Tat-D is actually a DNase rather than a membrane protein (37). This result is consistent with the recent finding that Tat-D has a role in apoptotic DNA degradation in *C. elegans* (19).

In the current study, we characterized the biochemical func-
tions of Tat-D using purified yeast recombinant Tat-D protein and determined its role in apoptotic DNA degradation in yeast cells. Tat-D was revealed to be a metal-dependent endo-/exonuclease with optimal activities under acidic conditions. The TAT-D gene knock-out in yeast has an insignificant effect on cell growth and DNA repair but shows alteration in apoptosis induced by hydrogen peroxide. Furthermore, overexpression of Tat-D in yeast cells facilitates cell death. These results indicate that Tat-D is involved in yeast apoptosis.

## MATERIALS AND METHODS

### Protein Blast Search and Sequence Alignment

For the protein Blast search, we obtained nine C. elegans (Cps-6 Nuc 1, Crn-1, Crn-2, Crn-3, Crn-4, Crn-5, Crn-6, and Cyp-13) and three human (DFF40/CAD, DNsI, and DNsα γ) nuclease proteins as query elements. We searched their counterparts in the *S. cerevisiae* data base (available on the World Wide Web at www.yeastgenome.org). In addition, we searched for all of the available Tat-D homologues in the NCBI data base using Crn-2 protein as the query sequence. The relevant proteins were aligned, and their sequence similarities were calculated using the ClustalW 1.8 multiple sequence alignment algorithm as published at the BCM Search Launcher, Baylor College of Medicine HGSC (available on the World Wide Web at searchlauncher.bcm.tmc.edu).

### Protein Overexpression, Purification, and Site-directed Mutagenesis

The ScTAT-D DNA fragment was generated by PCR using *S. cerevisiae* genomic DNA and the primers listed in Table I. The pET-28b plasmid with the insertion of yTat-D DNA was used as a template. Site-directed mutagenesis, overexpression, and purification of wild type and mutant FEN-1 enzymes were carried out based on previously published procedures (38–40).

### DNA Substrate Preparation and scTat-D Nuclease Activity Assays

Protocols for DNA substrate preparation and nuclease activity assays were performed as previously published (41). Oligonucleotides used to construct the substrates are listed in Table I. Briefly, oligonucleotides shown in the figures were individually phosphorylated at the 5′-end. This was done by incubating 40 pmol of the oligonucleotide with 10 μCi of γ-<sup>32</sup>PATP and 1 μl (10 units/μl) of polynucleotide kinase at 37 °C for 60 min. Polynucleotide kinase was then inactivated by heating at 72 °C for 10 min. 80 pmol of each remaining oligonucleotide comprising the substrates were added to the labeled oligonucleotides. The samples were incubated at 70 °C for 5 min followed by slow cooling to 25 °C, thus allowing the oligonucleotides to anneal and form the flap and nick-duplex substrates. Substrates were precipitated at −20 °C overnight after adding 20 μl of 3 M NaOAc and 1 ml of 100% ethanol. Substrates were collected by centrifugation and washed once with 70% ethanol and resuspended in sterile water.

Reactions were carried out with the indicated amount of ScTat-D and 0.1 or 0.2 μM of DNA substrates in a reaction buffer containing 50 mM Tris (pH 8.0) and 10 or 5 mM MgCl<sub>2</sub>. Each reaction was then brought to a total volume of 10 μl with water. All reactions were incubated at 30 °C for 15 min and terminated by adding an equal volume of stop solution (95% formamide, 20 mM EDTA, 0.05% broomphenol blue, 0.05% xylene cyanol). An aliquot of each reaction was then run on a 15% denaturing polyacrylamide gel at 1900 V for 1 h. The gel was dried at 70 °C for 50 min, and the bands were visualized by autoradiography.

### Yeast Strains and Gene Disruption

Two yeast strains, RKY2672 and BY4741, were used for the disruption of scTAT-D. The gene was knocked out using the PCR-mediated gene disruption method (41, 42). We used the plasmid pFA6a-KanMX4 (kindly provided by Dr. K. Kuchler) for amplifying the marker gene, Kan<sup>R</sup>. A DNA fragment of the marker gene flanked on the upstream side by a 53-bp upstream sequence of ScTAT-D and on the downstream side by a 53-bp downstream sequence of scTAT-D was produced by PCR and used to transform yeast cells as indicated. The transformed cells were plated on selection medium, YPD with 200 μg/ml G418. scTAT-D disruption was confirmed by PCR for the G418-resistant colonies. The RKY2672-based strains were used to examine the spontaneous mutation status due to the disruption of ScTAT-D. The By4741-based strains were used for assays on cell growth and survival rate with H<sub>2</sub>O<sub>2</sub>, 4,6-diamidino-2-phenylindole, and annexin V staining and TdT-mediated dUTP nick end labeling (TUNEL) assays.

### Yeast Cell Survival Tests

Yeast cells were grown in YPD medium overnight and adjusted to identical density at A<sub>600</sub>. The cells were diluted by 100 times and grown in 10 ml of YPD medium until they reached exponential phase (A<sub>600</sub> = 0.6). H<sub>2</sub>O<sub>2</sub> was added to the desired concentrations. After a 2-h treatment with H<sub>2</sub>O<sub>2</sub>, cells were washed twice with distilled water, and the sample volume was adjusted to 5 ml. Cells treated with 0, 0.5, 1, 2, 3, and 6 mM H<sub>2</sub>O<sub>2</sub> were diluted by 4000, 2000, 1000, and 500 times, respectively. 100 μl of diluted sample was plated onto YPD plates. The number of surviving colonies was determined after a 2-day incubation at 30 °C. The survival rate was calculated using the number of colonies with H<sub>2</sub>O<sub>2</sub> treatment, multiplying by the dilutions during cell plating, and dividing by the number of control cells (without H<sub>2</sub>O<sub>2</sub> treatment). Assays were repeated three times.

### TUNEL Assays on Yeast Cells

The procedure for the TUNEL assay follows Madeo et al. (29). Cells were grown to early log phase (A<sub>600</sub> = 0.6), and hydrogen peroxide was then added with the indicated amounts in Fig. 7. After 2 h, cells were fixed by adding 3.7% formaldehyde into the cell cultures. Cells were fixed for at least 1 h and then collected, washed, and digested by lyticase. The digested cells were applied to a polylysine-coated slide as described for immunofluorescence (43). The slides were rinsed with PBS and incubated with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 min at room temperature to block endogenous peroxidases. The slides were further rinsed with PBS, incubated in

### Changes in *S. cerevisiae* Apoptotic Tat-D Nuclease

# Table I

| Oligonucleotide name | Oligonucleotide sequence | Oligonucleotide use |
|----------------------|--------------------------|---------------------|
| D1                   | 5′-GAGACTTCTCCCTCAAGACCTGGATA-3′ | Substrate           |
| D2                   | 5′-CAGTTATCTACCCCTCAAGACCTGGATA-3′ | Substrate           |
| D3                   | 5′-TTAGGACAGACTGCCCTCAAGACCTGGATA-3′ | Substrate           |
| D4                   | 5′-GAGACTGCTGCCTCAAGACCTGGATA-3′ | Substrate           |
| D5                   | 5′-AGGCCTCTTCCCTCAAGACCTGGATA-3′ | Substrate           |
| D6                   | 5′-AAGCTTCTCCCTCAAGACCTGGATA-3′ | Substrate           |
| E158A-F              | 5′-AAGCTTCTCCCTCAAGACCTGGATA-3′ | E158A mutation      |
| E158A-R              | 5′-GAGACTGCTGCCTCAAGACCTGGATA-3′ | E158A mutation      |
| D352A-F              | 5′-GAGACTGCTGCCTCAAGACCTGGATA-3′ | D352A mutation      |
| D352A-R              | 5′-GAGACTGCTGCCTCAAGACCTGGATA-3′ | D352A mutation      |
| D372A-F              | 5′-GAGACTGCTGCCTCAAGACCTGGATA-3′ | D372A mutation      |
| D372A-R              | 5′-GAGACTGCTGCCTCAAGACCTGGATA-3′ | D372A mutation      |
| 1257NF               | 5′-TGGGACACTTTCTCCCTGCAAGACCTGGATA-3′ | ScTAT-D CCAGTACTGTTATAGGTCTGACGTAGGCTGAC-3′ disruption |
| 1257NR               | 5′-TGGGACACTTTCTCCCTGCAAGACCTGGATA-3′ | ScTAT-D CCAGTACTGTTATAGGTCTGACGTAGGCTGAC-3′ disruption |
| 1257F                | 5′-TGGGACACTTTCTCCCTGCAAGACCTGGATA-3′ | ScTAT-D CCAGTACTGTTATAGGTCTGACGTAGGCTGAC-3′ disruption |
| 1257R                | 5′-TGGGACACTTTCTCCCTGCAAGACCTGGATA-3′ | ScTAT-D CCAGTACTGTTATAGGTCTGACGTAGGCTGAC-3′ disruption |

The abbreviations used are: TUNEL, TdT-mediated dUTP nick end labeling; PBS, phosphate-buffered saline.
permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate) for 2 min on ice, rinsed twice with PBS, incubated with 10 µl of TUNEL reaction mixture (terminal deoxynucleotidyl transferase 200 units/ml, fluorescein isothiocyanate-labeled dUTP 10 mM, 5 mM Tris-HCl, 200 mM sodium cacodylate, 5 mM cobalt chloride; Roche Applied Science) for 60 min at 37 °C, and then rinsed 3 times with PBS. For the detection of peroxidase, cells were incubated with 10 µl of Converter-POD (anti-fluorescein isothiocyanate antibody, Fab fragment from sheep, conjugated with horseradish peroxidase) for 30 min at 37 °C, and then stained with DAB-substrate solution (Roche Applied Science) for 10 min at room temperature. A coverslip was mounted with a drop of Kaiser’s glycerol gelatin (Merck). Since staining intensity varies, only samples from the same slide were compared. 4′,6-Diamidino-2-phenylindole staining was performed as described previously (25). To determine frequencies of TUNEL-positive cells, at least 300 cells of three independent experiments were evaluated.

RESULTS

Presence of Potential Apoptotic Nucleases in Yeast—In order to identify apoptotic nucleases in yeast cells, we performed a Blast search using the yeast protein data base based on 12 known apoptotic nucleases so far identified from multicellular organisms as query sequences. The 12 known nucleases are listed in Table II, including DFF40/CAD, Cps-6/endonuclease G, Nuc1/Dnase I, Crn-1/FEN-1, Crn-2/Tat-D, Crn-5/RNase T, Crn-4/RNase T, Crn-5/Rrp46, Crn-6/DNase I, Crn-6/DNase IIb, and Cyp-13/Cyp E (Table II). Except for Crn-5/RRP46, Crn-6/DNase I, and Cyp-13/Cyp E (Table II). Except for Crn-2/Tat-D, Crn-5/RNase T, and Crn-6/DNase IIb, the remaining five nucleases were shown to form a degradesome in yeast cells, including Cps-6/endonuclease G, Nuc1/Dnase I, Crn-1/FEN-1, Crn-2/Tat-D, Crn-5/RNase T, Crn-4/RNase T, Crn-5/Rrp46, Crn-6/DNase I, and Cyp-13/Cyp E (Table II). Except for Crn-2/Tat-D, Crn-5/RNase T, and Crn-6/DNase IIb, the remaining five nucleases were shown to form a degradesome in yeast cells (26). Since Cps-6/endonuclease G is translocated from the mitochondrial to the nucleus during apoptosis (27, 44), its sequence appears only in the eukaryotic cells. It contains multiple positively charged amino acid residues, which is similar to the nuclear localization signal of nuclear proteins such as FEN-1 (45).

Fig. 1B shows the reconstruction of evolutionary relationships among the 12 Tat-D proteins based on the above aligned sequences. Overall, the tree exhibits an evolutionary trend from lower to higher organisms. The bacterial Tat-Ds are more ancestral except for E. coli Tat-D that groups with C. elegans. In eukaryotes, the two yeast Tat-Ds are more ancestral to those from mammalian, plant, and fruit fly. This result indicates that Tat-D is evolutionarily conserved, possibly due to its important biological functions.

Purified Yeast Tat-D Has a Nuclease Activity—In order to better understand the biochemical and biological roles of Tat-D, we cloned the S. cerevisiae TAT-D gene, overexpressed it, and purified its protein (Sc-Tat-D) to homogeneity (Fig. 2A). Although the overall overexpression level was low in the BL21 (DE3) expression system, the His-tagged protein is soluble and can be purified through a nickel column. As shown in Fig. 2A, the purified protein is about 45 kDa in size. We then tested whether this protein could cleave circular plasmid DNA, which would allow us to easily determine whether it has endonuclease activity. Our result shows that Sc/Tat-D can efficiently degrade plasmid DNA, indicating that Sc/Tat-D is a DNase (Fig. 2B). This result implies that Tat-D could be a candidate for apoptotic DNA degradation.

TABLE II

| Previously proposed apoptotic nucleases | Identified or possible activities | Corresponding proteins | Human | C. elegans | Yeast | Similaritya |
|----------------------------------------|---------------------------------|------------------------|-------|------------|------|-------------|
| Cps-6/endonuclease Gb | Endonuclease | + | + | + | 57 |
| Nuc1/Dnase Ia | Endonuclease | + | + | – | 53 |
| Crn-1/FEN-1b | Flap endonuclease and (possibly) exonuclease | + | + | + | 53 |
| Crn-2/Tat-D | Nuclease | + | + | + | 55 |
| Crn-5/RNase Tb | Ribonuclease | + | + | + | 53 |
| Crn-4/RNase Tb | Ribonuclease | + | + | + | 39 |
| Crn-5/Rrp46b | Ribonuclease | + | - | - | 48 |
| Crn-6/DNase Ibb | Endonuclease | + | + | - | 33 |
| Cyp13/CypEb | Nuclease | + | + | + | 63 |
| DFF40/CAD | Endonuclease | + | + | - | 33 |
| DNase I | Endonuclease | + | + | - | 33 |
| DNase γ | Endonuclease | + | + | + | 39 |

a Protein sequence similarity between C. elegans and yeast proteins.
b Summarized from Ref. 19.

"+", presence.

"−", absence.
Tat-D Has Both Endonuclease Activity Making Random Cleavages and Exonuclease Activity Excising from 3' to 5' — To reveal the cleavage pattern of ScTat-D endonuclease activity and to determine if the enzyme also has an exonuclease activity, we used single-stranded or blunt end double-stranded DNA substrates labeled at the 5'- or 3'-end. Fig. 3A shows the DNA
cleavage pattern of ScTat-D using substrates labeled at the 5'-end. Clearly, ScTat-D has enzyme activity on both the double- and single-stranded substrates, whereas the activity on the single-stranded DNA is at least 20 times weaker than that on the double-stranded substrate. In addition, the cleavage pattern could rule out the 5’ to 3’ enzyme activity, since no single nucleotides were cut from the 5’-end. Once the endonuclease activity cleaves, the products could be substrates suitable for exonuclease activity as well. Fig. 3B shows the DNA cleavage pattern of ScTat-D using substrates labeled at the 3’-end. It is clear that ScTat-D makes a strong excision at the 3’-end. The activity on the double-stranded substrate is about 5 times stronger than that on the single-stranded substrate. This result suggests that ScTat-D has 3’ to 5’ exonuclease activity.

To confirm that ScTat-D has both endo- and exonuclease activities, we performed nuclease activity assays based on a substrate with a biotin at the 3’-end. Since the biotin is able to interact with streptavidin, we used it to block the 3’ to 5’ exonuclease activity of ScTat-D in the presence of bound streptavidin. Our result (Fig. 4A) shows that with increasing amounts of streptavidin, the 3’ to 5’ exonuclease activity of ScTat-D was completely abolished, whereas its endonuclease activity was not affected. This result indicates that ScTat-D is indeed an endo-/exonuclease.

ScTat-D Makes Cleavages at Recessive, Overhanging, or Nicked DNA Substrates—To investigate the substrate specificity of ScTat-D, we designed additional substrates with a recessive end, a 3’ overhang end, or a nicked site. The single-stranded DNA was used as a control in this experiment. For each substrate, three concentrations of ScTat-D protein were applied. The results are shown in Fig. 4B. ScTat-D could efficiently cut the recessive or 3’ overhang end (fewer than three nucleotides). For the overhang substrate, however, if the overhang length is longer than three nucleotides, the exonuclease activity of ScTat-D is significantly decreased. ScTat-D is also able to cleave the nicked DNA substrate efficiently. The cleavage is comparable with those based on recessive and overhang substrates. Together with its endonuclease and exonuclease activities as revealed above, ScTat-D is clearly a multifunctional protein. The endonuclease and exonuclease activities might be required to further cleave DNA intermediates with nicks or different kinds of ends formed during DNA breakage or due to cleavage made by endonucleases, such as DFF40/CAD and endonuclease G.

Mutation of Glu185, Asp325, or Glu327 Affects ScTat-D Nuclease Activities—To determine whether the nuclease activities of Tat-D assayed above are intrinsic and to test if the 21 fully conserved amino acid residues have critical roles in the catalytic activities of Tat-D, we mutated three of the 21 conserved amino acid residues into alanine by site-directed mutagenesis. The mutant proteins were overexpressed in an E. coli system and purified to homogeneity as done for the wild type ScTat-D. We then assayed their endonuclease (Fig. 5A) and exonuclease (Fig. 5B) activities and calculated the percentage cleavage for the exonuclease activity (Fig. 5C). It is evident that the three purified
mutants E185A, D325A, and E327A have less enzymatic activity than the wild type. Among them, the enzyme activities of E327A are reduced most significantly, almost by 95% in comparison with those of the wild type ScTat-D. The other two mutants have a decrease of 50% in their enzyme activities. These results indicate that the three conserved amino acid residues are indeed critical for the enzyme activities of ScTat-D. Meanwhile, this also confirmed that the endo- and exonuclease activities are intrinsic to the Tat-D protein, not introduced by contamination of E. coli proteins during protein purification.

ScTat-D Nuclease Activity Is Metal-dependent and Acid-optimal—In order to determine the optimal conditions for the enzyme activities of ScTat-D, we performed metal-dependent assays and examined effects of various pH and temperature conditions on enzyme activities. Table III shows the result of metal-dependent assays. This protein requires metals for its enzyme activities, since there is no substrate cleavage observed without the addition of a metal ion. In addition, we determined that the optimal metal for the maximal enzyme activities of ScTat-D is the magnesium ion (Mg$^{2+}$) based on the comparison of seven metal ions, including Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, Co$^{2+}$, Cu$^{2+}$, Ni$^{2+}$, and Zn$^{2+}$ (See Table III). They were selected for this study by referring to the characterization of other nucleases, such as FEN-1. Although Mn$^{2+}$ and Ca$^{2+}$ are the second and third optimal metals for ScTat-D, respectively, they showed over 3-fold less activity compared with that with Mg$^{2+}$. The optimal concentration of Mg$^{2+}$ for ScTat-D is $\sim$0.3 mM. ScTat-D retains a notable enzyme activity in a wide range of Mg$^{2+}$ concentrations from 1 m to over 14 m. Contrary to Mg$^{2+}$, Zn$^{2+}$ is the worst among the seven tested metals for the enzyme activities of ScTat-D. It even has an inhibitory effect on other metal ions such as Mg$^{2+}$, Ca$^{2+}$, and Mn$^{2+}$ for the enzyme activity (data not shown).

The results of the assays using various pH and temperature conditions are shown in Table IV. Surprisingly, ScTat-D was determined to be an acid-optimal nuclease. The optimal pH for ScTat-D is approximately pH 5. When pH changed from 5 to 4.5, the enzyme activity of ScTat-D was reduced to $\sim$82%. When pH increased from 5 to 9.6, the enzyme activity was reduced to $\sim$10%. However, at pH 8, ScTat-D still had a notable enzyme activity, about 55% of that at pH 5. In addition to the assays on optimal pH conditions, we determined the optimal temperature for ScTat-D. Based on a total of eight temperature conditions from 22 to 55 °C, ScTat-D was revealed to have an optimal temperature around 30 °C (Table IV), which is consistent with the optimal growth temperature for S. cerevisiae cells. Furthermore, from Table IV, we may also notice that Tat-D retains a notable enzyme activity in a large range of temperature conditions from 22 to 42 °C, indicating that this protein tolerates temperature changes.

Knock-out of the ScTAT-D Gene in Yeast Has No Effect on Cell Growth or DNA Repair but Increases TUNEL-positive Signals and Improves Cell Survival to Hydrogen Superoxide—In order to determine biological functions of ScTat-D in yeast cells, we disrupted this gene in S. cerevisiae BY4741 and

![Image](http://example.com/image.png)
to play a role in apoptotic DNA degradation (19), we speculated that ScTat-D might also be involved in apoptosis in *S. cerevisiae*. To test our hypothesis, we treated the relevant yeast cells, including BY4741 (wild type) and its TAT-D null mutant, with hydrogen peroxide (H$_2$O$_2$), which was revealed to be a good inducer of yeast apoptosis at low concentrations (29). Since it was previously observed that the optimal concentration of H$_2$O$_2$ for the induction of yeast apoptosis is about 3 mM, we employed three concentrations (0.5, 1, and 3 mM) of this reagent for the treatment of relevant yeast cells. After culture for 2 h in the medium with hydrogen peroxide, cells were examined for their apoptotic DNA fragmentation status with TUNEL assays. The result is shown in Fig. 6, A and B. The ScTAT-D knock-out strain has more TUNEL-positive cells in comparison with the wild type. This result is similar to that based on a TAT-D or DNase II knock-out strain of *C. elegans* (19), indicating that ScTat-D might produce TUNEL-negative DNA ends as DNase II does. When cells were treated with 0.5 mM H$_2$O$_2$, the percentage of TUNEL-positive cells was increased from about 9% in wild type to about 20% in the null mutant. This is an over 1-fold increase. In addition, with higher concentrations of H$_2$O$_2$ (1 and 3 mM), the percentage of TUNEL-positive cells increases in both wild type and the null mutant but is still significantly higher in the mutant than in the wild type. Overall, this experiment demonstrates that mutation of ScTAT-D could affect DNA fragmentation status and the TUNEL signal of yeast cells, which is similar to the result based on a TAT-D knock-out *C. elegans* strain and is indicative of the involvement of ScTat-D in yeast apoptosis.

Since apoptosis finally leads to cell death, we additionally assayed cell survival of yeast cells after H$_2$O$_2$ treatment. Interestingly, our result shows that the ScTAT-D null mutant has a higher cell survival rate than does the wild type (Fig. 6C). The difference became larger when a higher concentration of H$_2$O$_2$ was used. However, when the concentration of H$_2$O$_2$ is too high (e.g., 12 mM), no cells in either the wild type or the null mutant can survive. The higher cellular survival rate in the null mutant might be due to incomplete degradation of the chromosomal DNA. Furthermore, we overexpressed ScTat-D in yeast cells. It appeared that the overexpression of ScTat-D makes yeast cells be more sensitive to H$_2$O$_2$ treatment (Fig. 6D). These results are consistent overall with the observation based on TUNEL assays, further suggesting that ScTat-D is involved in yeast apoptosis.

**DISCUSSION**

As demonstrated in multicellular organisms, nucleases are required for apoptotic DNA fragmentation and degradation. This is also true for unicellular organisms such as yeast. Our current study has revealed eight nuclease candidates that are potentially required for apoptotic DNA fragmentation and degradation. However, some other known nucleases previously proposed to be involved in mammalian apoptosis were not found in yeast cells, especially the nuclease DFF40/CAD (Table II). Because it is the only nuclease regulated by caspases to degrade genomic DNA in the nucleus, DFF40/CAD is a critical component of the caspase-dependent apoptotic pathway. Besides DFF40/CAD, other classical elements, such as Bcl-2 family members, Apaf-1, and caspases of the caspase-dependent pathway, are also missing in yeast cells (46, 47). It seems to be true that the whole caspase-dependent pathway is not present in yeast cells. This is evolutionarily conceivable, since the caspase-dependent pathway may have been evolved with the increase of organismic complexity. Alternatively, yeast cells might have a different (caspase-activity-dependent) pathway with use of proteins functionally similar but not homologous to caspases. This is supported by the finding of a metacaspase yCA1 in yeast cells.

**FIG. 5.** Nuclease activities of wild type (wt) and mutant ScTat-D proteins. A, endonuclease activity; B, exonuclease activity of Tat-D proteins. *, $^{32}$P labeling; lane M, DNA marker; nt, nucleotide. The shaded triangles represent the increasing concentrations of ScTat-D proteins from 0, 0.2, 0.4, and 0.6 to 0.8 μM. 0.2 μM labeled substrate was used for each reaction. C, cleavage quantification based on the exonuclease activity of Tat-D proteins as indicated in B.

RKY2672 strains. Disruption of this gene has neither an effect on cell growth nor sensitivity to UV and γ-ray irradiation. In addition, the mutant has a similar spontaneous mutation frequency based on the Can$^+$ forward mutation assay to the wild type (data not shown). It seems that either ScTat-D is not involved in cell growth or DNA repair, or there might be other redundant nucleases that can complement ScTat-D functions when ScTAT-D is disrupted.

Since the *C. elegans* homologue of ScTat-D was determined...
The eight putative apoptotic nucleases identified in yeast cells during worm development. The degradesome, including apoptosis-inducing factor (data not shown), have been revealed to have corresponding candidates in yeast cells (Table II). We therefore propose that yeast cells have a similar degradesome for the apoptotic DNA fragmentation and degradation. Because of the absence of the critical DFF40/CAD nuclease in yeast, the degradesome could play more important roles in yeast than in multicellular organisms.

Although the regulatory mechanism of how Tat-D is involved in apoptotic DNA degradation is still unknown, the current study provides good evidence for the involvement of this evolutionarily conserved nuclease in apoptotic DNA fragmentation and degradation in yeast. Biochemical properties of Tat-D make it a good candidate for its biological functions in apoptosis. Tat-D cleaves the double-stranded DNA in both endonucleolytic and exonucleolytic manners. This is in contrast to endonuclease G, which only possesses endonuclease activity and requires the recruitment of other endo-/exonucleases to form a degradesome complex in order to degrade DNA efficiently in the apoptotic cells (19, 44).

Like some of the apoptotic nucleases, Tat-D is metal-dependent. Its optimal metal is Mg$^{2+}$ (Table III). Recently, apoptotic nucleases have been classified into three categories based on metal dependence and the preference of pH conditions (18). One category is Ca$^{2+}$/Mg$^{2+}$-dependent endonucleases, represented by human CAD and DNase I; the second is Mg$^{2+}$-dependent DNases, represented by mouse CAD; and the third is acid endonucleases or cation-independent DNases, exemplified by DNase II. Clearly, Tat-D can be placed into the second category, since its optimal metal is Mg$^{2+}$ or the third category based on its optimal pH of ~5. However, unlike DNase II, which is activated by acidic conditions, Tat-D has enzyme activity from pH 4.5 to 9 (Table IV). Although its optimal pH is around 5, it retains about 60% enzyme activity at pH 8. This could be a suitable biochemical characteristic for Tat-D to be involved in apoptotic DNA degradation, since the cellular pH condition was reported to change from weak basic to acidic during the process of apoptosis.

The more direct evidence for the involvement of Tat-D in apoptosis comes from in vivo assays. In C. elegans, Parrish and Xue have shown that knockdown of the Tat-D homologue by small interfering RNA delayed DNA degradation of apoptotic cells during worm development. The tat-D mutant showed an

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**Table III**

| Metal   | Concentration | RA | ROA |
|---------|---------------|----|-----|
| MgCl$_2$ | 0.50          | 30 |     |
|         | 1.00          | 60 |     |
|         | 2.00          | 68 |     |
|         | 4.00          | 85 |     |
|         | 8.00          | 100| 100 |
|         | 12.0          | 90 |     |
|         | 16.0          | 80 |     |
| CaCl$_2$ | 0.13          | 40 |     |
|         | 0.25          | 70 |     |
|         | 0.50          | 100| 27  |
|         | 1.00          | 76 |     |
|         | 2.00          | 20 |     |
|         | 4.00          | 5  |     |
|         | 8.00          | 0  |     |
| MnCl$_2$ | 0.13          | 28 |     |
|         | 0.25          | 100| 23  |
|         | 0.50          | 50 |     |
|         | 1.00          | 6  |     |
|         | 2.00          | 0  |     |
|         | 4.00          | 0  |     |
|         | 8.00          | 0  |     |
| CoCl$_2$ | 0.13          | 70 |     |
|         | 0.25          | 80 |     |
|         | 0.50          | 100| 18  |
|         | 1.00          | 84 |     |
|         | 2.00          | 73 |     |
|         | 4.00          | 52 |     |
|         | 8.00          | 0  |     |
| CuCl$_2$ | 0.05          | 63 |     |
|         | 0.13          | 100| 5   |
|         | 0.25          | 87 |     |
|         | 0.50          | 72 |     |
|         | 1.00          | 53 |     |
|         | 2.00          | 30 |     |
|         | 4.00          | 13 |     |
| NiCl$_2$ | 0.05          | 43 |     |
|         | 0.13          | 100| 5   |
|         | 0.25          | 81 |     |
|         | 0.50          | 61 |     |
|         | 1.00          | 43 |     |
|         | 2.00          | 20 |     |
|         | 4.00          | 5  |     |

$a$ RA, relative enzyme activity.

$b$ ROA, relative optimal activities of Tat-D with an indicated metal in comparison with that with MgCl$_2$; ZnCl$_2$ is not listed in the table, since ScTat-D has no activity at all using ZnCl$_2$.

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**Table IV**

| pH effect | Temperature effect |
|-----------|--------------------|
| pH        | RA  | Temperature  | RA  |
| 4.5       | 82  | 22            | 89  |
| 5.0       | 100 | 27            | 95  |
| 5.3       | 91  | 30            | 100 |
| 6.5       | 76  | 33            | 97  |
| 7.2       | 65  | 37            | 87  |
| 8.0       | 55  | 42            | 55  |
| 8.8       | 30  | 47            | 20  |
| 9.6       | 10  | 55            | 0   |

$a$ RA, relative enzyme activity.

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Metal Concentration RA ROA

| pH | RA  | Temperature  | RA  |
|----|-----|--------------|-----|
| 4.5| 82  | 22           | 89  |
| 5.0| 100 | 27           | 95  |
| 5.3| 91  | 30           | 100 |
| 6.5| 76  | 33           | 97  |
| 7.2| 65  | 37           | 87  |
| 8.0| 55  | 42           | 55  |
| 8.8| 30  | 47           | 20  |
| 9.6| 10  | 55           | 0   |
increased number of TUNEL-positive cells. Similarly, in the current study, we observed that disruption of the ScTAT-D gene in yeast cells could increase the frequency of TUNEL-positive cells, induced by a low concentration of hydrogen peroxide. This consistency strongly suggests that Tat-D is involved in apoptosis for both C. elegans and yeast. Disruption of the ScTAT-D gene in yeast cells also increased survival, which may indicate that disruption of ScTAT-D results in less degradation of the genomic DNA in apoptotic cells, allowing the cells to have a better chance to repair their DNA and recover from apoptosis. Although it is easy to understand the relationship between less degradation and more repair and recovery of genomic DNA, it is intriguing how the reduced DNA degradation results in a signal to the cells to recover from their apoptotic status.

In summary, this is the first work involving the apoptotic nucleases in yeast cells. Although we identified eight potential nucleases for yeast apoptotic DNA degradation, we focused our attention on the characterization of ScTat-D. Our biochemical data shows that ScTat-D is an endo-exonuclease suitable for apoptotic DNA degradation; however, further analysis using genomic DNA with nucleosomes as substrate, in order to mimic apoptotic DNA degradation, in order to understand the mechanisms of apoptotic DNA fragmentation and degradation in yeast cells, it is necessary to knock out more apoptotic nuclease genes in yeast cells, especially the yeast homologue of endonuclease G, which is the typical apoptotic nuclease in mammalian cells. Epistatic analyses of different apoptotic nucleases in yeast may allow us to determine the pathways of DNA fragmentation and degradation in yeast cells and reveal in which pathway ScTat-D might be involved.

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