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DNA Helicase III of *Saccharomyces cerevisiae*, Encoded by YER176w (*HEL1*), Highly Unwinds Covalently Closed, Circular DNA in the Presence of a DNA Topoisomerase and yRF-A

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Previously, we have purified and characterized DNA helicase III from the yeast *Saccharomyces cerevisiae* [Shimizu, K. and Sugino, A. (1993) *J. Biol. Chem.* 268, 9578-9584]. Here, we have further characterized DNA helicase III activity. It was found that the combined action of the helicase III, yeast DNA topoisomerase I (yTop I), and yeast RPA protein on a covalently closed, circular DNA generates a highly underwound DNA species that has been called form I* or form U. Furthermore, these underwound structures can be accessed by yeast DNA polymerase I (α)-primase to initiate DNA synthesis. These reactions mimic *in vivo* initiation of chromosomal DNA replication. In order to clone the gene encoding DNA helicase III, a partial amino acid sequence of the purified DNA helicase III polypeptide was determined. Using a mix oligonucleotides synthesized based on the amino acid sequence of the helicase, we cloned the gene encoding the helicase III and found it to be identical to *YER176W* (*HEL1*) on chromosome V. The amino acid sequence predicted from the nucleotide sequence of the gene has conserved DNA helicase domains that are highly homologous to those of DNA helicases required for DNA replication. However, complete deletion of the gene from the chromosome did not result in any growth defect, suggesting that the gene product is not required for DNA synthesis or that it is functionally substituted by other helicase(s). Furthermore, the deletion strain does not exhibit sensitivity to any DNA-damaging reagents, although it is hypersensitive to calcofluor white, hygromycin, and papulacandin.

Key words: DNA helicase, DNA polymerase α-primase, DNA topoisomerase, highly unwound DNA, yeast *Saccharomyces cerevisiae*.

DNA replication requires the concerted action of many enzymes and proteins (*1, 2*). Before DNA synthesis occurs, the two strands of the DNA helix must be unwound to provide DNA polymerases with single-stranded DNA. Unwinding of the DNA is an essential process not only for chromosomal DNA replication (*1, 2*) but also for DNA repair and recombination (*3*). An activity, called DNA helicase or DNA unwinding protein, that catalyzes unwinding of double-stranded DNA in the presence of either NTP or dNTP has been identified and purified from various organisms (*3*). In the prokaryote *Escherichia coli*, many different DNA helicases are known: rep protein, dnaB protein, primosomal protein n′, DNA helicase I, DNA helicase II (or uvrD gene product), DNA helicase III, DNA helicase IV, RecBCD enzyme, and UvrAB complex (see Refs. *2* and *3* for a review). It has been shown genetically and biochemically that the dnaB protein and primosomal protein n′ are required for *E. coli* chromosomal oriC-dependent DNA replication (*2*). *E. coli* bacteriophages T4 and T7 also encode their own DNA helicases, gene 41 (*4*) and dda protein (*5*), and gene 4 protein (*6*), respectively.

In eukaryotes, on the other hand, only fragmentary information on DNA helicase activity is currently available (see Ref. *3* for a review). The yeast *Saccharomyces cerevisiae* offers a system that can combine biochemical studies with classical genetic and molecular genetic approaches. Furthermore, the total nucleotide sequence of the chromosomal DNA has been determined. This makes it an attractive model eukaryote with which to explore the role of helicase in various DNA metabolic pathways. However, few DNA helicases have been purified and characterized from yeast cell extracts (*7-14*). Among these, only Dna2 helicase is known to be required for DNA replication (*13*). This helicase is required for maturation of Okazaki fragments, rather than unwinding DNA strands during DNA replication (*15*). The best characterized DNA helicase from eukaryotes is the SV40-encoded T-antigen, a multifunctional protein with DNA helicase activity (*16*). It recognizes its own origin of DNA replication and unwinds not only the double-stranded DNA at the origin but also the DNA strand during DNA replication (*16*). It is also known that herpes simplex virus 1 (HSV1) encodes its own DNA helicase, which also exhibits a DNA primase activity and is required for its DNA replication (*17, 18*). Although many species of DNA helicase activity have been identified and

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purified from higher eukaryotes (*3, 19-25*), only helicase B of mouse is known to be required for chromosomal DNA replication (*26*). However, it is not known where helicase B is required during DNA replication. Recently, it has been shown that a form of the MCM protein complexes that are required for chromosomal DNA replication exhibits DNA helicase activity (*27*).

To identify and purify the yeast DNA helicase that is required for chromosomal DNA replication, we have fractionated the crude extracts from *S. cerevisiae* cells and have so far purified ATPase III, which has DNA helicase activity and stimulates *S. cerevisiae* DNA polymerase I (a homolog of mammalian DNA polymerase α) (*7*) and DNA helicase III (*10*). Here, we present further characterization of DNA helicase III and identification of the gene encoding DNA helicase III. We show that the combined action of the helicase III, *E. coli* DNA gyrase, and yeast RPA protein on covalently closed, circular DNA generates a highly underwound DNA species that has been called form I* or form U. Furthermore, these underwound structures can be accessed by yeast DNA polymerase I (α)-primase to initiate DNA synthesis. The isolated gene encoding the helicase III was found to be identical to YER176w (*HEL1*). Although helicase III resembles replicative helicases in its biochemical characteristics, deletion of the gene does not cause any noticeable defect in cell proliferation.

MATERIALS AND METHODS

Yeast and Bacterial Strains—*Saccharomyces cerevisiae* CB001 (*MAT***a** *leu2 trp1 ura3 prb pep4Δ::URA3*) and YHA1 (*MAT***a** *ade5 leu2-3, 112 trp1-289 ura3-52 his7-2/MATα ade5 leu2-3, 112 trp1-289 ura3-52 can1*) were previously described (*28*). YYK1 (*MAT***a** *leu2 trp1 ura3 prb pep4Δ::URA3 Δhel3::LEU2*) and YYK2 (*MAT***a** *ade5 leu2-3, 112 trp1-289 ura3-52 his7-2*) were the DNA helicase III deletion mutants isolated in this study and isogenic to CB001 and CG379 (*28*), respectively. *E. coli* DH5α (*29*) was used for the manipulation of plasmid DNA.

DNA—The following nucleotides were synthesized on a ABI automated DNA synthesizer: ϕX174 oligonucleotide 5′-CGCAAAGTAAGAGCTTCTCGAGCTGCGCAAGGATAGGTC-3′ (corresponding to nucleotides 183 to 145 in Ref. *30*), 5′-CAAAGCCTCTACGCGATTTCATAGTGGAGGCCTCCAGCAATCTT-3′ (nucleotides 4518 to 4473 in Ref. *30*), and *HEL3* PCR primers, 5′-TAPyCCNGAPyCTNAAPyAC-3′ (corresponding to amino acid sequence YPDLNT), 5′-ATPyTTNAAPuTTPyTGNACNAAPyTCPuTTPyTC-3′ (complementary nucleotide sequence corresponding to amino acid sequence ENEFVQNFNI), 5′-GGTTGTAGAAATATTCTGCAGGAAAATTCGC-3′ (−995 to −965 from A of the first initiation codon of *HEL3*), 5′-TACTTCTTAGTCTGTCGACCTCCTTATATTG-3′ (−8 to −48 from A of the first initiation codon of *HEL3*), 5′-CTTACTAGCCGGCTAGGCCTCGGCAAGAAAA-3′ (3359 to 3379 from A of the first initiation codon of *HEL3*), and 5′-TCTTATAGTAGAAAACCCGGGACTCGCCAGT-3′ (4297 to 4267 from A of the first initiation codon of *HEL3*), where the underlined nucleotides were changed from three wild-type sequences. ϕX174 ssDNA and RF-I DNA were from BRL or New England Biolabs. *S. cerevisiae* DNA was extracted from *S. cerevisiae* CB001 cells as described (*28*).

Chemicals—[γ-^{32}P]ATP (specific act. ≥5,000 Ci/mmol) was from Amersham. Mono Q and Mono S FPLC columns and heparin-Sepharose were from Pharmacia, hydroxylapatite was from Bio-Rad, and single-stranded DNA cellulose was from Sigma Chemicals. All nucleoside triphosphates were "Ultrapure" from Pharmacia. Preformed 4-20% gradient polyacrylamide gels were purchased from Daiichi Pure Chemicals. A singly primed ϕX174 single-stranded viral DNA was prepared as follows. Three times molar excess of the chemically synthesized 18-mer 5′-CTTCTGCGTCATGGAAGC-3′ (which is complementary to nucleotides 11 to 28 of ϕX174 ssDNA in Ref. *30*) was mixed with ϕX174 ssDNA (from New England Biolabs) in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 250 mM NaCl, heated at 80°C for 10 min, and incubated at 56°C for 15 min. Then the mixture was slowly cooled to room temperature.

Enzymes—DNA helicase III was purified as described before (*10*). Yeast DNA polymerase I (α)-primase complex and DNA polymerase II* (ε) were the same as previously described (*31*). Yeast DNA polymerase III (δ), RF-C complex, RF-A (RPA), and PCNA were as described (*32*). *E. coli* DNA gyrase (*33*) was provided by Drs. K. Mizuuchi and M. Gellert (NIH). Yeast type I DNA topoisomerase (Top I) was purified from CB001 cells as described (*34*).

Protein Sequencing—DNA helicase III purified from 1 kg of *S. cerevisiae* CB001 cells (*10*) was fractionated by electrophoresis in a 5% SDS-polyacrylamide gel. After staining the gel with Coomassie Brilliant Blue, the 120-kDa band was excised and electroeluted. The eluted polypeptide (about 50 μg) was digested in 0.2 M Tris-HCl (pH 9.5) containing 5 pmol of lysylendopeptidase (Wako) at 37°C overnight. To terminate the proteolysis, 1/10 volume of 10% trifluoroacetic acid was added, and the supernatant was collected. Oligopeptides generated by the endopeptidase were separated by reversed-phase high pressure liquid chromatography, then subjected to amino acid sequencing with a PSQ-10 protein sequencer (Shimadzu).

Partial Purification of DNA Helicase III—Yeast cells [CB001 (wild-type) and YYK1 (*Δhel3*)] were grown in 18 liters of YPD medium at 30°C to 5×10^7 cells/ml, harvested by centrifugation (about 150 g), suspended in 500 ml of buffer A [50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% (w/v) glycerol, and 1 mM PMSF], and disrupted as described (*10, 31*). Crude lysate was loaded on an SP-Sepharose column (80 ml) equilibrated with buffer A containing 0.1 M NaCl. The column was washed with 200 ml of the same buffer, and protein retained on the column was eluted with 200 ml of buffer A containing 0.5 M NaCl. To the eluate, 50% saturation of $(NH_4)_2SO_4$ was added, and the precipitated proteins were collected by centrifugation at 15,000 rpm for 20 min. The precipitates were resuspended in 16 ml of buffer A and dialyzed against 2 liters of buffer A containing 0.1 M NaCl for 4 h. The conductivity was adjusted to that of 0.1 M NaCl in buffer A, and the samples were applied to a Mono Q HR 10/10 column (Pharmacia) equilibrated with 0.1 M NaCl in buffer A. The column was washed with 20 ml of 0.1 M NaCl in buffer A. Since DNA helicase III passed through the column, the flow-through fractions were collected and loaded on a Mono S column (HR10/10) equilibrated with 0.1 M NaCl in buffer A. The column was washed with 20 ml of 0.1 M NaCl in buffer A, then developed with a 120-ml linear gradient of 0.1-0.6 M NaCl in buffer A.

Other Methods—Protein gels, Western blots, and protein concentration determinations were carried out as described (*32*). Single-stranded DNA-dependent ATPase activity associated with DNA helicase III was measured as described (*10*).

RESULTS

Further Characterization of DNA Helicase III—In the previous study (*10*), we purified and characterized DNA helicase III. The helicase requires a divalent cation, Mg^{2+} or Mn^{2+}, ATP or dATP, and a single-stranded portion on the duplex substrate. It moves in the 5′ to 3′ direction on the single-stranded portion of the substrate and unwinds the strand of DNA in the 3′ to 5′ direction. It also has an intrinsic DNA-dependent ATPase (dATPase) activity that hydrolyzes either ATP or dATP to ADP or dADP and orthophosphate in the presence of DNA. From these properties, we speculated that DNA helicase III is one of the DNA helicases that unwind double-stranded DNA during chromosomal DNA replication in yeast. Some DNA helicases required for DNA replication also act in combination with DNA topoisomerase and single-stranded DNA binding protein on covalently closed, circular DNA to generate a highly underwound DNA species that has been called form I* or form U (*2, 34*). It is also known that form I* or form U is a good substrate for the DNA polymerase α-primase complex, which is required for initiation of DNA replication (*35, 36*). Thus, we tested whether DNA helicase III has a similar activity using yeast DNA polymerase I (α)-primase and single-stranded DNA binding protein RF-A (RPA) by measuring incorporation of a radioactive dNTP into plasmid DNA. As shown in Table I, we detected a significant amount of DNA synthesis on a super-coiled, double-stranded DNA in the presence of yeast RPA, DNA

TABLE I. **Requirement for an *in vitro* DNA synthesis on supercoiled, covalently closed DNA.**

Omission or addition	DNA synthesis (pmol/30 min)	(%)
Complete	167	100.0
−DNA	<1	<0.6
−ATP, CTP, GTP, and UTP	5	3.0
−yRPA	33	19.8
−yRPA, DNA gyrase, and helicase III	35	20.9
−helicase III	71	42.5
−helicase III, −DNA gyrase	38	22.7
−DNA gyrase	97	58.1
−DNA gyrase, +yTopI	105	62.9
−yPolI-primase	<1	<0.6
−yPolI-primase, +yPolII and yPolIII	<1	<0.6
+yPolIII, PCNA, and yRF-C	250	149.7
+yPolII, yPolIII, PCNA, and yRF-C	275	164.7

The complete reaction mixture (50 μl) contained 50 mM Tris-HCl, pH 7.8, 35 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 100 μg/ml BSA, 1.54 nmol (total nucleotide concentration) of ARS1 plasmid DNA (*45*), each dNTP at 50 μM ([α-^{32}P]dTTP was present at 100–400 cpm/pmol), 1 mM ATP, three other rNTPs at 100 μM, 10 U of *E. coli* DNA gyrase (*33*), 2 μg of yRPA, 0.25 μg (43 U) of helicase III, and 0.5 μg of yPolI-primase complex. The mixtures were incubated at 30°C for 30 min and ^{32}P-labels incorporated into acid-insoluble materials were measured as described (*31*).

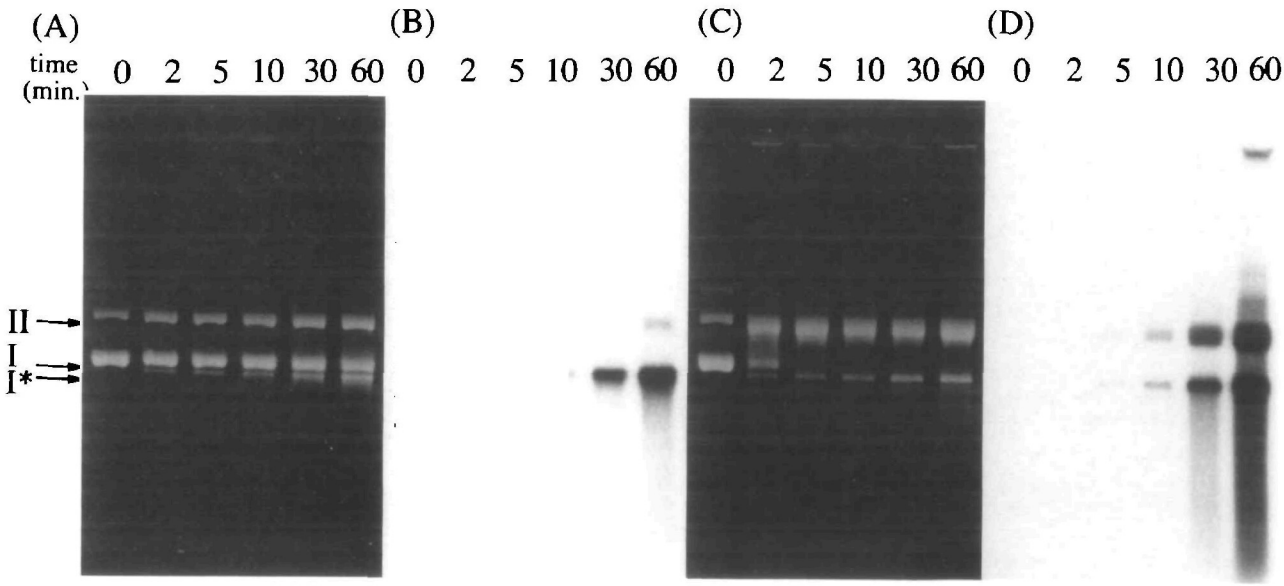

Fig. 1. **DNA helicase III produces highly unwound DNA in the presence of DNA gyrase and yRF-A.** [A and B] The plasmid ARS1 DNA was incubated with DNA polymerase I (α)-primase, DNA helicase III, DNA gyrase, and yRF-A in the presence of rNTPs and dNTPs ([α-^{32}P]dTTP was included) at 30°C. At the indicated times, an aliquot was withdrawn, the reaction was stopped by addition of 1% SDS and 10 mM EDTA, and the sample was subjected to agarose gel electrophoresis. After electrophoresis, the gel was stained with ethidium bromide and photographed under UV light. Then the gel was dried and autoradiographed. [C and D] For control, the plasmid ARS1 DNA was incubated with DNA polymerase I (α)-primase, yRF-A, and the Mono Q fractions of the *S. cerevisiae* CB001 cell extracts, which contained yTopI and unknown DNA helicase activity, in the presence of rNTPs and dNTPs ([α-^{32}P]dTTP was included) at 30°C. The reaction products were analyzed as (A) and (B). Arrows in the figure indicate the position of form I (supercoiled), II (open circle) and I* or U form of ARS1 DNA, respectively. (A) and (C) show the photographed gel stained with ethidium bromide, while (B) and (D) are autoradiographs of the dried gel. It was not clear whether the radioactivity was found in the I* or U form of ARS1 DNA in (A) and (B). However, in (C) and (D), it was very clear that the radioactivity was found in the I* or U form of the DNA as well as in form II DNA, although the supercoiled DNA was quickly relaxed by topoisomerase activity.

helicase III, and yeast DNA polymerase I-primase. This activity was further stimulated by addition of *E. coli* DNA gyrase, which is considered to be a swivelase (*37*), and was dependent on the presence of yeast DNA polymerase I-primase, since the activity could not be substituted by either DNA polymerase II (ε) or DNA polymerase III (δ), which are two additional DNA polymerases required for chromosomal DNA replication in yeast (*38*). However, these two DNA polymerases along with their accessory proteins, PCNA and RF-C complex, further stimulated the activity (Table I). As this DNA synthesis activity is dependent on the presence of rNTPs, DNA primase activity associated with DNA polymerase I-primase complex could play a crucial role in initiation of DNA synthesis, presumably RNA primer synthesis.

When the products were analyzed by agarose gel electrophoresis, one additional DNA band which migrated faster than the super-coiled DNA was detected in the presence of DNA helicase III, RPA, DNA polymerase I-primase, and DNA gyrase (Fig. 1A). ^{32}P-labels incorporated into DNA were found exclusively in this band (Fig. 1B). When the products were analyzed by alkaline agarose gel electrophoresis, DNA fragments of about 150 nucleotides in length and others of more than 150 nucleotides were detected in the reaction catalyzed by DNA polymerase I-primase, DNA helicase III, DNA gyrase, and yeast RPA (Fig. 2). On the other hand, the major products produced in the reaction without DNA helicase III were about 150 nucleotides long (Fig. 2f). When DNA polymerase II (ε) or III (δ) was further added, products of about 150 nucleotides were further extended and the majority of the products were more than 150 nucleotides long (Fig. 2, h-j). These results strongly suggest that DNA helicase III generates a highly underwound DNA species from covalently closed, supercoiled DNA in the presence of RPA and DNA gyrase, DNA primase activity synthesizes RNA primers, and DNA polymerase I extends RNA primers to make short RNA-DNA molecules that resemble *in vivo* Okazaki fragments. Finally, DNA polymerase III (δ) or II (ε) extends short RNA-DNA molecules to make longer DNA. Note that DNA gyrase was not fully substituted with yeast TopI (Table I and Fig. 2j). Thus, the role of DNA gyrase in the reaction may introduce further negative supercoils into the substrate, thereby locally generating a single-stranded region which is sufficient for the DNA helicase III loading.

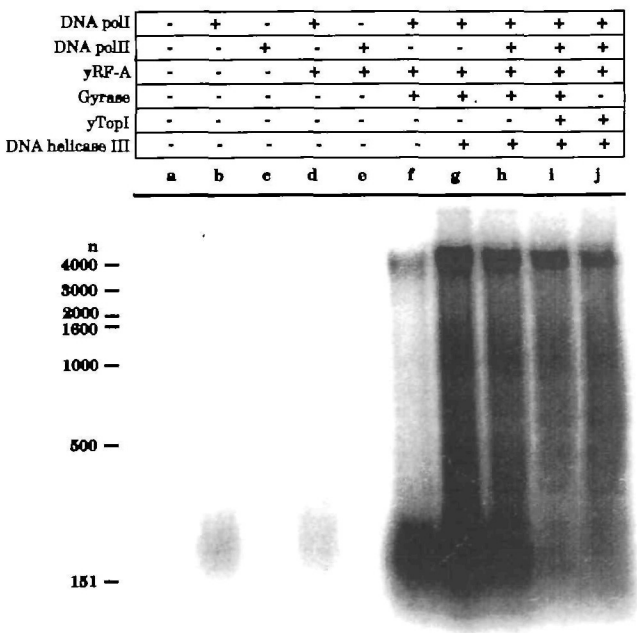

Fig. 2. **Highly unwound, covalently closed DNA becomes a good substrate for DNA polymerase I (α)-primase.** The plasmid ARS1 DNA incubated with DNA polymerase I (α)-primase, DNA helicase III, DNA gyrase, and yRF-A in the presence of rNTPs and dNTPs ([α^{32}P]dTTP was included) at 30°C for 30 min was denatured with 0.2 N NaOH and applied on an alkaline agarose gel. After electrophoresis, the gel was dried on a DEAE-cellulose sheet and autoradiographed. In the figure, − and + represent omission and addition of each protein in the reaction. The numbers shown on the left-hand side of the figure are the marker DNA in nucleotides.

Identification of the Gene Encoding DNA Helicase III— To identify and clone the gene (*HEL3*) encoding DNA helicase III, the 120-kDa polypeptide copurified with the DNA helicase III activity was isolated and its partial amino acid sequence was determined as described in "MATERIALS AND METHODS." As shown in Table II, amino acid sequences were obtained from the eight fragments (AP-1 to AP-8) generated from the polypeptide by Lys-C endopeptidase digestion, followed by reverse phase HPLC column chromatography. Amino acid sequences of the fragments were used for searching GenBank and *Saccharomyces* Genome databases. We found these sequences matched perfectly amino acid sequence predicted from YER176w (*HEL1*), which encodes a DNA helicase (Table II) (*39*), suggesting that DNA helicase III is encoded by YER176w (*HEL1*).

To confirm that DNA helicase III is encoded by YER176w (*HEL1*), two mix-oligonucleotides corresponding to amino acid sequences YPDLNT (5′-TAPyCCNGAPyPyTNAAPy-AC-3′) and ENEFVQNFNI (5′-ATPuTTPuAAPuTTPyTG-NACPuAAPyTCPuTTPyTC-3′), respectively, were synthesized and used for PCR amplification of yeast genomic DNA. The 92-bp DNA fragment predicted from the nucleotide sequence of YER176w (*HEL1*) was amplified. The nucleotide sequence of the 92-bp fragment perfectly matched that of YER176w (*HEL1*) (data not shown). These data indicate that DNA helicase III is encoded by YER176w (*HEL1*).

The predicted amino acid sequence includes the sequences GTGKT (amino acid residue 673-677) and DEAT (amino acid residue 820-823), which are considered to be Walker type A and B motifs, respectively, and are found in many NTP-binding proteins. The sequence also contains

TABLE II. **Protein sequence of the 120-kDa polypeptide copurified with DNA helicase III.**

Peptide number	Amino acid sequence	Amino acid residue-number predicted from the *YER176w* gene
AP-1	TVFDTSNDEDICCEECQDK	28-46
AP-2	GKK	339-341
AP-3	STLSYPDLNTYLNDYSFALE NEFVQNFNILWPR	472-510
AP-4	NLSPDMQVVANK	752-763
AP-5	FYK	778-780
AP-6	IYNGELK	898-904
AP-7	NVVINPKQISMQQEY	993-1007
AP-8	HGLIVVGN	1083-1090

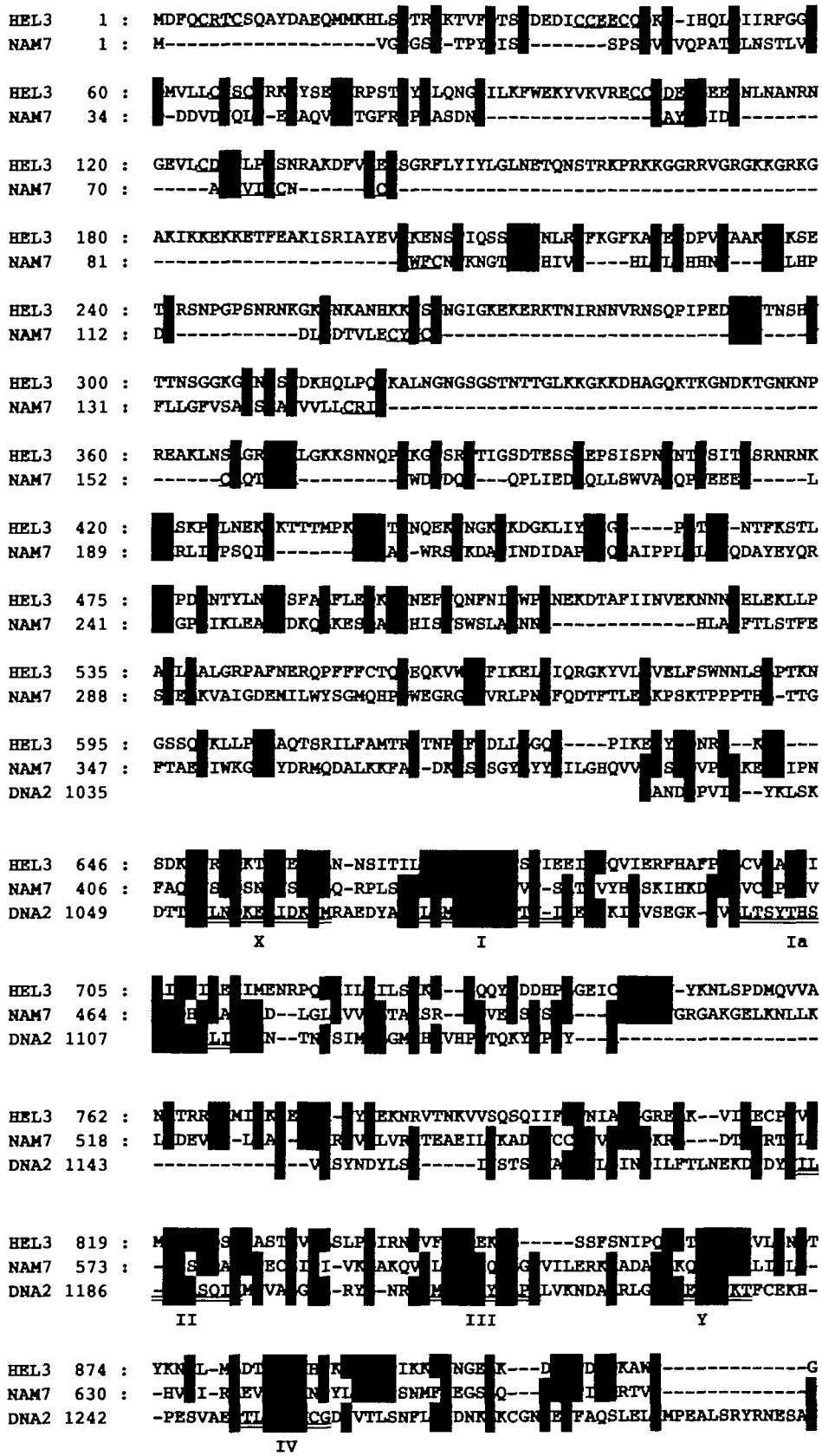

Fig. 3 (continued on next page)

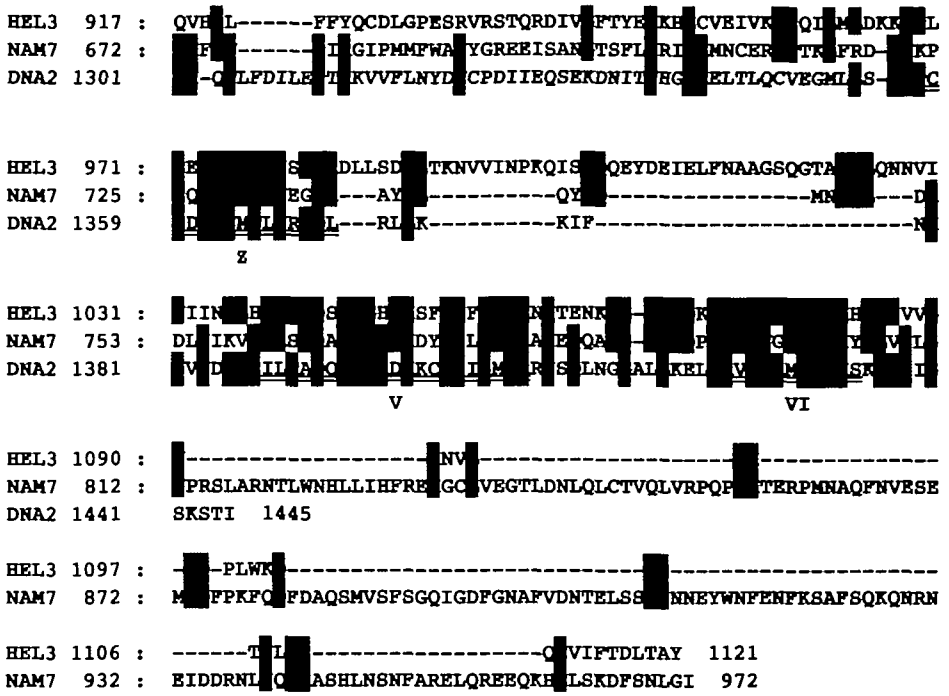

Fig. 3. **Amino acid sequence of DNA helicase III (YER176w/*HEL1*).** The predicted amino acid sequence of the YER176w (*HEL1*) gene is aligned with those of *NAM7* (*40*) and *DNA2* (*41*). Identical amino acids are shaded, and the DNA helicase motifs (I to VI) conserved among various DNA helicases are double-underlined. Other sequences (shown by X to Z) that were noticed to be well conserved between DNA helicase III, Nam7, and Dna2 proteins are also double-underlined. Potential Zn-ligand structures are single-underlined.

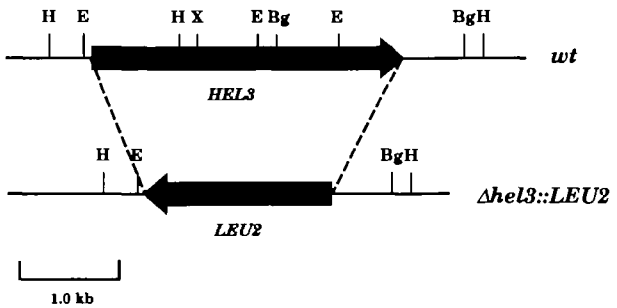

Fig. 4. **Construction of DNA helicase III deletion mutant.** The open reading frame of YER176w (*HEL1*), which encodes DNA helicase III, was deleted and replaced with *S. cerevisiae LEU2* gene, resulting in *Δhel3::LEU2*. The YER176w (*HEL1* or *HEL3*) gene in wild-type cells was replaced with *Δhel3::LEU2* by one-step gene replacement (*42*). The figure also shows representative restriction endonuclease sites (H, *Hin*dIII; E, *Eco*RI; Bg, *Bgl*II; X, *Xba*I).

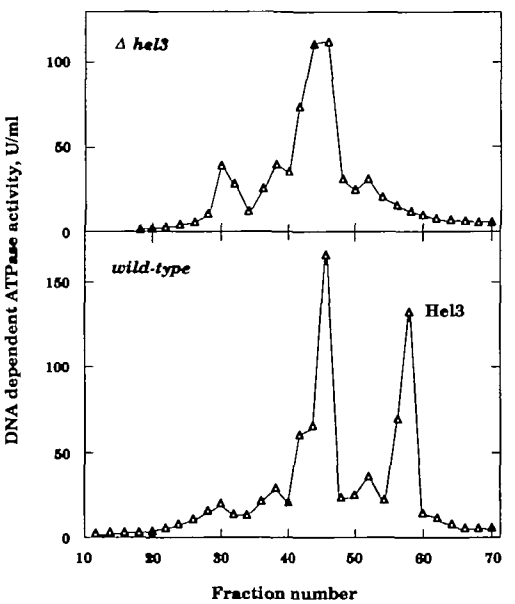

Fig. 5. **DNA helicase III activity is missing in the YER176w (*HEL1*) deletion (*Δhel3*) mutant cell extracts.** The YER176w (*HEL1*) deletion (*Δhel3*) mutant (YYK1) and wild-type (CB001) cell extracts were applied on a S-Sepharose column, and the retained proteins were eluted with 0.5 M NaCl in buffer A as published (*10*). The 0.5 M NaCl eluate was dialyzed against 0.1 M NaCl in buffer A, passed through a Mono Q column, and applied on a MonoS column as shown in "MATERIALS AND METHODS."

several motifs that are typical of proteins interacting with nucleic acids: (a) six motifs (I to VI in Fig. 3) diagnostic for a superfamily of helicases are found in the same order and with similar distances; (b) the N-terminal portion of the protein possesses Zn-ligand structures (Fig. 3). Besides the six helicase motifs (I to VI), three additional highly conserved motifs (X to Z in Fig. 3) were detected between Nam7 (*40*) and Dna2 protein (*41*). Thus, from these amino acid sequence similarities, YER176w (*HEL1*) belongs to the Nam7/Dna2 sub-superfamily of helicases (Fig. 3).

Disruption of YER176w (HEL1)—To investigate the function of DNA helicase III, the gene of YER176w (*HEL1*) was disrupted by removing its entire coding region and inserting the yeast *LEU2* gene as described in "MATERIALS AND METHODS." One of the wild-type genes in a diploid strain was replaced with the disrupted gene (Fig. 4) by one-step gene disruption (*42*). After confirming the chromosomal gene disruption by Southern blot analysis, the Leu$^+$ transformant was sporulated and dissected. Of 10 tetrads dissected, all showed four viable spores (data not shown), indicating that the gene is not required for cell viability.

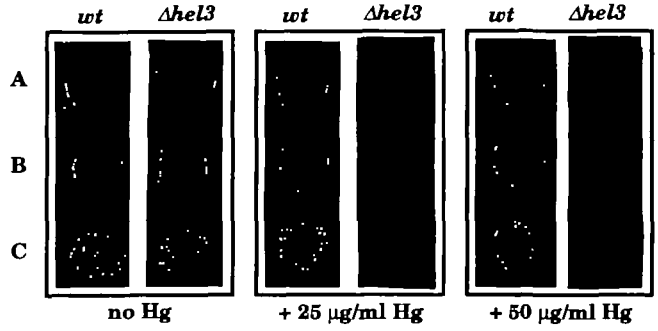

Fig. 6. **The YER176w (*HEL1*) deletion (*Δhel3*) mutant is sensitive to hygromycin.** CG379 (wild type) and YYK2 (*Δhel3*) cells were grown in YPD medium to about 1×10^7 cells/ml at 30°C (A) and diluted with YPD medium to 1×10^6 cells/ml (B) and 1×10^5 cells/ml (C). Samples of 10-μl were spotted on a YPD plate, a YPD plate containing 25 μg/ml hygromycin (Hg), or a YPD plate containing 50 μg/ml Hg and incubated at 30°C for three days. The cells grown on these plates were photographed.

Taking one of the disruption mutant strains (YYK1, isogenic to CB001), we tested whether DNA helicase III is missing in mutant cells. Extracts from either wild-type (CB001) or *Δhel3::LEU2* (YYK1) cells were subjected to S-Sepharose, Mono Q, and Mono S column chromatographies as described in "MATERIALS AND METHODS." Two peaks of ssDNA-dependent ATPase activity were detected in wild-type cells after Mono S column chromatography (Fig. 5). The second peak is DNA helicase III (*10*). In the mutant cells, however, only one peak of ssDNA-dependent ATPase activity was detected, which corresponded to the first peak of the activity in wild-type cells. Thus, the mutant cells lack the DNA helicase III activity, consistent with the notion that the DNA helicase III is encoded by YER176w (*HEL1*).

Phenotype of the YER176w (HEL1) Disruption Mutant—The YER176w (*HEL1*) disruption mutant was cultivated at various temperatures between 15 and 37°C and in various media, but in all cases it failed to show any detectable growth defect (data not shown). It also exhibited no significant sensitivity to UV light, hydroxyurea, or MMS treatment (data not shown), suggesting that helicase III is not required for repair of DNA damage by these treatments. However, the mutant exhibited hypersensitivity to hygromycin (Fig. 6) and to calcofluor and papulacandin (data not shown). Since these drugs are not related to each other (*43*), the sensitivity could be due to permeability change of the mutant.

DISCUSSION

In this paper, we have described one additional property of DNA helicase III of *S. cerevisiae*: in combination with DNA topoisomerase and single-stranded DNA-binding protein, the helicase acts on covalently closed, circular DNA to generate a highly underwound DNA species that has been called form I* or form U (Fig. 1A). This highly unwound DNA species provided a good template for DNA synthesis catalyzed by yeast DNA polymerase I-primase complex, which is believed to be required not only for initiation of chromosomal DNA replication but also for the lagging strand synthesis, namely, Okazaki fragment synthesis (Figs. 1B and 2). Yeast DNA polymerase I-primase complex could not be substituted by two other DNA polymerases [DNA polymerases II (ε) and III (δ)] which are also required for chromosomal DNA replication in yeast (*38*). Since DNA synthesis occurring on form I* was dependent on the presence of rNTPs, it is highly possible that DNA primase activity associated with DNA polymerase I-primase complex synthesizes RNA-primer, and DNA polymerase I subsequently elongates RNA primer to form Okazaki fragment-like products on plasmid DNA. In the absence of DNA helicase, we still observed small amount of DNA synthesis on a supercoiled DNA (Table I and Fig. 2). The major products of this reaction were about 150 nucleotides long (Fig. 2). However these small DNA were not further extended by DNA polymerase. This is due to local single-strandedness of a supercoiled DNA, and this provides a template for DNA primase action. At the same time, DNA helicase III can recognize this single-stranded region, bind there, and further unwind DNA. The observed DNA synthesis catalyzed by DNA polymerase I-primase complex in the presence of yeast RF-A, DNA helicase III, DNA gyrase (or DNA topoisomerase I), and a supercoiled DNA was extensive, and the products of the reaction were mostly double-stranded DNA, as the products were digestible with restriction endonucleases (data not shown). Therefore, the reaction seen in Fig. 2 is very much like the reaction seen in the reconstituted SV40 DNA replication (*36*), except for specific initiation at the origin.

These results suggested that DNA helicase III is required for chromosomal DNA replication. After cloning the gene (*HEL3*) encoding the DNA helicase III, it was found that the gene is identical to YER176w (*HEL1*) (*39*). A partial amino acid sequence determined from oligopeptides generated by digestion with the endopeptidase perfectly matched the amino acid sequence predicted from the nucleotide sequence of the cloned gene, thus confirming that the gene encodes DNA helicase III. The amino acid sequence of the cloned gene contains an NTP-binding motif and seven conserved DNA helicase motifs that are highly homologous to those of yeast *NAM7* and *DNA2* (Fig. 4), further supporting the notion that the cloned gene encodes DNA helicase III. The deletion of YER176w (*HEL1*) did not result in death of the cell, suggesting that the DNA helicase is not required for DNA metabolic pathways that are essential for cell growth, or that its function is taken over by other DNA helicases. Based on amino acid sequence similarity of DNA helicase III to other DNA helicases, DNA helicase III belongs to the Nam7/Dna2 helicase family, which is very different from those families already recognized (*44*). Thus, either Nam7 or Dna2 helicase may substitute functionally for DNA helicase III. However, the *dna2* mutation was not suppressed by overproduction of *HEL1* (our unpublished results), suggesting that it is less likely that Dna2 helicase can substitute for DNA helicase III.

DNA helicase is known to be required for DNA repair and DNA recombination as well as transcription (see Ref. *3* for a review). Therefore, we checked whether the DNA helicase III deletion strain is sensitive to DNA-damage by UV, hydroxyurea, and MMS treatments. The mutant strain did not show any sensitivity to these treatments, suggesting that DNA helicase is not required for either DNA repair or recombination.

The deletion mutant of DNA helicase III exhibits hypersensitivity to calcofluor white, hygromycin, and papulacandin. As these drugs are not structurally related, the sensitivity could be due to permeability change of the mutant strain. Therefore, it is likely that DNA helicase III has a role in expression of genes involved in cell surface biosynthesis and architecture. In fact, large-scale screening of genes involved in cell surface biosynthesis and architecture using the same drugs has also identified YER176w (*HEL1*) (*43*).

By inserting the isolated DNA helicase III gene (YER-176w/*HEL1*) under the yeast Gap promoter, we constructed a yeast overproducer of DNA helicase III. Using this overproducer, more than 5 mg of the DNA helicase III can be purified from 1 liter of culture. This will facilitate biophysical and crystal structural studies of DNA helicase III.

Finally and more interestingly, it was found that partially purified fractions of *S. cerevisiae* cell extracts that lacked DNA helicase III activity, promoted the formation of I* or U form without addition of *E. coli* DNA gyrase (Kawasaki, Y. and Sugino, A., unpublished observation). This suggests that these fractions may contain a DNA helicase activity that is similar to SV40 Large T-antigen and works at the replication fork.

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