Articles

Purification and Characterization of the DNA Polymerase α Associated Exonuclease: The *RTH1* Gene Product

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Received November 22, 1996; Revised Manuscript Received March 19, 1997[⊗]

ABSTRACT: We report here the purification and mechanistic characterization of a 5′−3′ exonuclease associated with DNA polymerase α from the yeast *Saccharomyces cerevisiae*. Earlier, we identified a 5′ → 3′ exonuclease activity that copurified with yeast DNA polymerase α−primase in a multiprotein complex [Biswas, E. E., et al. (1993) *Biochemistry, 32*, 3020−3027]. Peptide sequence analysis of the purified 47 kDa exonuclease was carried out, and the peptide sequence was found to be identical to the *S. cerevisiae* gene YKL510 encoded polypeptide, which is also known as yeast *RAD2* homolog 1 or *RTH1* nuclease. The native exonuclease also had strong flap endonuclease activity similar to that observed with *RTH1* nuclease and homologous yeast (*RAD2*) and mammalian enzymes. During our studies, we have discovered certain unique features of the mechanism of action of the native *RTH1* nuclease. Studies presented here indicated that the exonuclease had specific pause sites during its 5′−3′ exonuclease nucleotide excision. These pause sites were easily detected with long (∼50 bp) oligonucleotide substrates during exonucleolytic excision by the formation of a discontinuous ladder of excision product. We have further analyzed the mechanism of generation of the pause sites, as they could occur through a number of different pathways. Alignment of the pause sites with the nucleotide sequence of the oligonucleotide substrate indicated that the pause sites were dependent on the nucleotide sequence. Our analysis revealed that *RTH1* nuclease pauses predominantly at G:C rich sequences. With poly(dA):oligo$(dT)_{50}$ as substrate, the exonucleolytic products formed a continuous ladder with no evidence of pausing. The G:C rich DNA sequences are thermodynamically more stable than the A:T rich sequences, which may be in part responsible for pausing of the *RTH1* 5′ → 3′ exonuclease at these sites.

Replication of chromosomal DNA requires a number of proteins and enzymes with a variety of enzymatic activities (Kornberg & Baker, 1992). In the lagging strand of the replication fork, an exonuclease is required for removal of the primer once the synthesis of Okazaki fragments is complete. In addition, an exonuclease may also be involved in maintaining the fidelity of DNA replication. The presence of a 5′ → 3′ exonuclease that interacts with DNA polymerases have been demonstrated by various laboratories (Ishimi et al., 1988; Goulian et al., 1990; Siegal et al., 1992). Earlier, we have shown that a 5′ → 3′ exonuclease copurifies with DNA polymerase α−primase (pol α)[1] complex (Biswas et al. 1993). Recent genetic studies, especially in mammalian systems, indicate that a structure-specific endonuclease, with a 5′ → 3′ exonuclease activity, known as flap endonuclease (FEN) is involved in various nuclear processes including DNA replication and DNA repair (Harrington & Lieber, 1994; Prakash et al., 1993; Habraken et al., 1993, 1994; Murray et al., 1994; Sommers et al., 1995). The yeast enzyme is a homolog of the *RAD2* gene and was called *RAD2* homolog 1 or *RTH1* nuclease (Sommers et al., 1995; Johnson et al., 1995). *In vitro* studies in mammalian systems indicated that the *RTH1* nuclease is involved in maturation of the Okazaki fragment and plays a role similar to that observed with the 5′ → 3′ exonuclease component of the DNA polymerase I of *Escherichia coli* (Ishimi et al., 1988; Turchi et al., 1994; Waga et al., 1994).

It has been shown that tumors from hereditary nonpolyopsis colorectal carcinoma (HNPCC) and a large number of other sporadic cancer cell lines are associated with instability of microsatellite DNA. Some of these tumors and instability of microsatellite DNA are associated with a defect in mismatch repair pathway (Boyer et al., 1995: Fishel et al., 1993; Leach et al., 1993; Peinado et al., 1993; Thibodeau et al., 1993; Liu et al., 1995; Kim et al., 1994; Lothe et al., 1994); however, many of these tumors may be associated with defects in other repair genes including *RTH1* nuclease. Possible evidence for such involvement has been provided by Prakash and co-workers (Johnson et al., 1995). They have shown through a series of interesting *in vivo* genetic studies that, in the yeast *Saccharomyces cerevisiae*, *RTH1* nuclease is involved in the growth and maintenance of certain repetitive DNA elements and stability of microsatellite DNA. In addition, they have also demonstrated that the *RTH1* gene

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[⊗] Abstract published in *Advance ACS Abstracts,* May 1, 1997.

[1] ABBREVIATIONS: ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl flouride; pol α, DNA polymerase α; RPA, replication protein A; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; TLCK, *p*-tosyllysyl chloromethylketone; TPCK, *p*-tosylphenylalanine chloromethylketone; Tris, tris(hydroxymethyl)aminomethane.

S0006-2960(96)02889-9 CCC: $14.00

is dispensable at a permissive temperature; however, at a restrictive temperature of 37 °C, the *RTH1* gene deletion mutants of *S. cerevisiae* fail to grow. Their studies clearly established the importance of *RTH1* nuclease in cell growth and also pointed to the possibility that an alternate 5′ → 3′ exonuclease may replace *RTH1* nuclease in cell growth, which is distinct from the *RAD2* protein. Tishkoff et al. (1997) have shown that mutations in *RTH1* gene result in a strong mutator phenotype and the mutations are lethal in combination with mutations in *RAD51* and *RAD52*. However, it does not play a role in *MSH2*-dependent mismatch repair pathway. Recent evidence indicates that the *RTH1* nuclease plays a central role in DNA replication presumably by maintaining the fidelity of DNA replication and integrity of the genome. In eukaryotic DNA replication, it appears to function in a manner analogous to *E. coli* DNA polymerase I in removing the RNA−DNA hybrid primers from Okazaki fragments (Depamphilis, 1993; Goulian et al. 1990; Ishimi et al., 1988; Taljanidisz et al., 1987; Turchi et al., 1994; Vishwanath et al., 1986; Waga et al., 1994).

We have described here the purification of the DNA polymerase α associated 5′ → 3′ exonuclease protein from *S. cerevisiae* to homogeneity and established its identity as that of the YKL510 gene product *RTH1* nuclease. We have analyzed the mechanism of action of the native *RTH1* 5′ → 3′ exonuclease and described its mechanistic features, and in the following paper (Biswas et al., 1997) we have discussed the stimulation of *RTH1* nuclease by yeast replication protein A.

MATERIALS AND METHODS

Yeast. Protease deficient yeast, *S. cerevisiae*, BJ 2168 strain from the Yeast Genetic Stock Center (Berkeley, CA), was grown in a laboratory fermenter to midlog phase, and the cells were chilled to 4 °C then harvested by centrifugation. The cells were then resuspended in 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA and 10% glycerol to OD 400 (λ = 600 nm) and stored frozen at −80 °C until further use.

Nucleic Acids, Enzymes, and Other Reagents. Oligonucleotides were synthesized commercially and obtained from either Integrated DNA Technologies (Coralville, IA) or Oligos Inc. (Portland, OR). When needed, the oligonucleotides were further purified by gel filtration. Ultrapure deoxy- and ribonucleotides were obtained from Pharmacia (Piscataway, NJ) and were used without further purification. [α-^{32}P]ATP, [α-^{32}P]dATP, and [γ-^{32}P]ATP were obtained from DuPont/NEN (Boston, MA). RPA used in this study was purified as described (Biswas et al. 1993a). Terminal deoxynucleotidyl transferase was purchased from United States Biochemical Corp, (Cleveland, OH). T4 polynucleotide kinase was obtained from New England Biolabs (Beverly, MA). Yeast polymerase α−primase complex used in this study was purified as described (Plevani et al., 1985). The anti-pol α monoclonal antibody (MAb)-agarose column, used in the pol α−primase purification, was received as a gift from Dr. Lucy M. S. Chang of Uniformed Services Health Sciences University. All chemicals used to prepare buffers and solutions were reagent grade and were purchased from Fisher Chemical Company (Pittsburgh, PA). Protease inhibitors were from Bachem (Los Angeles, CA). Polyethyleneimine−cellulose strips for thin layer chromatography were from Fisher Chemical Co.

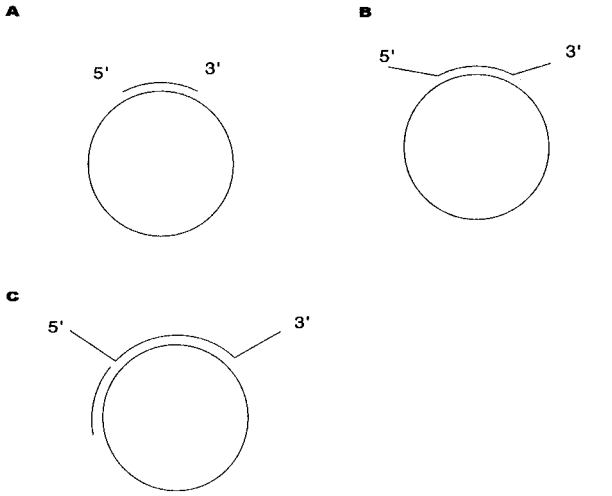

FIGURE 1: Schematic representation of nuclease substrates utilized in this study. Details on the preparation of these substrates are given in Materials and Methods. (A) M13 recessed substrate, (B) M13 pseudo Y substrate, and (C) M13 flap substrate.

Oligonucleotides. The oligonucleotides were analyzed for purity by polyacrylamide-7 M urea gel electrophoresis. The final purity of the oligonucleotides were >99% as judged by autoradiography of the phosphorylated products. The sequences of the oligonucleotides used in this study were (**1**) 5′-TCACGACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCGGTACCCGGGG-3′; (**2**) 5′-GGGTCTCACGACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCGGTACCCGGGGTAGGA-3′; and (**3**) 5′-TTAAGTTGGGTAACGCCAGGGTTTTCCCAG-3′. Oligonucleotides **1** and **2** were used to prepare the recessed and forked substrates, respectively, as described below. Oligonucleotides **2** and **3** were used to prepare the M13 FLAP substrate. A schematic diagram of the substrates is given in Figure 1 and details of the substrate preparation are given below.

Buffers. Buffer A contained 25 mM Tris-HCl (pH 7.5), 10%(v/v) glycerol, 0.01% (v/v) NP40, 1mM EDTA, and NaCl as indicated. Buffer B contained 10% glycerol, 0.01% (v/v) NP40, and potassium phosphate (pH 7.5) as indicated. Buffer C was analogous to Buffer A, except that it contained 25 mM Hepes instead of Tris-HCl. Buffer D contained 50 mM Tris-HCl, 1 mM EDTA, and 10% glycerol. Buffer E contained 25 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 0.1 mg/mL BSA, and 5 mM DTT. Buffer F contained 95% formamide, 10 mM EDTA, 1 mg/mL bromophenol blue, and 1 mg/mL xylene cyanol.

Purification of the 5′ → 3′ Exonuclease. All purification steps were carried out at 4 °C using a BIOCAD automated chromatography system. Unless otherwise indicated, all buffers used in the various chromatographic steps contained 5 mM DTT and the following protease inhibitors: 1 μg/mL each of leupeptin, pepstatin A, antipain, chymostatin; 0.1 mM each of benzamidine hydrochloride, $NaHSO_3$; and 2.5 μg/mL each of TPCK and TLCK, unless indicated otherwise. A flow chart of the purification is shown below (Scheme 1).

Yeast extract (Fr I) was prepared from 400 g of log phase protease-deficient yeast (strain BJ2168, Yeast Genetic Stock Center, Berkeley, CA) as previously described (Biswas et al., 1993a). The initial purification of DNA polymerase α−primase complex, with associated 5′ → 3′ exonuclease, DNA-dependant ATPase and helicase activities, was carried

Scheme 1

Yeast extract (Fraction I)
⇓
Phosphocellulose chromatography (Fraction II)
⇓
SS-DNA-cellulose Chromatography (Fraction III)
⇓
POROS-Q HPLC (Fraction IV)
⇓
POROS-S HPLC (Fraction V)
⇓
POROS Phenyl (HP) HPLC (Fraction VI)

out as described earlier (Biswas et al., 1993a). The isolation of the 5′ → 3′ exonuclease was then carried out as follows. The conductivity of the pol α fraction (Fr II) was adjusted to that of buffer A-100 by dialysis against buffer A-0 and loaded onto a 10 mL ssDNA−cellulose column equilibrated with buffer A-100. The column was washed extensively with A-100, and the nuclease was eluted with a 120 mL gradient of buffers A-100 to A-1000. Preliminary studies indicated that the separation of the 5′ → 3′ exonuclease from the polymerase activity can be achieved using ssDNA cellulose chromatography. Fractions were assayed for polymerase, ATPase, and nuclease activities. The active 5′ → 3′ exonuclease fractions were pooled (Fr III) and dialyzed against buffer A-0 until it reached the conductivity of buffer A-100. The dialyzate was then loaded on to a 2 mL (0.46 × 10 cm) of POROS-HQ (Perseptive Biosystems Inc., Cambridge, MA) column equilibrated with buffer A-100. The column was washed with 20 mL of buffer A-100 and eluted with a 40 mL gradient of buffer A-100 to A-500. The 5′ → 3′ exonuclease did not bind to the HQ column at this salt concentration and was found in the flow-through (Fr 4). Fraction IV was then loaded on a 2 mL (0.46 × 10 cm) of POROS-HS column (PerSeptive Biosystems, Cambridge, MA) equilibrated with buffer C-100. The 5′ → 3′ exonuclease was eluted with a 40 mL gradient of buffers C-100 to C-500. The peak exonuclease fractions were pooled (Fr V), dialyzed against buffer D for 6 h, and then loaded onto a 2 mL (0.46 × 10 cm) of phenyl−sepharose column. The column was eluted with a 40 mL gradient of buffer D + 1.6 M $(NH_4)_2SO_4$ to buffer D + 100 mM NaCl. The fractions were analyzed for nuclease activities and by SDS−PAGE. Approximately 90 μg (Fr VI) of the 5′→ 3′ exonuclease protein was obtained from 400 g of yeast cells.

Peptide Sequencing. The *RTH1* polypeptide (Fr VI) was resolved on a 5 → 18% gradient SDS−PAGE then transferred to polyvinylidene difluoride (PVDF) membrane. The *RTH1* nuclease was visualized with 1% ponceau S, and the stained band was excised. The immobilized polypeptide was subjected to *in situ* digestion and peptide sequencing as described earlier (Biswas et al., 1995). Six of the potentially important peaks from microbore HPLC fractionation were collected and analyzed for identifying the mass of the peptides by MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight mass spectrometry). In order to identify the exonuclease, mass data of all six peptides were submitted to the Mass Spectrometry Facility at the Univerity of California at San Francisco and searched against the NCBI nonredundant protein database.

Preparation of Exonuclease Substrates: (i) Recessed Substrate for Exonuclease Activity. Oligonucleotide **1**, which is a 50-mer complementary to the 50 bp sequence between nucleotides 6268 and 6317 of M13mp19 ssDNA was labeled at its 3′ end using terminal deoxynucleotidyl transferase. The oligo was hybridized to M13mp19 (Figure 1A) as previously described (Biswas et al. 1993b). Excess unhybridized labeled oligonucleotide was removed by spin column (Promega, Madison, WI) purification. The purified substrate was diluted to 17 fmol/μL (10000−20000 cpm/μL) with 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. *(ii) Forked Substrate for Endonuclease and Exonuclease Activities.* This substrate was prepared exactly as described for the recessed substrate except that a 60-mer oligonucleotide, oligo **2**, with sequence complementary to the sequence between nucleotides 6268 and 6317 of M13mp19 was used (Figure 1B). The resulting substrate thus had five nucleotide forks at both 5′ and 3′ ends. The 5′ fork was a substrate for the structure-specific endonuclease activity, and once the fork is removed it then became a substrate for 5′ → 3′ exonuclease activity. *(iii) M13 Flap Substrate for Endonuclease Activity*. This substrate is similar to that described by Harrington and Lieber (1994) in that it possesses a 5′ flap which acts as a substrate for the structure specific endonuclease activity of *RTH1*; however, the duplex region of the substrate is larger (50 bp as opposed to 14 bp) (Figure 1C). Oligonucleotides **2** and **3** were used to prepare this substrate following the method previously described (Harrington and Lieber, 1994).

Nuclease Assays: (A) Exonuclease Assay. Reactions were set up on ice, and a standard 10 μL reaction mixture in buffer E contained 9 mM $MgCl_2$, 4 mM ATP, 100 pmol substrate, and *RTH1* nuclease as indicated. The reactions were allowed to proceed for 30 min and terminated by the addition of an equal volume of buffer F, followed by boiling and chilling in an ice water bath. Half of the reaction products were analyzed by electrophoresis on a 15% polyacrylamide, 7 M urea gel in 89 mM Tris-borate (pH 8.3) containing 1 mM EDTA (1 × TBE buffer). Following electrophoresis, the gel was dried and exposed to Fuji RX film for 12 h at −80 °C.

(B) Endonuclease Assay. The reactions were set up on ice, and the standard 10 μL reaction mixture in buffer E contained 9 mM $MgCl_2$, 100 pmol of substrate DNA, and native *RTH1* nuclease, as indicated. The reactions were incubated for 5 min at 37 °C and were terminated by the addition of and equal volume of buffer F, boiling for 3 min, followed immediately by chilling in an ice water bath. The reaction products were analyzed by polyacrylamide gel electrophoresis as described for "exonuclease assay" above.

Other Methods. Protein concentrations were estimated according to the method of Bradford (l976), using bovine serum albumin as a standard. The ATPase assays were carried out as previously described (Biswas et al. 1993b).

RESULTS

Purification of the 5′ → 3′ Exonuclease. Purification was carried out from 400 g of frozen yeast cells. Fraction VI was analyzed by SDS−PAGE, and the protein appeared to be purified to homogeneity. Approximately 90 μg (Fr VI) of the 5′ → 3′ exonuclease protein was obtained from 400 g of yeast cells.

Characterization of the 5′ → 3′ Exonuclease Associated with the DNA Polymerase α Complex. Previously we have purified a complex of DNA polymerase α with an associated 5′ → 3′ exonuclease activity (Biswas et al., 1993a). In order to characterize this nuclease activity, we have purified the

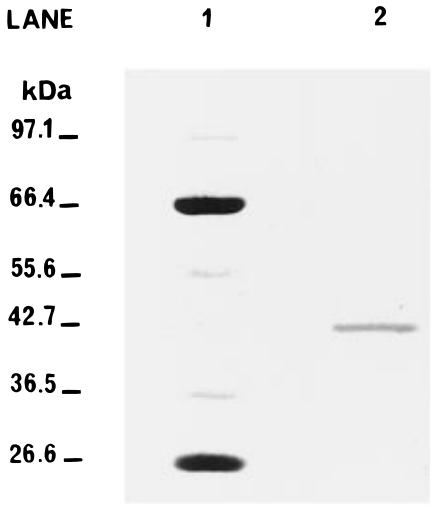

FIGURE 2: SDS−PAGE analysis of native *RTH1* nuclease. Samples of native nuclease (Fr VI), which were utilized in these studies were analyzed by electrophoresis on a 5 → 18% polyacrylamide gel followed by silver staining. Details of the purification are given in Materials and Methods. Lane 1, molecular weight standards (New England Biolabs); lane 2, 1 μg of native *RTH1* nuclease (Fr VI).

protein to homogeneity using a multistep purification procedure, as described above. The purified 5′ → 3′ exonuclease is a 47 kDa protein, which is similar in size to several recently described exonucleases from various systems. SDS−PAGE analysis of the purified exonuclease is shown in Figure 2 and clearly demonstrates its homogeneity. The molecular mass of 47 kDa was determined by a least square analysis of the molecular mass markers. The polypeptide size and its nuclease activity suggested that the purified exonuclease was similar to the mammalian flap endonuclease (Ishimi et al., 1988; Goulian et al., 1990; Siegal et al., 1992). Analysis of the exo- and endonuclease activities across the fractions for the final step of purification (phenyl sepharose) is shown in Figures 3 and 4. These data clearly show the coexistence of these two activities with the 47 kDa peptide. In order to determine the true identity of the purified exonuclease, we have analyzed the peptide sequence of the 47 kDa polypeptide. Following immobilization on PVDF membrane, the polypeptide was subjected to *in situ* digestion with trypsin. The mass of the tryptic fragments were determined using a MALDI-TOF apparatus. The peptides were separated by microbore reversed phase chromatography, and the individual peptide peaks were collected. One of the purified peptides was sequenced by automated Edman degradation using an Applied Biosystem gas phase peptide sequencer. The sequence of the peptide was as follows:

NH_2-M L F L D P E V I D G N E I N-COOH

A GENBANK search using the peptide sequence indicated that this protein is identical to the hypothetical gene product of the open reading frame (ORF) YKL510 of the yeast *S. cerevisiae* (Harrington & Lieber, 1994; Murray et al., 1994). Harrington and Lieber (1994) and Prakash and co-workers (Johnson et al., 1995; Sommers et al., 1995) identified the YKL510 ORF coding for a polypeptide of 382 amino acids, as the yeast homolog of the mammalian flap endonuclease (FEN). In addition, the NCBI database was also searched with mass of six peptides from MALDI-TOF analysis. All six peptide masses matched completely with the same

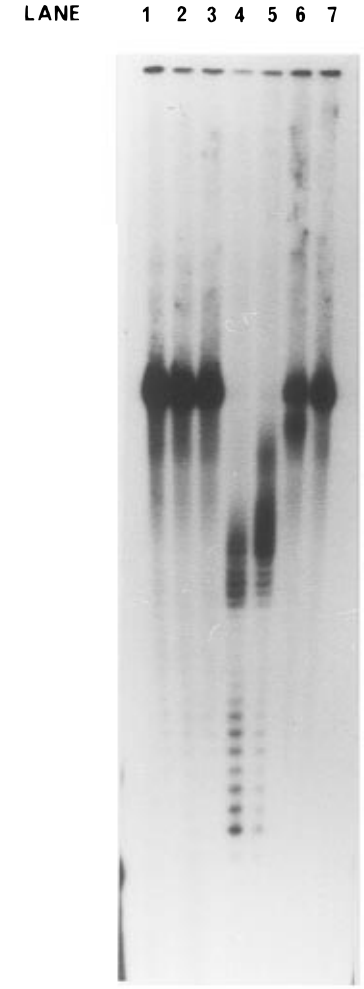

FIGURE 3: Analysis of the exonuclease activity following phenyl sepharose fractionation. A standard exonuclease assay, utilizing the 3′ labeled forked substrate was carried out across the fractions. In each case, 1 μL of sample was tested. Details of the fractionation are given in Results. Lane 1, Fr 19; lane 2, Fr 21; lane 3, Fr 23; lane 4, Fr 25; lane 5, Fr 27; lane 6, Fr 29; lane 7, Fr 31.

polypeptide (Table 1). These six peptides encompassed 20% (78 out of a total of 382 aminoacid residues) of the deduced protein sequence. The yeast gene was later called by Sommers et al. (1995) as the yeast *RAD* 2 homolog 1 or *RTH1*. Therefore, it appears that the purified 5′ → 3′ exonuclease from the DNA polymerase α multiprotein complex is identical to the YKL510 ORF gene product or *RTH1* nuclease.

Mechanism of the Exonuclease Activity of Native RTH1. The exonuclease activity of *RTH1* nuclease was examined using the forked and recessed substrates as described earlier. The forked substrate requires a endonucleolytic cleavage and removal of the 5′ fork in order to be utilized as a substrate for the exonuclease, whereas the recessed substrate can be utilized directly as an exonuclease substrate and does not undergo endonucleolytic cleavage. Kinetic analyses of the native *RTH1* nuclease with forked and recessed substrates in the presence of $MgCl_2$ and $MnCl_2$ are shown in Figure 5. The rate of exonucleolytic digestion of both substrates was not significantly different. Presumably, the endonuclease activity of *RTH1* nuclease is kinetically much faster than the exonuclease activity and thus, is not a rate-limiting step. As a result, the kinetics of exonucleolytic degradation of both substrates appear identical. It has been shown that the recombinant *RTH1* endonuclease activity is stimulated in the

FIGURE 4: Analysis of the endonuclease activity following phenyl sepharose fractionation. A standard endonuclease assay, utilizing the 3′ labeled forked substrate was carried out across the fractions. In each case, 1 μL of sample was tested. Details of the fractionation are given in Results. Lane 1, Fr 19; lane 2, Fr 21; lane 3, Fr 23; lane 4, Fr 25; lane 5, Fr 27; lane 6, Fr 29; lane 7, Fr 31.

presence of 1 mM $MnCl_2$ (Harrington & Lieber, 1994). In the case of native exonuclease activity, there was at least 3-fold stimulation with either substrate in the presence of Mn^{2+}. Although the native *RTH1* nuclease could utilize Mg^{2+} as a metal cofactor, it appeared to prefer Mn^{2+}.

One of the most striking features of the products produced by digestion of either substrate in the presence of either $MgCl_2$ or $MnCl_2$, was the formation of specific product size groups as shown in Figure 5. With a recessed substrate, the products were separated into size groups that centered around (1) 18 bp, (2) 24 bp, (3) 32 bp, (4) 40 bp. Products which were longer than 40 bp were not very well resolved and difficult to estimate. With the forked substrate, a similar distribution was observed, although the individual product groups were smaller by approximately 5 nucleotides. This was most likely due to the absence of the five nucleotide 3′ forks in the recessed substrate. With either of these substrates, the products of exonuclease activity were of specific size groups and were definitely not continuous. The distribution of products remained unaltered with different metal cofactors. The observed product groups could be formed due to pausing of the exonuclease on the template, low processivity, or both. However, the oligonucleotides in both substrates were labeled at the 3′-terminus with terminal deoxynucleotidyl transferase (TdT) and [α-^{32}P]-dATP. This enzyme is capable of incorporating a number of nucleotides, and the observed product groups could be due to multiple dATP incorporation and thus simply an artifact of labeling. In order to rule out this possibility, we labeled the substrates with TdT and [α-^{32}P]cordycepin triphosphate (ddATP), which can be incorporated at the 3′-terminus only once due to the lack of a 3′-OH group in cordycepin triphosphate. We also labeled the forked substrate with TdT using [α-^{32}P]dATP under conditions that result in the incorporation of only one dATP per 3′-terminus. Kinetic studies of *RTH1* nuclease and these substrates are shown in Figure 6. With either of the two substrates, the formation of the exonuclease product groups were comparable.

An alignment of the products with their sequence (generated by Maxam−Gilbert method) suggested that these pause sites may be formed in a sequence-dependent manner. A predominantly G:C rich sequence appeared to correlate best with the pause sites. The G:C base pair is thermodynamically more stable than an A:T base pair. In addition, G:C rich sequences can also induce bending in DNA (Brukner et al., 1993), both of which could force the exonuclease to pause at these sites or dissociate and reassociate. In order to further explore this possibility, we have examined the kinetics of exonuclease activity on a poly(dA):oligo(dT)$_{50}$ template. The result of this experiment is shown in Figure 7. The data clearly demonstrated that the products of this exonuclease digestion form a continuous ladder, and formation of any discreet size groups of digestion product or pause sites were not observed. These results suggest that the *RTH1* nuclease acted as a highly processive exonuclease on the poly(dA):oligo(dT)$_{50}$ template. Even at the earliest time point of 5 min, products <15 bp were observed, indicating a rapid and processive exonuclease action. In general, we have not observed products smaller than 10 bp in most of our studies. This was likely due to the fact that the exonuclease reactions were carried out at 37 °C and short duplex structures were unstable at this temperature and spontaneously melted. Consequently, we always observed that the final products of the exonuclease reaction were 10−15 nucleotides depending on the template.

Ribonuclease H Activity Associated With RTH1 Exonuclease. The 5′ → 3′ exonuclease of *RTH1* nuclease is quite similar in structure and function to the 5′ → 3′ exonuclease of DNA polymerase I of *E. coli*. In chromosomal DNA replication of *E. coli*, DNA polymerase I removes the RNA primers from Okazaki fragments (Kornberg & Baker, 1992). We have examined the RNase H activity of *RTH1* nuclease on a RNA-primed DNA template. The primed DNA template was prepared by priming poly(dT) DNA templates with yeast DNA pol α−primase and an autoradiogram of the primer synthesis reaction is shown in Figure 8A. Pol α−primase synthesized multimeric primers on poly(dT) template ranging in size from 20 to 60 nucleotides. The RNA-primed poly(dT) template was incubated with *RTH1* nuclease between 0 and 30 min. *RTH1* nuclease effectively digested the RNA primers to less than 20 nucleotides long. The reaction was essentially complete in 15 min (Figure 8B). These results indicated that *RTH1* nuclease can function as a RNase H.

DISCUSSION

We reported earlier the presence of a 5′ → 3′ exonuclease activity that copurified with DNA polymerase α−primase complex from the yeast *S. cerevisiae* (Biswas et al., 1993a). In order to decipher the role(s) of the exonuclease in the chromosomal DNA replication, it was essential to develop a method of purification of this exonuclease activity. Through a multistep chromatographic procedure, we developed a purification scheme that provided us with approximately 90 μg of highly purified nuclease starting from 400 g of yeast. Peptide sequence analysis of the 47 kDa polypeptide was carried out, and the data indicated the protein's identity with that of the YKL510 gene product (Harrington & Lieber, 1994; Sommers et al., 1995). This gene product has already been identified as the yeast homolog of the mammalian nuclease that is involved in the maturation

Table 1: Corrlation of the Peptide Masses as Determined by MALDI-TOF Analysis to Calculated Mass Data of *RTH1* Nuclease as Determined by the UCSF Mass Spectrometry Facility[a]

peptide	mass submitted	mass matched	ΔDa[b]	start[c]	end[c]	peptide sequence	modification
1	1366.0000	1364.6066	1.3934	112	123	(K)LAEATTELEKMK(Q)	None
2	1961.5000	1961.2926	0.2074	284	300	(R)MLFLDPEVIDGNEINLK(W)	None
3	1977.3000	1977.2919	0.0082	284	300	(R)MLFLDPEVIDGNEINLK(W)	1-Met-ox
4	2670.7000	2672.9219	−2.2220	48	71	(R)QQDGGQLTNEAGETTSHLMGMFYR(T)	none
5	2686.8000	2688.9212	−2.1212	48	71	(R)QQDGGQLTNEAGETTSHLMGMFYR(T)	1-Met-ox
5	2686.8000	2688.2325	−1.4324	142	166	(K)LLGLMGIPYIIAPTEAEAQCAELAK(K)	none
6	2703.5000	2704.9206	−1.4206	48	71	(R)QQDGGQLTNEAGETTSHLMGMFYR(T)	2-Met-ox
6	2703.5000	2704.2318	−0.7318	142	166	(K)LLGLMGIPYIIAPTEAEAQCAELAK(K)	1-Met-ox

[a] All six peptides matched 100% with the peptides from a 43279.4 Da polypeptide coded by ORF YKL113c or *RTH1* nuclease. Met-ox refers to oxidation of methionine(s) in the peptide sequence. The matched peptides accounted for 20% (78/382 amino acid residues) of thr *RTH1* nuclease. [b] ΔDa = (mass submitted − mass matched). [c] Start and end refers to the positions of the first and last positions in the polypeptide chain.

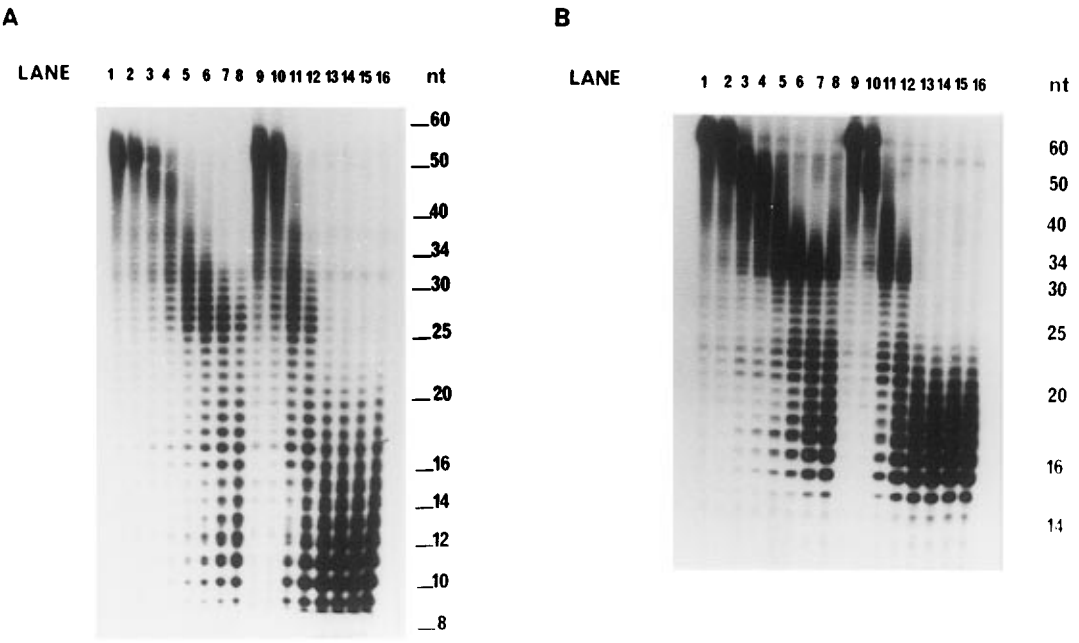

FIGURE 5: Kinetic analysis of the exonuclease activity of *RTH1* nuclease on pseudo Y and recessed substrates. A standard exonuclease assay was carried out with 2 ng of *RTH1* (Fr 4) using either a recessed (panel A) or a forked (panel B) substrate. In both panels A and B, lanes 1−8 were reactions containing 9 mM $MgCl_2$ and lanes 9−16 correspond to reactions containing 9 mM $MnCl_2$. Lanes 1 and 9, blank; lanes 2 and 10, 0 min; lanes 3 and 11, 5 min; lanes 4 and 12, 10 min; lanes 5 and 13, 20 min; lanes 6 and 14, 30 min; lanes 7 and 15, 45 min; lanes 8 and 16, 60 min.

of the Okazaki fragments during lagging strand DNA replication (Ishimi et al., 1988; Goulian et al., 1990; Turchi et al., 1994; Waga et al., 1994). Recent studies by Prakash and co-workers (Johnson et al., 1995; Sommers et al., 1995) demonstrated that this exonuclease is important for cellular DNA replication, stability of certain repetitive DNA elements, and some mutagen induced DNA repair processes. Because of the structural as well as functional similarities between the this exonuclease and the *RAD2* gene and its gene product, it was named *RTH1* nuclease.

We have explored the mechanism of action of the purified native yeast *RTH1* nuclease. Nearly all previous studies, particularly those involving yeast *RTH1* nuclease, were carried out with recombinant enzyme expressed in *E. coli*. It is quite possible that the folding of the protein and/or the post-translational processing of the native enzyme in yeast are different from that in *E. coli*. As a result, investigation of the mechanistic details of the native protein, that has been purified to homogeneity, would likely be worthwhile.

We have used a substrate for the exonuclease with a long duplex region (50 bp) unlike the more commonly used exonuclease substrates with shorter duplex regions (e.g., <20 bp). Due to the length of our substrates, we were able to detect that the *RTH1* exonuclease produced a ladder of products. However, the product ladder was discontinuous and we observed the presence of distinct pause sites. Pause sites have been described for other replication enzymes such as T4 DNA polymerase (Charette et al., 1987) and RNA polymerase (Kornberg & Baker, 1992). Enzyme pausing is likely related to the mechanism of action of the enzyme. Our investigation of *RTH1* nuclease revealed that most likely the enzyme can excise an A or T much faster than a G or C. One possible explanation for this phenomenon is the fact that hydrogen bonds in a G:C pair are far stronger than those in an A:T pair; the exonuclease action requires cleavage of the phosphodiester bond and melting of the hydrogen bonds. However, differential recognition or binding of various bases by the exonuclease or dissociation of the exonuclease from the template at a certain sequences could not be ruled out at the present time. The results of the experiments with poly (dA):oligo $(dT)_{50}$ in Figure 7 clearly indicated a sequence dependence for the *RTH1* nuclease.

In lagging strand DNA replication, the initial products of DNA synthesis are the Okazaki fragments (Kornberg & Baker, 1992; Vishwanath et al., 1986; Taljanidisz et al., 1986). Completion of the lagging strand DNA replication

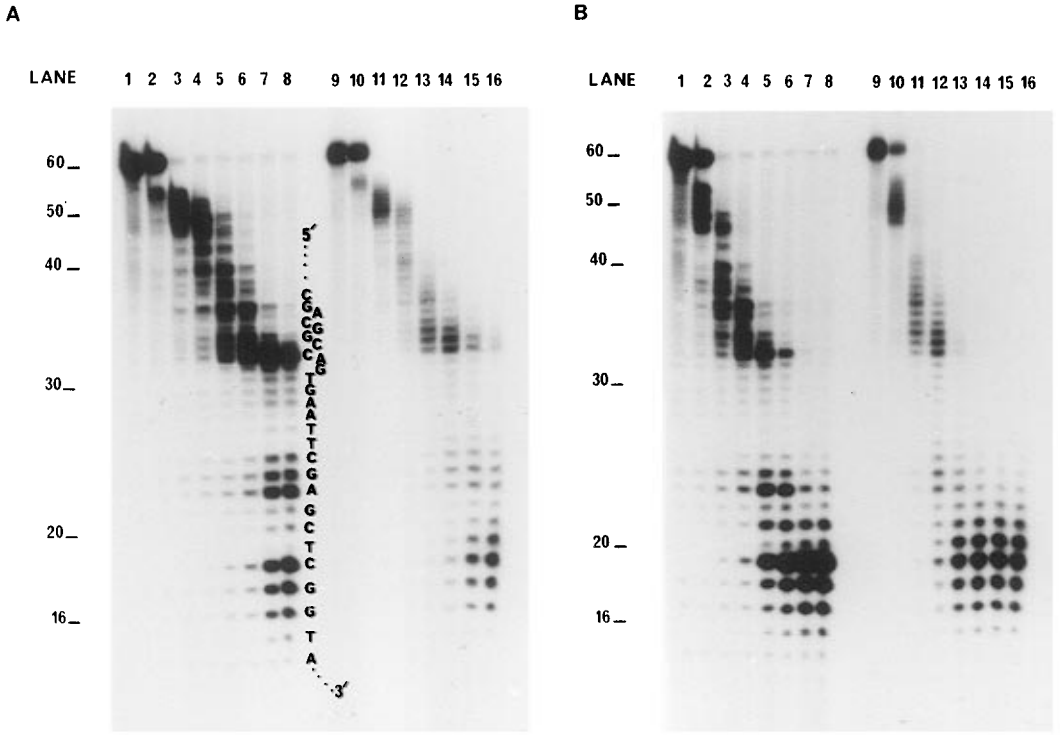

FIGURE 6: Kinetic analysis of the exonuclease activity of *RTH1* on forked substrates labeled with cordycepin 5′-triphosphate (ddATP) or deoxyadenosine 5′-triphosphate (dATP). A standard exonuclease assay was carried out with 2 ng of *RTH1* (Fr VI) using a forked substrate, which was labeled at the 3′ end with either ddATP (lanes 1−8) or dATP (lanes 9−16). Reaction mixtures in panel A contained 9 mM $MgCl_2$ and those in panel B contained 9 mM $MnCl_2$. Lanes 1 and 9, blank; lanes 2 and 10, 0 min; lanes 3 and 11, 5 min; lanes 4 and 12, 10 min; lanes 5 and 13, 20 min; lanes 6 and 14, 30 min; lanes 7 and 15, 45 min; lanes 8 and 16, 60 min.

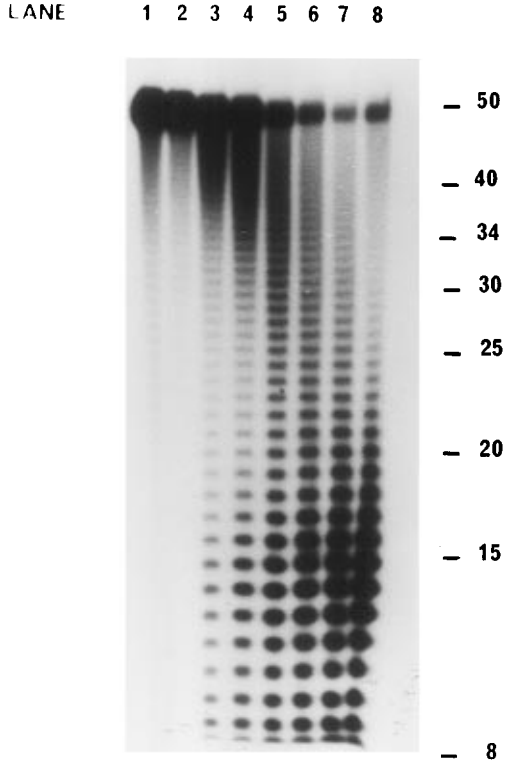

FIGURE 7: Kinetic analysis of the exonuclease activity of *RTH1* nuclease on poly(dA):oligo(dT)50 template. A standard exonuclease assay was carried out with 2 ng of *RTH1* using 3′-labeled poly(dA):oligo$(dT)_{50}$. Lane 1, blank (no *RTH1*); lane 2, 0 min; lane 3, 5 min; lane 4, 10 min; lane 5, 20 min; lane 6, 30 min; lane 7, 45 min; lane 8, 60 min.

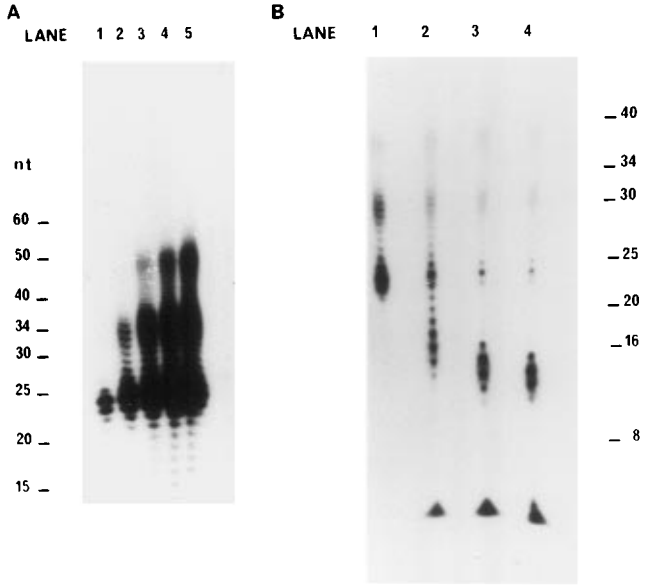

FIGURE 8: Digestion of RNA primed DNA templates by native *RTH1* nuclease. Using immunoaffinity purified yeast pol α−primase complex, primed poly(dT) templates were generated to be used subsequently as substrates for *RTH1* digestion. (A) Time course of primed DNA synthesis by pol α−primase. Lane 1, 1 min; lane 2, 5 min; lane 3, 10 min; lane 4, 15 min; lane 5, 30 min. (B) Digestion of primed poly(dT) by *RTH1*. A standard exonuclease assay was carried out using 2 ng of *RTH1* and primed template corresponding to a 5 min reaction as shown in panel A. Lane 1, blank; lane 2, 5 min; lane 3, 15 min; lane 4, 30 min.

requires removal of the primers followed by gap filling and ligation. RNase H is needed for the removal of the RNA primers; however, in *E. coli* the 5′ → 3′ exonuclease of DNA polymerase I can carry out removal of the primers. In eukaryotes, the primers synthesized by pol α−primase are mixed ribo-deoxynucleotide primers. Consequently, the enzyme whose role it is to remove the synthesized Okazaki fragments must be able to digest mixed ribo−deoxynucle-

otide primers. We have synthesized RNA primers using immunoaffinity purified yeast pol α−primase on a poly(dT) and examined the activity of *RTH1* exonuclease on these primers. Our results indicated that *RTH1* 5′ → 3′ exonuclease can efficiently remove these primers. The primers shorter than 15 nucleotides were not degraded, which was likely due to the fact that under our reaction conditions these primers would melt from the template and would no longer be substrates for the exonuclease. The *RTH1* nuclease is structurally homologous to DNA polymerase I of *E. coli*, T4 RNase H, and the RNase H domain of HIV reverse transcriptase (Hollingsworth & Nossal, 1991; Mueser, 1996). The active site structure of the *RTH1* nuclease, as determined by computer modeling, is closely comparable to these and other ribonuclease H proteins from heterologous sources (Biswas et al., 1997). Thus, there appears to be a structural basis for the RNase H like activity of the *RTH1* nuclease.

In conclusion, it appears that the *RTH1* gene product can fulfill the requirements of a chromosomal replicative nuclease. However, further studies are required to fully analyze the nuclease activities which take place at the replication fork, as well as to analyze the multiple roles which the *RTH1* nuclease may play.

ACKNOWLEDGMENT

This work was supported by a grant (GM 36002) from the National Institute of General Medical Sciences, National Institutes of Heath. We thank Dr. Rameshwar Sharma of this University for a critical review of the manuscript and Mr. Kim Sokoloff of the Media Services of this university for excellent photography. We thank Dr. John Leszyk of Worcester Foundation of Experimental Biology for peptide sequencing and MALDI-TOF analysis.

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BI962889V