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The second subunit of DNA polymerase III (δ) is encoded by the *HYS2* gene in *Saccharomyces cerevisiae*

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ABSTRACT

DNA polymerase III (δ) of *Saccharomyces cerevisiae* is purified as a complex of at least two polypeptides with molecular masses of 125 and 55 kDa as judged by SDS–PAGE. In this paper we determine partial amino acid sequences of the 125 and 55 kDa polypeptides and find that they match parts of the amino acid sequences predicted from the nucleotide sequence of the *CDC2* and *HYS2* genes respectively. We also show by Western blotting that Hys2 protein co-purifies with DNA polymerase III activity as well as Cdc2 polypeptide. The complex form of DNA polymerase III activity could not be detected in thermosensitive *hys2* mutant cell extracts, although another form of DNA polymerase III was found. This form of DNA polymerase III, which could also be detected in wild-type extracts, was not associated with Hys2 protein and was not stimulated by addition of proliferating cell nuclear antigen (PCNA), replication factor A (RF-A) or replication factor C (RF-C). The temperature-sensitive growth phenotype of *hys2-1* and *hys2-2* mutations could be suppressed by the *CDC2* gene on a multicopy plasmid. These data suggest that the 55 kDa polypeptide encoded by the *HYS2* gene is one of the subunits of DNA polymerase III complex in *S.cerevisiae* and is required for highly processive DNA synthesis catalyzed by DNA polymerase III in the presence of PCNA, RF-A and RF-C.

INTRODUCTION

DNA polymerases have a central role in replication of cellular chromosomal DNA. The complexity and importance of these processes to the cell are reflected in the multiple species of DNA polymerases found in both prokaryotic and eukaryotic cells, where specific functions are performed predominantly by one or another species of polymerase (for a review see 1). In the yeast *Saccharomyces cerevisiae* three distinct nuclear DNA polymerases [I (α), II (ε) and III (δ)] have been purified and characterized and all three DNA polymerases are essential for cell growth and are required for chromosomal DNA replication (for a review see 2). These polymerases are purified as complexes consisting of several polypeptides. DNA polymerase I (α) consists of four polypeptides: the 180 kDa polypeptide with DNA polymerase activity, the 86 kDa polypeptide with unknown function and the 58 and 48 kDa polypeptides with DNA primase activity (3). The genes encoding all four polypeptides have been cloned and shown to be required for chromosomal DNA replication. DNA polymerase II (ε) is also purified as a complex of 256, 80, 34, 30 and 29 kDa polypeptides. While the 256 kDa polypeptide encoded by *POL2* is the catalytic subunit having DNA polymerase and 3′→5′ exonuclease activities, the functions of the other polypeptides remain unknown (4–7), although the second subunit of the polymerase is also required for chromosomal DNA replication. Recently an additional possible subunit of DNA polymerase II has been identified and shown to be required for DNA replication as well as for S phase checkpoint control (8). DNA polymerase III (δ) is purified as a complex of at least two polypeptides with molecular masses of 125 and 55 kDa as judged by SDS–PAGE (4,9). It is widely believed that the 125 kDa polypeptide, which is encoded by the *CDC2* (*POL3*) gene, contains the DNA polymerase and 3′→5′ exonuclease activities (4,9–12). Three accessory protein complexes, replication factor A (RF-A), replication factor C (RF-C) and proliferating cell nuclear antigen (PCNA), associate with DNA polymerase II or III to promote highly processive DNA synthesis *in vitro* (13–15). RF-A is a single-stranded DNA (ssDNA) binding protein and consists of heterotrimeric subunits (70, 34 and 14 kDa respectively) (16,17). PCNA is a homotrimer with a molecular mass of 29 kDa. The crystal structure of yeast PCNA shows that the trimer forms a closed ring with the appropriate dimensions and electrostatic properties to encircle double-stranded DNA (dsDNA) and to interact with it by non-specific contacts (18). Processivity in DNA synthesis is achieved by protein–protein interactions between PCNA and the polymerase, thereby tethering the DNA polymerase at the primer terminus (19). RF-C is a multiprotein complex consisting of 95, 40, 40, 38, 36 kDa subunits (13,15,20) and aids in processivity by binding preferentially to the 3′-end of a DNA primer bound on template DNA. This complex of RF-C and DNA is recognized by PCNA, which binds to it in an ATP-dependent manner. Finally, DNA polymerases II and III recognize the complex of RF-C and PCNA

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bound to primer–template. In addition to stimulating polymerase loading, PCNA and possibly RF-C form a holoenzyme together with DNA polymerase III (14). The genes encoding PCNA and all five subunits of RF-C have been cloned and shown to be required for cell growth, possibly for chromosomal DNA replication (20–22; Noskov,V., Araki,H. and Sugino,A., unpublished results). Although DNA polymerase III activity has been well studied, its precise subunit structure and the function of each subunit have not been well elucidated in *S.cerevisiae*.

In this work we isolated the 125 and 55 kDa polypeptides in purified DNA polymerase III fractions from *S.cerevisiae* cell extracts and demonstrated that they are encoded by the *CDC2* and *HYS2* genes respectively. Furthermore, we showed that they constitute a form of DNA polymerase III activity capable of highly processive DNA synthesis mediated by RF-A, RF-C and PCNA.

MATERIALS AND METHODS

Yeast and bacterial strains

Saccharomyces cerevisiae CB001 (MAT**a** *leu2 trp1 ura3 prb pep4*Δ::*URA3*) and *pol3-t*-DM (MATα *leu2-2 trp1-*Δ*1 ura3-X pol3-t*) were previously described (4,23). KSH542 (MAT**a** *hys2-1 ade1 his2 his3 trp1 ura3 leu2*) was from K.Sugimoto (24). N22 (MAT**a** *hys2-2 ade1 his2 trp1 ura3 leu2 bar1*) was isolated by the plasmid shuffling method described previously (7). KSH542-2 (MAT**a** *hys2-1 ade1 his2 his3 trp1 ura3 leu2 pep4*Δ::*URA3*) and N51-1 (MAT**a** *hys2-2 ade1 his2 trp1 ura3 leu2 bar1 pep4*Δ::*URA3*) were constructed by one step replacement (25) of the *PEP4* gene in strains KSH542 and N22 respectively with *pep4*Δ::*URA3* (26). Strains 370 (MAT**a** *ade1 ade2 cdc2-1 ura1 his7 trp1 lys2 gal1*) and 336 (MAT**a** *ade1 ade2 cdc2-2 ura1 his7 trp1 lys2 gal1*) were obtained from B.Garvik (University of Washington). *Escherichia coli* DH5α (5) was used for manipulation of plasmid DNA. BL21 (DE3), which harbors a lysogen containing the T7 RNA polymerase gene under control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *lac* UV5 promoter, was used for expression of the *HYS2* gene product (27).

DNA

A singly primed ϕX174 viral ssDNA was prepared as follows. A three times molar excess of the chemically synthesized 18mer 5′-CTTCTGCGTCATGGAAGC-3′ (complementary to nt 11–28 of ϕX174 ssDNA; 28) was mixed with ϕX174 ssDNA (New England Biolabs) in 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 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. Poly(dA)$_{300}$ and oligo(dT)$_{10}$ (Pharmacia) were mixed at a weight ratio of 20:1 and annealed in 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 300 mM NaCl at a concentration of 0.2 mg/ml at 30°C for 1 h to make the poly(dA)$_{300}$:oligo(dT)$_{10}$ template–primer.

DNA polymerase assays

Polymerase activity was assayed by measuring incorporation of [α-^{32}P]dTTP into trichloroacetic acid-insoluble material as described (4). When poly(dA)$_{300}$:oligo(dT)$_{10}$ was used as template the reaction mixture (50 µl) contained 50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 50 µM [α-^{32}P]dTTP (100–500 c.p.m./pmol; Amersham), 50 µM each dATP, dGTP and dCTP and 10 µg/ml poly(dA)$_{300}$:oligo(dT)$_{10}$. One unit of DNA polymerase III incorporates 1 nmol nucleotide/h. When a singly primed ϕX174 ssDNA was used as template (Table 2) the reaction mixture (50 µl) contained 50 mM Tris–HCl, pH 7.5, 8 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 50 µM [α-^{32}P]dTTP (100–500 c.p.m./pmol; Amersham), 50 µM each dATP, dGTP and dCTP, 0.5 mM ATP and 4 µg/ml singly primed ϕX174 ssDNA.

Enzymes

DNA polymerase III was purified at 4°C as follows. Two or three kilograms of *S.cerevisiae* CB001 cells were disrupted and fraction III was prepared as previously published (4). The conductivity of fraction III was adjusted to that of 0.1 M NaCl in buffer A [50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 10 mM $NaHSO_3$, 10% glycerol, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF)] and applied to a Poros Q column (5 × 10 cm; Perseptive) or a Q Sepharose high performance (HP) BioPilot column (6 × 10 cm; Pharmacia) equilibrated with 0.05 M NaCl in buffer A. The column was washed with 400 or 800 ml 0.05 M NaCl in buffer A and then proteins were eluted from the column with a 2.2 or 2.8 l linear gradient of 0.05–0.5 M NaCl in buffer A. DNA polymerase III activity was eluted at 0.2 M NaCl. Ammonium sulfate (0.516 g/ml) was added to the active fractions and the precipitates collected by centrifugation in a Beckman JA10 rotor at 9500 r.p.m. for 50 min. The pellet was resuspended in buffer A and dialyzed against 3 l buffer A for 3 h. The dialysate was adjusted to 0.1 M NaCl in buffer A and clarified by centrifugation in a Beckman JA20 rotor at 10 000 r.p.m. for 10 min. The supernatant was applied to a Mono S HR 16/10 column (Pharmacia) equilibrated with 0.1 M NaCl in buffer A. The column was washed with 60 ml 0.1 M NaCl in buffer A and then subjected to a 400 ml linear gradient of 0.1–0.5 M NaCl in buffer A. DNA polymerase III fractions eluting at 0.3 M NaCl were pooled, dialyzed against 4.5 l buffer B (0.05 M KPO_4, pH 7.0, 10 mM $NaHSO_3$, 10% glycerol, 10 mM 2-mercaptoethanol, 1 mM PMSF, 0.01% Nonidet P-40) for 4.5 h and applied to a Bio-Scale CHT 10-I column (BioRad) equilibrated with buffer B. The column was washed with 30 ml buffer B and then subjected to a 180 ml linear gradient of 0.05–0.5 M KPO_4 in buffer B. The active fractions were pooled, dialyzed against 3 l 0.05 M NaCl in buffer A^{NP} (buffer A containing 0.01% Nonidet P-40) for 6 h and applied to a HiTrap heparin column (5 ml; Pharmacia) equilibrated with 0.05 M NaCl in buffer A^{NP}. The column was washed with 15 ml 0.05 M NaCl in buffer A^{NP} and then subjected to an 85 ml linear gradient of 0.05–1 M NaCl in buffer A^{NP}. DNA polymerase III activity, eluting at 0.6 M NaCl, was pooled, dialyzed against 25 mM NaCl and 50% glycerol in buffer A^{NP} for 3.2 h, dispensed into small aliquots and frozen in liquid nitrogen. Purity of DNA polymerase III was >50% as judged by SDS–PAGE followed by silver staining. This preparation had a specific activity of 3.6 × 10^3 U/mg.

For purification of RF-C, 1 kg *S.cerevisiae* CB001 cells was disrupted and fraction III prepared (4). The conductivity was adjusted to 0.4 M NaCl in buffer A and applied to an Affi-Gel Blue column (2.6 × 12 cm; BioRad) equilibrated with 0.4 M NaCl in buffer A. The column was washed with 200 ml 0.4 M NaCl in buffer A and eluted with 200 ml 1.2 M NaCl in buffer A. Ammonium sulfate (0.516 g/ml) was added to the 1.2 M NaCl eluate and the precipitates collected by centrifugation in a Beckman JA20 rotor at 18 000 r.p.m. for 30 min. Half of the pellet

was resuspended in buffer A^{NP} and dialyzed against 2 l buffer A^{NP} for 4.3 h. The dialysate was adjusted to 0.1 M NaCl in buffer A^{NP} and clarified by centrifugation in a Beckman JA20 rotor at 10 000 r.p.m. for 10 min. The supernatant was applied to a ssDNA cellulose column (1.6 × 8 cm; Sigma) equilibrated with 0.1 M NaCl in buffer A^{NP}. The column was washed with 60 ml 0.1 M NaCl in buffer A^{NP} and then subjected to a 200 ml linear gradient of 0.1–0.7 M NaCl in buffer A^{NP}. Active fractions, eluting at 0.45 M NaCl, were pooled, dialyzed against 3 l 0.05 M NaCl in buffer A^{NP} for 3 h and applied to a HiTrap heparin column (1 ml; Pharmacia) equilibrated with 0.1 M NaCl in buffer A^{NP}. The column was washed with 6 ml 0.1 M NaCl in buffer A^{NP} and then subjected to a 20 ml linear gradient of 0.1–1 M NaCl in buffer A^{NP}. Active fractions, eluting at 0.65 M NaCl, were pooled, dialyzed against 3 l 0.05 M NaCl in buffer A^{NP} for 4.3 h and applied to a Mono Q HR 5/5 column (Pharmacia) equilibrated with 0.05 M NaCl in buffer A^{NP}. The column was washed with 5 ml 0.05 M NaCl in buffer A^{NP} and then subjected to a 10 ml linear gradient of 0.05–0.5 M NaCl in buffer A^{NP}. Fractions containing RF-C activity, eluting at 0.2 M NaCl, were dispensed into small aliquots and frozen in liquid nitrogen. Purity of RF-C was >50% as judged by SDS–PAGE followed by silver staining.

RF-A and PCNA were as previously described (29).

Protein sequencing

Purified DNA polymerase III from 4 kg *S.cerevisiae* CB001 cells was concentrated on a Mono S PC 1.6/5 column (Pharmacia) and fractionated by electrophoresis in a SDS–polyacrylamide gel as shown in Figure 1. After staining the gel with Coomassie brilliant blue the appropriate bands were excised and transferred to a 1.5 ml Eppendorf tube. Enough 0.2 M Tris–HCl, pH 9.5, 50% acetonitrile was added to the tube to cover the gel piece and the tube was incubated at 30°C with mixing for 30 min. This washing was repeated twice. Next, using disposable plastic pestles, the gel piece was ground until it was fine enough to pass through a 200 µl pipette tip and the polypeptide was eluted by incubation at 37°C overnight. The polypeptide eluted was digested in 0.2 M Tris–HCl, pH 9.5, containing 5 pmol lysyl endopeptidase (Wako) at 37°C overnight. To terminate proteolysis a 1/10 vol. 10% trifluoroacetic acid was added and the supernatant collected. Oligopeptides generated by the endopeptidase were separated by reversed phase high pressure liquid chromatography and subjected to amino acid sequencing in a PSQ-10 protein sequencer (Shimadzu).

Expression of Hys2 protein

The *Nco*I–*Eco*RI fragment of plasmid pAS1-CYH2 containing the *HYS2* gene (from K.Sugimoto, Nagoya University) was inserted into plasmid pET15b (Novagen). The constructed plasmid (pET15b-HYS2) contains the *HYS2* gene lacking 5% of its coding region at the C-terminus. *Escherichia coli* BL21 (DE3) cells transformed with pET15b-HYS2 plasmid DNA were grown at 37°C in LB + 0.1 mg/ml ampicillin (900 ml) to 8×10^8 cells/ml and 0.5 mM IPTG was added to the cell culture to induce the protein. After 3 h incubation cells were collected by centrifugation, suspended in 10 ml buffer A (lacking PMSF) and sonicated at 0°C. The cell lysate was then centrifuged at 8000 r.p.m. for 20 min in a Beckman JA20 rotor. The precipitates were resuspended in 10 ml of the same buffer and recentrifuged at 10 000 r.p.m. for 20 min. This step was repeated once. The precipitates were resuspended in the sample buffer and subjected to SDS–PAGE (30). The induced 35 kDa polypeptide (smaller than the expected size of 52 kDa for unknown reasons) was eluted from the gel and proved to be a part of the Hys2 protein by protein sequencing.

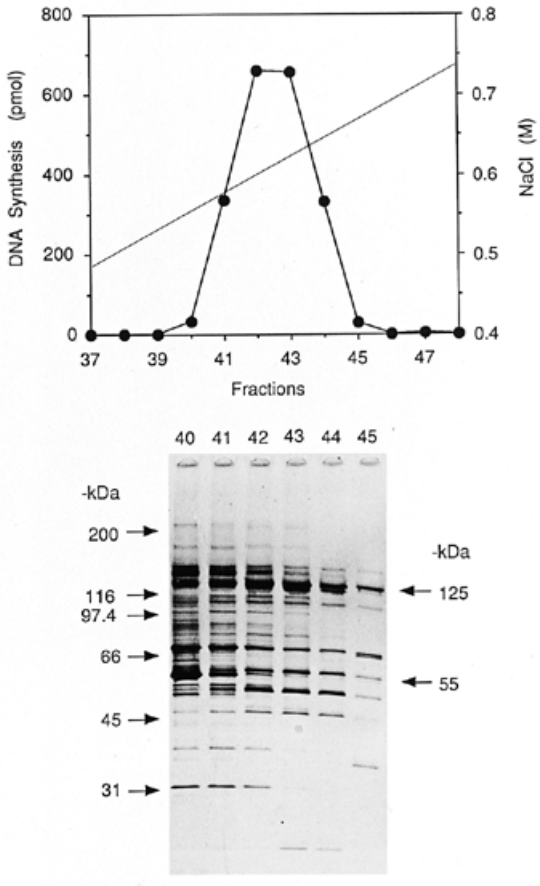

Figure 1. HiTrap heparin chromatography of DNA polymerase III. DNA polymerase III activity purified from yeast CB001 cells was chromatographed on a HiTrap heparin column (5 ml) as described in Materials and Methods. Aliquots of 5 µl of the column fractions were assayed for DNA polymerase activity at 30°C for 30 min using poly(dA)$_{300}$:oligo(dT)$_{10}$ (20:1) as template. Aliquots of 7.5 µl were electrophoresed in a 4–20% SDS–polyacrylamide gel and the proteins visualized by silver staining. Migration of the protein markers (myosin, *E.coli* β-galactosidase, rabbit muscle phosphorylase b, bovine serum albumin, hen egg white ovalbumin and bovine carbonic anhydrase) is indicated by the arrows on the left. The 125 and 55 kDa polypeptides co-eluting with DNA polymerase activity are indicated by the arrows on the right and were used for microsequencing.

Partial purification of DNA polymerase III

Yeast cells were grown in 6 l YPD medium at 25°C to 5×10^7 cells/ml, harvested by centrifugation, disrupted in a Waring Blendor HGB-SS in liquid nitrogen and fraction III prepared as described (4). The conductivity was adjusted to that of 0.05 M NaCl in buffer A and samples applied to a Mono Q HR 5/5 column (Pharmacia) equilibrated with 0.05 M NaCl in buffer A. The column was washed with 5 ml 0.05 M NaCl in buffer A and then subjected to a 20 ml linear gradient of 0.05–0.5 M NaCl in buffer A. The fractions containing DNA polymerase III activity (eluting at 0.2 M NaCl) were pooled, the conductivity adjusted to that of 0.1 M NaCl in buffer A and then applied to a Mono S PC 1.6/5 column (Pharmacia) equilibrated with 0.1 M NaCl in buffer A. The column was washed with 0.5 ml 0.1 M NaCl in buffer A and then subjected to a 2 ml linear gradient of 0.1–0.5 M NaCl in buffer A.

Isolation of multicopy suppressor genes of the temperature-sensitive *hys2-2* mutant

Saccharomyces cerevisiae strain N22 (*hys2-2 leu2*) was transformed to Leu$^+$ with a genomic library constructed in YEp13 (obtained from Y.Ohya, University of Tokyo) as described before (8) and incubated at 25°C for 1 day and then at 35.5°C for another 4 days. From ~80 000 Leu$^+$ transformants, 30 were able to grow at 35.5°C. Plasmid DNA was recovered from these transformants, analyzed by digestion with restriction endonucleases and found to be classified into two forms, pL13-127 and pL13-129 respectively. Both ends of the insert of the two plasmid DNAs were sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). It was found that pL13-127 contained the DNA fragment spanning nt 445 988–454 764 of chromosome X, including the *HYS2*, *SUI2*, YJR008w and *TDH2* genes, and pL13-129 contained the DNA fragment consisting of nt 271 429–282 482 of chromosome IV, including the *CDC2*, *DUN1*, *QRI1*, *QRI2* and *QRI7* genes. From this, suppression by pL13-127 should be due to complementation by *HYS2*. Therefore, pL13-129 was further characterized in this study. To determine which gene(s) of the insert is responsible for suppression of the mutant, subcloning of the fragments generated from the insert DNA were carried out and suppression was tested as described above.

Sequencing of mutant *hys2-1* and *hys2-2* alleles

The *HYS2* coding region of chromosomal DNA from yeast strain KSH542 (*hys2-1*) and N22 (*hys2-2*) was amplified by PCR using a range of oligonucleotide primers located within the open reading frame of *HYS2* synthesized by a Beckman automated DNA synthesizer. The amplified DNA fragments were purified from agarose gels and sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). In each case a single nucleotide change was detected, giving rise to a single amino acid change in each of the mutant proteins.

Other methods

Protein gels, Western blots and protein concentration determinations were carried out as described (4,29).

RESULTS

Identification of the 125 and 55 kDa polypeptides co-purified with DNA polymerase III activity

Unlike mammalian DNA polymerase δ, *S.cerevisiae* DNA polymerase III (δ) shows significant DNA polymerase activity on poly(dA)$_{300}$:oligo(dT)$_{10}$ in the absence of PCNA (4,9). Therefore, we used this template–primer for purification of DNA polymerase III as described in Materials and Methods. A major difference from our previous method (4) was to avoid performing hydrophobic interaction chromatography. The 125 and 55 kDa polypeptides, possibly as well as the 50 and 46 kDa polypeptides, co-purified with DNA polymerase III activity on HiTrap heparin chromatography, which is the last step of purification (Fig. 1). It was previously concluded that the catalytic polypeptide is encoded by the *CDC2* gene (10,11) and Cdc2 protein has both DNA polymerase and 3′→5′ exonuclease activities (12). However, the amino acid sequence has not been directly determined from the purified fractions of DNA polymerase III. Therefore, we determined the partial amino acid sequence of the 125 kDa polypeptide as well as the 55 kDa polypeptide (shown by arrows on the right hand side of Fig. 1). Amino acid sequences were obtained from the two oligopeptides derived from the 125 kDa polypeptide (Table 1). The sequences of peptides 1 and 2 correspond to amino acids 438–445 and 1086–1095 of Cdc2 protein (11,31) respectively. Thus this is the first demonstration of a direct link between the *CDC2* gene (EMBL accession no. X61920) product and the catalytic polypeptide of DNA polymerase III.

Table 1. Protein sequencing of the 125 and 55 kDa polypeptides co-purified with DNA polymerase III activity

Samples used for sequencing	Peptide number	Amino acid sequence
125 kDa polypeptide	1	ESVFSSKA
	2	ELQEKVEQLS
55 kDa polypeptide	3	SLPQQPFHK
	4	SLFDK

The polypeptide from Figure 1 was digested with lysyl endopeptidase and the resulting oligopeptides separated by reversed phase HPLC column chromatography. Each oligopeptide peak was subjected to microsequencing using a Shimadzu Protein Sequencer. Amino acids are represented by the single letter code.

Amino acid sequences of two oligopeptides were also obtained from the 55 kDa polypeptide, as shown in Table 1 and used for searching GenBank and *Saccharomyces* Genome databases. We found that the obtained sequences of peptides 3 and 4 completely matched with the sequence of the 14 amino acids 309–322 of the *HYS2* gene (DDBJ accession no. D50324) product, which is essential for cell viability and is suggested to play a role in DNA replication in *S.cerevisiae* (24). We therefore conclude that the 55 kDa polypeptide purifying with DNA polymerase III is encoded by *HYS2*.

Cdc2 and Hys2 proteins co-purify with DNA polymerase III activity

Polyclonal antibodies were generated against Hys2 polypeptide expressed in *E.coli* and were used to examine whether Hys2 protein co-purifies with DNA polymerase III activity during purification. CB001 wild-type cell extracts were fractionated by Q Sepharose HP column and subsequently by either Mono S or Superose 6 gel filtration column chromatography. DNA polymerase III activity eluted as the first and second peaks of DNA polymerase activity from the Q Sepharose HP column (Fig. 2A), while DNA polymerase I (α) eluted as the third peak and DNA polymerase II (ε) as the fourth and fifth peaks of DNA polymerase activity (4). The first peak (a) co-eluted with Cdc2 protein, but not with Hys2 protein. This activity was not related to either DNA polymerase I (α) or II (ε) as neither Pol1 nor Pol2 antibodies reacted with these fractions (data not shown). Therefore, these data strongly suggest that the activity present in the first peak represents a form of DNA polymerase III free from Hys2 protein and other unidentified DNA polymerases. We tried to further purify this activity by Mono S and HiTrap heparin chromatography, but because there was no significant DNA polymerase activity remaining with the purified Cdc2 polypeptide (data not shown) we do not know the exact nature of this activity. Cdc2 and Hys2 proteins were found in the second peak (b) of DNA polymerase activity by Western blotting with antiserum against Cdc2 and Hys2 proteins. When these fractions were further purified by either Mono S or Superose 6 column chromatography both Cdc2 and Hys2 proteins again co-purified

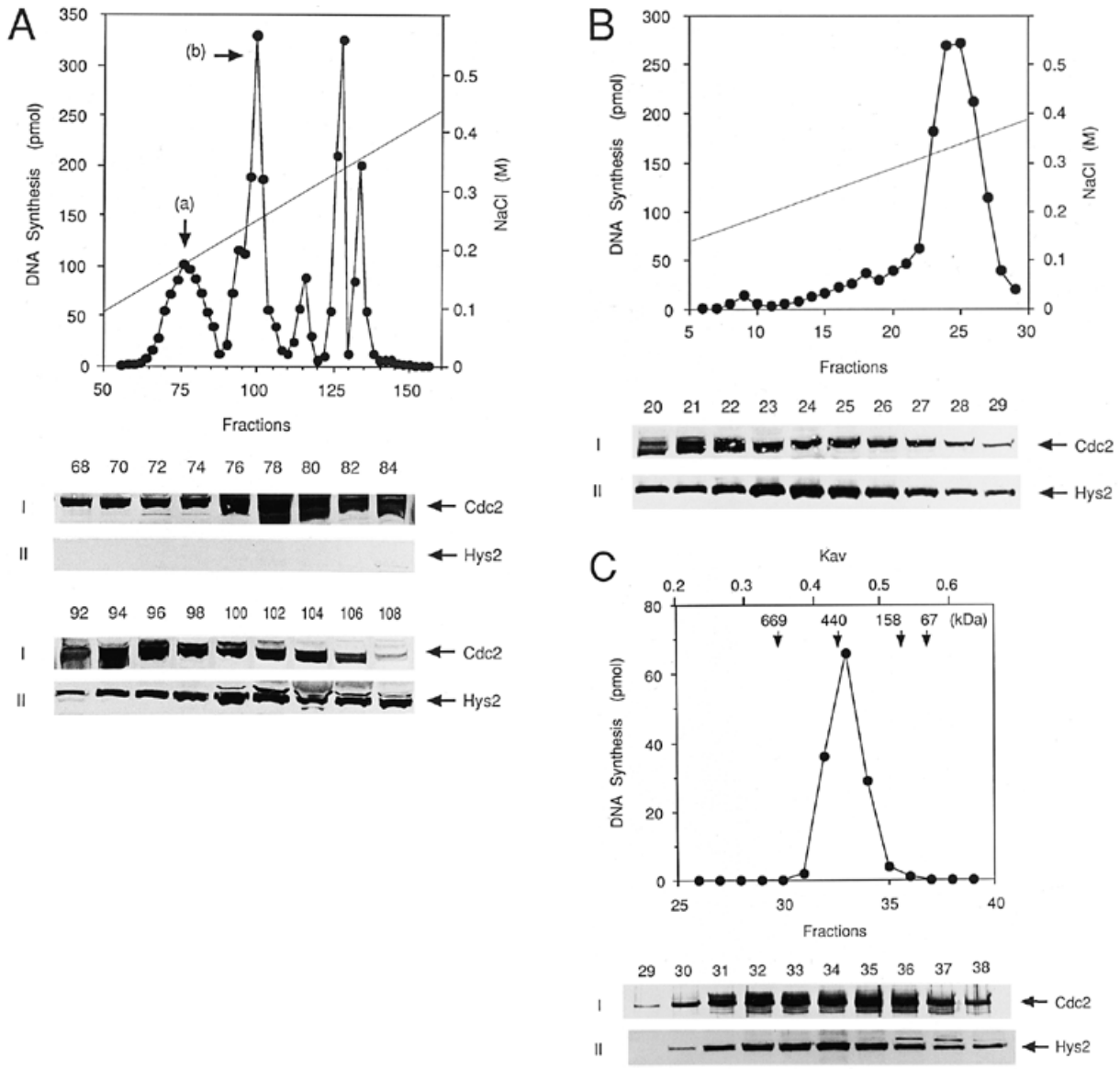

Figure 2. Q Sepharose HP, Mono S and Superose 6 chromatography of DNA polymerases from wild-type cells. (**A**) Yeast protein fraction enriched for DNA polymerase activity was isolated and chromatographed on a Q Sepharose HP BioPilot column as described in Materials and Methods. Aliquots of 5 µl of column fractions were assayed for DNA polymerase activity at 30°C for 30 min using poly(dA)$_{300}$:oligo(dT)$_{10}$ (20:1) as template. Aliquots of 7.5 µl were analyzed by Western blots with rabbit antiserum against the Hys2 and Cdc2 proteins. (**B**) DNA polymerase III activity from Q Sepharose HP peak b fractions (A) was chromatographed on a Mono S PC 1.6/5 column as described in Materials and Methods. DNA polymerase activity assay and Western blots were as in (A). (**C**) DNA polymerase III activity from Q Sepharose HP peak b fractions (A) was applied to a Superose 6 prep grade HR 16/50 column (Pharmacia) and eluted with 100 ml 0.2 M NaCl in buffer A. DNA polymerase activity assay and Western blots were as in (A). Elution of the protein markers (bovine thyroid thyroglobulin, horse spleen ferritin, rabbit muscle aldolase and bovine serum albumin) is indicated by the arrows.

with DNA polymerase III activity (Fig. 2B and C). The native mass of DNA polymerase III was estimated to be 300 kDa by Superose 6 gel filtration chromatography (Fig. 2C). Subsequent purification by HiTrap heparin column chromatography also showed co-purification of DNA polymerase III activity with Cdc2 and Hys2 proteins (Fig. 1). These results establish that Hys2 protein is a subunit of DNA polymerase III, together with Cdc2 protein.

DNA polymerase III activity from temperature-sensitive *hys2-1* mutant cells

Since *HYS2* is essential for cell growth and *hys2-1* temperature-sensitive cells are deficient in DNA replication (24), DNA polymerase III activity in *hys2* mutant cells may also be temperature sensitive. We fractionated DNA polymerase activities from thermosensitive KSH542-2 (*hys2-1 pep4*Δ) cell extracts. As shown in Figure 3A, two peaks (c and d) were obtained at ~0.2 M NaCl from *hys2-1* mutant cell extracts, as in wild-type cell extracts (Fig. 2A). However, the major peak of DNA polymerase activity which contained Hys2 and Cdc2 proteins could not be detected in *hys2-1* mutant cell extracts. Instead, Hys2 and Cdc2 polypeptides co-eluted in fractions which did not contain any significant DNA polymerase activity (Fig. 3A, fractions 18–20). Peaks c and d were shown to contain Cdc2 polypeptide but not Hys2 polypeptide by further purification by Mono S column chromatography (Fig. 3B). These results suggest that the Hys2-1 protein, although still found

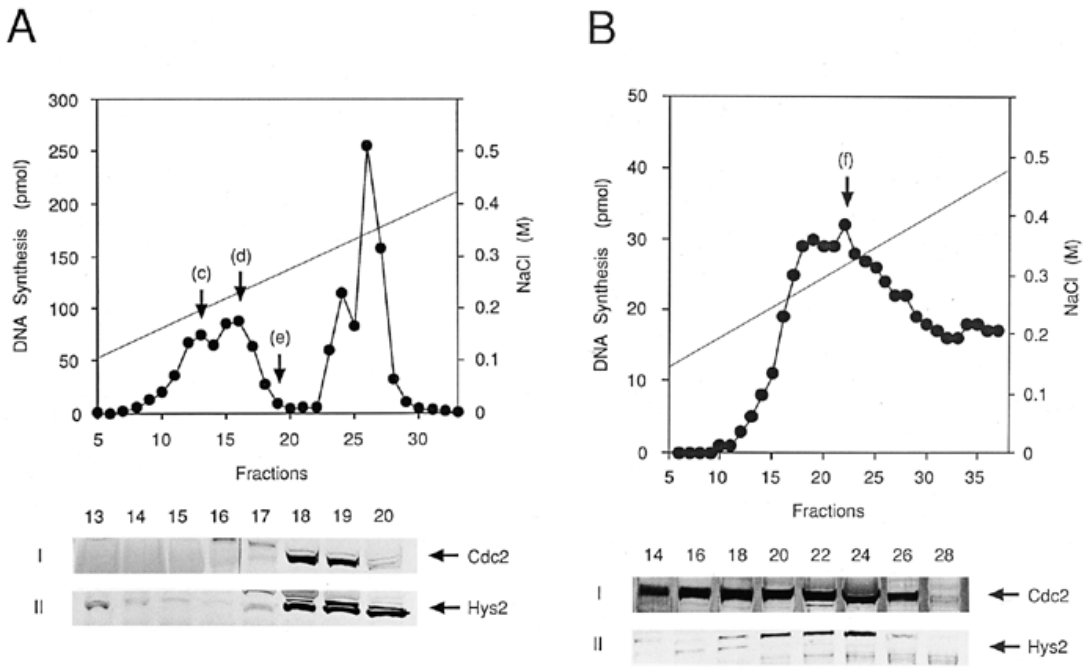

Figure 3. Mono Q and Mono S column chromatography of DNA polymerases from *hys2-1* mutant cells. (**A**) The extracts made from *hys2-1* mutant cells were subjected to column chromatography and DNA polymerase activity and Western blots were carried out as in Figure 2, except that Mono Q HR 5/5 was used instead of Q Sepharose HP BioPilot and DNA polymerase activity was assayed at 25°C for 60 min. (**B**) Peak d of the Mono Q fractions (fractions 15–17) was further applied to a Mono S column (PC1.6/5) and DNA polymerase activity was eluted as in Figure 2. DNA polymerase activity assay and Western blots were carried out as in (A). Peak c (fractions 12–14) of the Mono Q fractions was also subjected to further purification by Mono S column chromatography and similar results were obtained as for peak d (data not shown).

in a complex with Cdc2 polypeptide, deprives the complex of its polymerase activity. It may be possible that Hys2-1 protein is extremely thermosensitive and unstable, so that during either preparation of cell extracts or purification Hys2-1 protein is inactivated or denatured and the inactivated Hys2-1 protein inhibits DNA polymerase III activity.

We also fractionated DNA polymerase activities from thermosensitive *hys2-2* cell extracts as for the *hys2-1* mutant cell extracts. The results were very similar to Figure 3 (data not shown).

Highly processive DNA synthesis catalyzed by DNA polymerase III requires Hys2 protein

DNA polymerase III catalyzes highly processive DNA synthesis on poly(dA)$_n$:oligo(dT)$_{10}$ in the presence of PCNA (21). However, PCNA, RF-A and RF-C are required for processive DNA synthesis catalyzed by DNA polymerase III on a singly primed ϕX174 ssDNA template (14,15) and therefore protein–protein interactions between these protein complexes must be responsible for this processivity. As shown in Table 2, DNA synthesis activity of the peak b fraction, which contained co-eluting Cdc2 and Hys2 proteins (Fig. 2A), as well as that of purified DNA polymerase III (Fig. 1), was stimulated by PCNA, RF-A and RF-C (13–15). However, DNA synthesis catalyzed by other fractions (peaks a, c, d and f), which only contained Cdc2 protein, was not stimulated by addition of PCNA, RF-A and RF-C (Table 2). In addition, mixing fraction c or d with fraction e of *hys2-1* mutant cells, which contained both Hys2-1 and Cdc2 proteins, did not reconstitute PCNA, RF-A and RF-C-dependent DNA synthesis activity (Table 2). Therefore, we conclude that the highly processive DNA synthesis directed by PCNA, RF-A and RF-C requires Hys2 protein in the DNA polymerase III complex.

The *CDC2* gene on a multicopy plasmid suppresses temperature-sensitive cell growth phenotype of the *hys2* mutations

In order to isolate the gene products which interact with Hys2 protein we screened a yeast genomic library constructed on the multicopy plasmid YEp13 which suppress the temperature-sensitive growth phenotype of the *hys2* mutations as described in Materials and Methods. We isolated two different groups of plasmid which suppressed the mutations. We further characterized one of these plasmids. As shown in Figure 4A, pL13-129A and pL13-129B, but not pL13-129C, suppressed the *hys2-2* mutation. Furthermore, plasmids capable of suppressing the *hys2-2* mutation also suppressed the *hys2-1* mutation (Fig. 4), although the *hys2-1* mutant isolated by Sugimoto *et al.* (24) reverts at high frequency. On the other hand, plasmid YCp50, containing the *CDC2* gene, did not suppress the mutation (data not shown), suggesting that suppression is dependent on copy number of the *CDC2* gene on a plasmid. This suppression was very specific, as other DNA polymerase catalytic subunit genes, such as *CDC17* (or *POL1*) and *POL2*, on a multicopy plasmid did not suppress the mutations (data not shown). These results suggest that Cdc2 protein interacts with Hys2 protein and are consistent with the conclusion that Hys2 is the second subunit of DNA polymerase III (δ).

Table 2. DNA synthesis on a singly primed ϕX174 ssDNA template

Experiment	Polymerase fraction	Polymerase only (pmol)	+ RF-A (pmol)	+ RF-A + PCNA + RF-C (pmol)
1	Purified Pol III	9.3	24	129
2	Figure 2A peak a	35	8.8	6.9
3	Figure 2A peak b	23	9.1	108
4	Figure 3A peak c	27	9.3	3.6
5	Figure 3A peak d	23	8.7	4.3
6	Figure 3A peak e	8.1	2.9	3.1
7	Figure 3B peak f	30	8.4	5.7
8	1 µl peak c + 4 µl peak e	18	4.7	3.0
9	1 µl peak d + 4 µl peak e	19	4.5	3.0

Standard 50 µl DNA polymerase assays using singly primed ϕX174 ssDNA were carried out at 25°C for 15 min (experiments 1–7) or for 30 min (experiments 8 and 9) as described in Materials and Methods. They contained 220 ng purified DNA polymerase III (experiment 1), 2 µl (experiment 3) or 5 µl (experiments 2 and 4–9) DNA polymerase III fraction, RF-A (4.2 µg), PCNA (200 ng) and RF-C (130 ng).

Conversely, we tested whether the *HYS2* gene on a multicopy plasmid suppresses *cdc2* mutations. So far we have not been able to observe suppression of the temperature-sensitive cell growth phenotype of *cdc2* mutations (including *cdc2-1*, *cdc2-2* and *pol3-t*) by the *HYS2* gene on a multicopy plasmid (data not shown). When both *cdc2-2* or *pol3-t* and *hys2-2* mutations were combined mutant cells could not grow at 25°C, but did grow at 20°C (data not shown). These results are also consistent with the notion that Cdc2 protein interacts with Hys2 protein.

Sequence analysis of mutant *hys2* alleles

The mutation sites of the *hys2-1* and *hys2-2* alleles were determined by nucleotide sequencing as described in Materials and Methods. In each case we could only detect a single base change in the coding region of the *HYS2* gene, giving rise to a single amino acid change. In the *hys2-1* mutant a G at position 509 is changed to A, generating Gly170→Asp, while in the *hys2-2* mutant a G at position 910 is changed to A, producing Asp304→Asn (Fig. 5). The amino acid sequence of Hys2 protein is ~30% identical to the fission yeast Cdc1 protein and also to the small subunit of human and bovine DNA polymerase δ (32,33). Although the identical residues are spread throughout the proteins (32,33), both mutation sites found in the *hys2-1* and *hys2-2* alleles are at positions that are conserved in all species (Fig. 5), underlying their importance for Hys2 function.

DISCUSSION

Due to proteolysis and lack of a better purification procedure, *S.cerevisiae* DNA polymerase III has not been purified to homogeneity, unlike DNA polymerases I (α) and II (ε) (3,4),

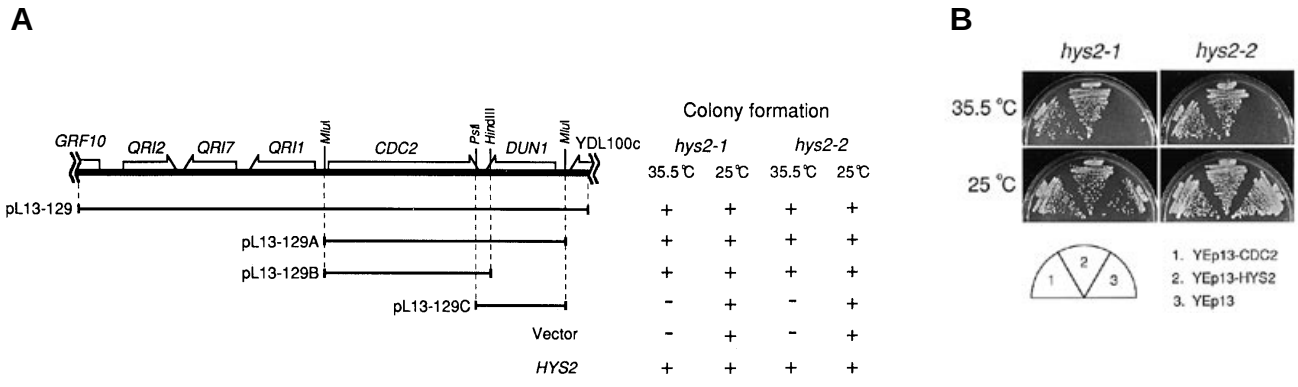

Figure 4. *CDC2* on a multicopy plasmid suppresses the temperature-sensitive growth phenotype of *hys2* mutations. (**A**) The insert DNA fragment of pL13-129 (shown by the top thin line) was digested with restriction endonucleases and subcloned into the multicopy plasmid yeast vector YEp13, resulting in pL13-129A, pL13-129B and pL13-129C. They were introduced into KSH542 (*hys2-1 leu2*) or N22 (*hys2-2 leu2*) cells by transformation and the transformants placed on SD-Leu plates at either 25 or 35.5°C for 3 days and cell growth examined. The figure also shows the genes located in the insert and their direction by an open arrow along with some representative restriction endonuclease sites. + indicates that cells grew at the indicated temperature, – indicates that cells did not grow at the temperature. (**B**) KSH542 (*hys2-1*) and N22 (*hys2-2*) transformed with either YEp13 vector, YEp13 vector containing the *CDC2* gene [*Mlu*I–*Hin*dIII fragment (A)] (YEp13-CDC2) or YEp13 vector containing the *HYS2* gene (24) (YEp13-HYS2) were streaked on SD-Leu plates, incubated at either 25 or 35.5°C for 3 days and then photographed.

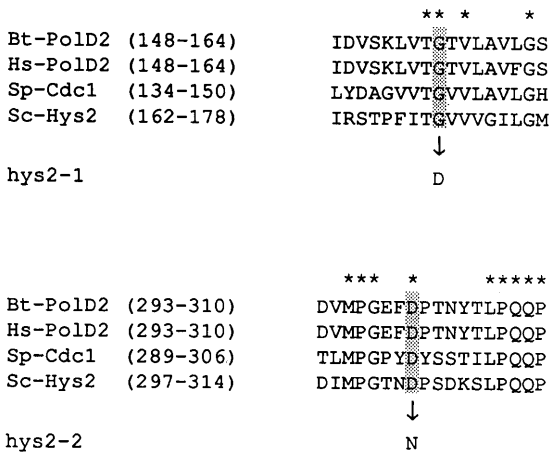

Figure 5. Mutation site of the *hys2-1* and *hys2-2* alleles. Mutation sites of the *hys2-1* and *hys2-2* alleles were determined as described in Materials and Methods. In each case a single base pair change in the coding region of *HYS2*, resulting in a single amino acid change, was detected as shown. Residues conserved in all four proteins are indicated by an asterisk above the aligned sequence and the number represents the amino acid residue number of each protein. Bt-PolD2, bovine DNA polymerase δ second subunit; Hs-PolD2, human DNA polymerase δ second subunit; Sp-Cdc1, *S.pombe* Cdc1 protein; Sc-Hys2, *S.cerevisiae* Hys2 protein.

although a few polypeptides were considered to be subunits of the polymerase (4,9). Our latest procedure for isolating *S.cerevisiae* DNA polymerase III (δ) yielded a highly purified protein sample as judged by SDS–PAGE followed by silver staining. Although several other protein bands were present that did not co-elute with DNA polymerase activity, we were able to identify and isolate 125 and 55 kDa polypeptides that did co-elute with DNA polymerase III activity (Fig. 1). Protein sequencing of the 125 kDa polypeptide provides the first direct demonstration that it is encoded by *CDC2*. Previous studies concluded that DNA polymerase III is encoded by *CDC2*, based upon the lack of DNA polymerase III activity in temperature-sensitive *cdc2-1* mutant cell extracts (10,11) and a thermosensitive DNA polymerase III activity present in temperature-sensitive *cdc2-2* mutant cell extracts (34).

The peptide sequences obtained from the 55 kDa polypeptide led us to conclude that it is encoded by the *HYS2* gene, an essential gene believed to be involved in DNA replication (24). Western blotting, using antibody raised against the *HYS2* gene product expressed in *E.coli*, confirmed this conclusion and also demonstrated that both Hys2 and Cdc2 proteins co-purify with DNA polymerase III activity, suggesting that the Hys2 and Cdc2 proteins are subunits of DNA polymerase III. Further support for identification of the Hys2 protein as a subunit of DNA polymerase III comes from homology between the *HYS2* gene and genes for the small subunit of DNA polymerase δ from bovine (32), human (32) and fission yeast (33,35).

We attempted to purify the DNA polymerase III complex from thermosensitive *hys2-1* and *hys2-2* mutant cells in order to demonstrate temperature-sensitive DNA polymerase activity. However, we could not detect any DNA polymerase III activity which contained both Hys2 and Cdc2 proteins in the mutant cell extracts after Mono Q chromatography (Fig. 3A and data not shown). Instead, we could detect fractions containing Cdc2 and Hys2 proteins, but no DNA polymerase III activity (Fig. 3A). Therefore, we could not demonstrate that DNA polymerase III is temperature-sensitive in *hys2* mutant cell extracts. These results may suggest that *hys2-1* and *hys2-2* proteins are extremely unstable and the proteins are inactivated during preparation of the cell extracts or column chromatography. As a result, inactivated *hys2-1* or *hys2-2* protein inhibits DNA polymerase III activity. Interestingly, DNA polymerase III activity that contained the Cdc2 polypeptide but not Hys2 protein could be detected in the mutant cell extracts (Fig. 3) as well as in wild-type cell extracts (Fig. 2). This activity was not temperature sensitive (data not shown). This is the first case in yeast that another form of DNA polymerase III, free from Hys2 protein, has been detected in yeast cell extracts and suggests that it may have a different function from the complex form of DNA polymerase III, such as DNA repair and/or recombination. This form of DNA polymerase III was not stimulated by PCNA, RF-C and RF-A (Table 2), suggesting that it does not participate in the highly processive DNA synthesis mediated by these factors. Alternatively, this form of DNA polymerase III might be a degradation product of intact DNA polymerase III complex which has lost Hys2 from the complex. Nevertheless, the complex form of DNA polymerase III activity containing both Cdc2 and Hys2 proteins was stimulated by addition of PCNA, RF-A and RF-C (Table 2). This result suggests that Hys2 protein in the DNA polymerase III complex plays a role in interaction between PCNA and RF-C. Our observation is similar to those of Goulian *et al.* for mouse DNA polymerase δ (36) and Zhou *et al.* for human DNA polymerase δ (37,38), but may differ from that of Brown and Campbell for *S.cerevisiae* DNA polymerase III (39). Brown and Campbell have shown that PCNA stimulates the bacterially produced catalytic subunit of DNA polymerase III and increases its processivity on poly(dA)$_n$:oligo(dT)$_{10}$. This difference may be due to it being bacterially produced DNA polymerase.

Screening of a yeast genomic library has so far yielded two different multicopy suppressors of the temperature-sensitive *hys2-2* mutation. One of them is the *CDC2* gene. When present on a multicopy plasmid, but not on a low copy YCp plasmid, the *CDC2* gene encoding the catalytic subunit of DNA polymerase III (δ) suppresses the temperature-sensitive *hys2-1* and *hys2-2* mutations (Fig. 4). Furthermore, the double mutant of *hys2-2* and *cdc2-2* or *pol3-t* was synthetic lethal, consistent with the conclusion that Hys2 protein is a subunit of DNA polymerase III (δ). Genetic interaction between *CDC2* and *HYS2* is similar to that seen in the fission yeast *Schizosaccharomyces pombe* between *pol3*$^+$, the Cdc2 homolog, and *cdc1*$^+$, the Hys2 homolog (33). Interestingly, the *cdc1*$^+$ gene also interacts with the *cdc27*$^+$ gene in *S.pombe* (33). This may suggest that the *cdc27*$^+$ gene product is the third subunit of DNA polymerase δ. Recently Zuo *et al.* showed that the *cdc27*$^+$ gene product is part of the DNA polymerase δ complex of *S.pombe* (35). However, the function of the *cdc27*$^+$ gene is not known and an obvious structural homolog has not been found in budding yeast. We observed that two additional polypeptides with molecular weights of ~50 kDa also seem to co-purify with DNA polymerase III activity as well as Cdc2 and Hys2 polypeptides (Fig. 1). Interestingly, the second multicopy suppressor of the *hys2-2* mutation was found to be the gene of *YJR043C*, which is located on chromosome X of *S.cerevisiae* and is not essential for cell growth (Nakashima,N. and Sugino,A., unpublished results). The predicted amino acid sequence of the gene product (expected mol. wt 40 312) shows ~18% homology to that of *S.pombe* Cdc27 protein (predicted mol. wt 42

350) and may correspond to the polypeptide of molecular weight ~46 kDa (Fig. 1). However, we do not know whether this is a homolog of *S.pombe* Cdc27 protein yet, as homology is scattered thoughout the polypeptides. Nonetheless, further genetic and biochemical studies are needed to clarify whether additional subunits of DNA polymerase III exist in *S.cerevisiae*.

By Western blotting with antiserum against Cdc2 and Hys2 polypeptides we could detect several additional polypeptides which strongly cross-reacted with the antiserum, besides the Cdc2 and Hys2 proteins (Figs 2 and 3). In the case of the Cdc2 antiserum some of them migrated faster than the Cdc2 protein on SDS–PAGE. These could be degradation products of the Cdc2 polypeptide and did not have DNA polymerase activity. In the case of the Hys2 antiserum the band slowly migrating on SDS–PAGE strongly cross-reacted with the antiserum and may be a post-translationally modified product of the protein. However, this form of the protein might not be functionally significant, as DNA polymerase III activity was not stimulated by addition of RF-C, RF-A and PCNA (Fig. 3B and Table 2). Alternatively, these bands might be unrelated to the protein, since we did not affinity purify the antiserum.

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