Cloning $DPB3$, the gene encoding the third subunit of DNA polymerase II of Saccharomyces cerevisiae

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ABSTRACT

DNA polymerase II purified from Saccharomyces cerevisiae contains polypeptides with apparent molecular masses of $>200, 80, 34, 30$ and $29$ kDa, the two largest of which (subunits A and B) are encoded by the essential genes $POL2$ and $DPB2$. By probing a $\lambda gt11$ expression library of yeast DNA with antiserum against DNA polymerase II, we isolated a single gene, $DPB3$, that encodes both the $34$- and $30$-kDa polypeptides (subunit C and C'). The nucleotide sequence of $DPB3$ contained an open reading frame encoding a $23$-kDa protein, significantly smaller than the observed molecular masses, $34$- or $30$-kDa, which might represent post-translationally modified forms of the $DPB3$ product. The predicted amino acid sequence contained a possible NTP-binding motif and a glutamate-rich region. A $dpb3$ deletion mutant ($dpb3\Delta$) was viable and yielded a DNA polymerase II lacking the $34$- and $30$-kDa polypeptides. $dpb3\Delta$ strains exhibited an increased spontaneous mutation rate, suggesting that the $DPB3$ product is required to maintain fidelity of chromosomal replication. Since a fifth, $29$-kDa polypeptide was present in DNA polymerase II preparations from wild-type cell extracts throughout purification, the subunit composition appears to be A, B, C (or C and C') and D. The $5'$ nontranscribed region of $DPB3$ contained the $MiU$-related sequence $ACGCGA$, while the $0.9$-kb $DPB3$ transcript accumulated periodically during the cell cycle and peaked at the G1/S boundary. The level of $DPB3$ transcript thus appears to be under the same cell cycle control as those of $POL2$, $DPB2$ and other DNA replication genes. $DPB3$ was mapped to chromosome II, $30$ cM distal to $his7$.

INTRODUCTION

The yeast Saccharomyces cerevisiae has three distinct, essential nuclear DNA polymerases (1–4). DNA polymerase I is similar in structure and activity to mammalian DNA polymerase $\alpha$ (5,6), which has been proposed to replicate the lagging strand of chromosomal DNA (7). DNA polymerase III is similar to mammalian DNA polymerase $\delta$ (8), which has been proposed as the leading strand replicase (7). Although DNA polymerase II was first described in 1970 (9–11), its structure and function were until recently unknown. DNA polymerase II purified to near-homogeneity contains polypeptides with apparent molecular masses, $>200, 80, 34, 30$ and $29$ kDa (12). The genes encoding the $>200$-kDa catalytic subunit A and the $80$-kDa subunit B have been cloned (4,13). Both genes are essential for cell growth and the thermosensitive mutants exhibit defective DNA synthesis at the restrictive temperature, suggesting that the DNA polymerase II holoenzyme participates directly in chromosomal DNA replication (4,13; and Araki et al., in preparation). Based on these results we have proposed a new model for the replication of DNA by three DNA polymerases (4). In this model, DNA polymerase I initiates synthesis at the origin and synthesizes DNA primers on the lagging strand, DNA polymerase II elongates the leading strand, and DNA polymerase III elongates the lagging strand. Yeast DNA polymerase II shares some similarities with mammalian DNA polymerase e (previously described as $62$ polymerase), an enzyme previously suggested to be involved in DNA repair in human cells (14).

In this report we describe the cloning, sequencing and genetic analysis of $DPB3$, the gene encoding the third subunit of DNA polymerase II. We found that both the $34$- and $30$-kDa polypeptides of purified DNA polymerase II are from the same gene, $DPB3$. Since $30$-kDa polypeptide seemed to be a proteolytic product of $34$-kDa polypeptide and the $29$-kDa polypeptide also appears to be a subunit of DNA polymerase II, DNA polymerase

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II thus contains four subunits. DPB3 is dispensable for cell replication, but is required for normal fidelity of chromosome replication, and the level of its transcript appears to be under the same cell cycle control as those of POL2 (Araki et al., in preparation). DPB2 (13) and other DNA replication genes (for reviews, see 15 and 16).

MATERIALS AND METHODS
Bacterial and yeast strains
Escherichia coli DH5α (17) was used for the propagation of plasmids. Y1090 (18) and LE392 (17) were used for λgt11 and λCharon28A phages, respectively. S. cerevisiae strains were YHA1 (MATa/MATa leu2-3,-112/leu2-3,-112 trp1-289/trp1-289 ura3-52/ura3-52 his7-2/+ +/can1) (4), BJ3501 (MATα pep4::HIS3 prb1Δ1.6 his3-Δ200 ura3-52 can1 gal7) (from E. W. Jones, Carnegie-Mellon University, PA), L155-1B (19), YHA3 (MATa ade5 leu2 metl-1 trp1 ura3-52), CG379(MATα ade5 his7-2 leu2-3,112 ura3-52) (from C. Giroux, Wayne State University, MI), XSS03-2C (MATα his1-7 hom3-10 leu2-3, 112 ura3-52 can1), and AMY50-1B (MATα lys1-1 his1-7 ade2-1 hom3-10 leu2-1 ura3-52).

Yeast genomic libraries and plasmid DNAs
λgt11 and λCharon28A S. cerevisiae genomic DNA libraries were described previously (4,20). Plasmid pBS(SK+) (Stratagene, La Jolla, CA) was used for subcloning and DNA sequencing. Plasmid pGB310 contains the S. cerevisiae URA3 gene, excisable by EcoRI, BamHI, HindIII, SmaI, and SacI (obtained from C. Giroux).

Biochemical and genetical methods
Antibody screening of λgt11 yeast genomic library (18), screening of λCharon 28A library with a DNA probe (17), double-stranded DNA sequencing by dideoxy-termination method (21), Western and Northern blottings were described previously (4). Partial purification of DNA polymerase II activity was as described (4,12). Synchronization of yeast cells by mating pheromone α, feed-starve, and elutriator centrifugation and the preparation of genomic DNA libraries were described previously (4,20). Plasmid pBS(SK+) DNA treated with KpnI, Bsrfl, EcoRI, HindIII, and 0.22-Kb HindIII fragment of λgt11-2 (lane 1) or with the antisera purified with antigens produced by λgt11-2 (lane 1) or λgt11-7 (lane 2) clones. The immuno-reacted polypeptides were visualized with alkaline-phosphatase conjugated anti-mouse IgG (Promega).

Figure 1. The purified DNA polymerase II (12) was separated by 4—20% SDS-PAGE and transferred to a Immobilon membrane filter as published (12). The filter was incubated either with 1000-fold diluted mouse antisem against polymerase II (lane 3) or with the antisem purified with antigens produced by λgt11-2 (lane 1) or λgt11-7 (lane 2) clones. The immuno-reacted polypeptides were visualized with alkaline-phosphatase conjugated anti-mouse IgG (Promega). On the right the polypeptide of DNA polymerase II is shown. Mouse antisem against polymerase II did not recognize the D subunit.

Rabbit antibodies against yeast DNA polymerase II
The mouse antisem against yeast DNA polymerase II (12) did not recognize the 29-kDa polypeptide of DNA polymerase II (12). Therefore, we have tried to raise rabbit antisem against DNA polymerase II (12) as described (24). This rabbit antisem recognizes not only the >200-kDa, 80-kDa, 34-kDa and 30-kDa polypeptides previously recognized by the mouse antisem, but also the 29-kDa polypeptide (see Fig. 6) by Western blotting.

Measurement of spontaneous mutation rates
In preliminary experiments, spontaneous reversion frequencies were monitored by a patch test, in which patches of cells (about 10 cm²) grown on YPDA were replica plated onto omission media and revertants counted after five to ten days. Patches of DPB3+ cells yielded an average of one Lys+ revertant, and 40 His+ revertants. Subsequently, spontaneous reversion rates were quantitated using the Leningrad test (25). Yeast cells grown in YPDA medium to stationary phase were washed, suspended in water at densities of approximately 10⁶-10⁷ cells/ml, and applied to plates using a multipronged replicator. Five-to-six hundred compartments were replicated for each determination. The plates contained synthetic medium either lacking or containing a limiting amount of either lysine, adenine or histidine. Limiting concentrations were 2 μg/ml histidine, 5 μg/ml lysine, 4868 Nucleic Acids Research, Vol. 19, No. 18
RESULTS

Cloning the gene for DNA polymerase II subunit C

It was very difficult to obtain enough DNA polymerase II to raise specific antibodies against each subunit of the polymerase (see ref. 12). Therefore, a λgt11 library of yeast genomic DNA was probed with mouse antiserum against DNA polymerase II (12). From approximately 6 x 10⁶ recombinant plaques, 29 positive λgt11 phages have been isolated. Antibody was affinity-purified using the positives as described by Snyder et al. (18) and used to probe a Western blot of DNA polymerase II to identify which of the subunits was being expressed by the recombinant λgt11 phage. Seventeen λgt11 phages had part of the gene for subunit B, as described previously (13). Eleven other positives generated affinity-purified antibody that reacted with both the 34- and 30-kDa polypeptides of DNA polymerase II but not with subunits, A and B. Figure 1 shows examples of such results. And one positive was not able to be assigned to any specific polypeptide of DNA polymerase II.

Since Western blotting indicated that each of these eleven λgt11 clones produced a fusion protein between β-galactosidase and either 34- or 30-kDa polypeptide, the clones seemed likely to clones produced a fusion protein between β-galactosidase and either 34- or 30-kDa polypeptide, the clones seemed likely to have only a part of the gene for either 34- or 30-kDa polypeptide. Thus intact gene was cloned from a XCharon28A yeast genomic DNA library by plaque lift hybridization using the 1.3-kb EcoRI insert of λgt11-2 (Fig. 2) as a probe. From approximately 10,000 recombinant plaques, twenty positives were obtained. All generated DNA containing a common 5-kb BamHI insert that hybridized to the 1.3-kb EcoRI fragment (Fig. 2). The restriction map of this BamHI fragment was identical to that of chromosomal DNA, suggesting that no rearrangement had occurred during cloning. Since the 5-kb BamHI fragment hybridized only to the same size BamHI fragment of yeast chromosomal DNA, this is a single-copy gene. The 5-kb BamHI fragment was subcloned into pBS(SK +) and the region encompassing the 1.3 kb EcoRI fragment was sequenced. The nucleotide sequence revealed one open reading frame encoding a protein of calculated molecular weight 23,005 (Fig. 3). We named this gene DPB3 (DNA Polymerase B subunit 3). The calculated molecular weight of the DPB3 gene product is substantially less than the observed molecular masses of the 34- or 30-kDa polypeptides, suggesting that the gene product either migrates anomalously in gel electrophoresis or is post-translationally modified. The 30-kDa polypeptide might be modified differently than the 34-kDa polypeptide, or might simply be a proteolytic product of the 34-kDa polypeptide.

The deduced amino acid sequence of DPB3 has a region of thirty-five residues (residues 120—154) containing 63% acidic residues—primarily glutamate—including a run of fourteen residues that contains eleven glutamate and one aspartate residue. Also, a possible nucleoside triphosphate-binding consensus sequence (26) occurs in the amino-terminal half of the predicted sequence (Fig. 4). However, the amino acid sequence of DPB3 had no significant similarity to any protein appearing in GenBank, NBRF-Nucleic acid and -protein Bank, EMBL Gene Bank, or SwissProt Bank, except for a kind of heat-shock proteins, such as chicken Hch08 (27), hamster glucose-regulated protein grp94 (28) and Trypanosoma cruzi heat-shock protein 90-kDa homolog (29). These similarities are limited to the acidic region of DPB3.

DPB3 is dispensable

The disrupted DPB3 gene, dpb3Δ, was constructed by replacing the 0.32-kb EcoRI-HindIII and 0.22-kb HindIII DNA fragments of DPB3 with a 1.2-kb fragment containing the URA3 gene from plasmid pGB310 (Fig. 2). One wild-type DPB3 copy of a homozygous diploid strain (YHA1) was replaced with the disrupted gene using the one-step gene replacement method (22). After confirming this construction by Southern blotting, a DPB3/dpb3Δ diploid was sporulated and the resulting tetrads were dissected. Out of twenty tetrads, twelve tetrads showed four viable spores, seven showed three viable spores and one gave two viable spores. The total numbers of Ura⁺ and Ura⁻ spores were 35 and 36, respectively, and therefore any defect in
germination of the dpb3Δ spores is unlikely. No growth rate difference was detected between wild type and dpb3Δ cells. Furthermore, as the homoallelic diploid (dpb3Δ/dpb3Δ) sporulated normally at 30°C and spore viability was more than 80%, DPB3 is also dispensable for sporulation.

Mapping of DPB3

Yeast chromosomes were separated by transverse alternating field electrophoresis, transferred to a nitrocellulose filter, and hybridized to 32P-labeled DPB3 DNA. Only chromosome II hybridized to the probe (data not shown). To map the DPB3 gene genetically, URA3 in the dpb3Δ disruptant was followed as described (30). The DPB3 gene was located 30 cm from his7 (the distribution of parental dihybrids: tetratype: non-paternal dihybrid tetrad was 36:1:41) and 52 cm from met8 (the tetrad distribution was 12:3:27) on the right arm of chromosome II. Since no gene maps at this locus (31), DPB3 is a newly identified gene.

dpb3Δ is a modest mutator

We initially used a semi-quantitative patch test to assess the effect of the dpb3Δ allele on spontaneous reversion. Three independent dpb3Δ derivatives of AMY50-1B were compared with YCp50 (30) transformants of AMY50-1B obtained in the same transformation. dpb3Δ increased the average frequency of lys1-1 revertants about six-fold (three experiments) and that of his7-1 about two-fold (two experiments). We observed no significant effect of dpb3Δ on growth, lethality of UV irradiation or methyl methane sulfonate, or on UV-induced reversion of lys1-1 or his7-1 (data not shown). Spontaneous reversion rates were quantitated using the Leningrad test (25) for a derivative of AMY50-1B for reversion of lys1-1, ade2-1, and his7-1, and for a dpb3Δ derivative of XS803-2C for reversion of his7-1. The results, shown in Table 1, indicate that dpb3Δ elevated the spontaneous reversion rate by factors of 2.2, 2.6 and 20 at his7-1, ade2-1 and lys1-1, respectively.

The abundance of the DPB3 transcript fluctuates during the cell cycle

A Northern blot of polyA-RNA separated by formaldehyde-agarose gel electrophoresis was probed with the 0.3 kb EcoRI-HindIII fragment of DPB3. A 0.9-kb transcript hybridized to the probe (data not shown). The steady-state levels of this 0.9-kb transcript were followed before and after synchronization of yeast cells with the α-factor mating pheromone. As shown in Fig. 5, the abundance of DPB3 mRNA fluctuated in a cell-cycle dependent manner and peaked at the same time as the transcript of CDC9 (32) as well as other DNA synthesis related gene transcripts (32). We also used two other methods of synchronizing yeast cells, namely the Feed-Starve protocol (33) and elutriator centrifugation (32). The results with these two synchronizations were essentially the same as with mating factor synchronization (data not shown). Therefore, it is unlikely that the periodic accumulation of the DPB3 transcript during the cell cycle is a synchronization artifact.

DNA polymerase II from dpb3Δ cells

DNA polymerase II (DNA polymerase II* in ref. 12) activity was partially purified from cells bearing the dpb3Δ gene. The activity eluted reproducibly earlier (at 0.29 M NaCl) than wild-type polymerase II (at 0.34 M NaCl) from a MonoQ column and later (at 0.32 M NaCl) than wild-type polymerase II (at 0.27 M NaCl) from a MonoS column (data not shown). The activity recovered in polymerase II fraction from dpb3Δ cells were reproducibly less than 50% of that from wild-type cells. The active fractions from the MonoQ column (Fig. 6A) were subjected to Western blotting followed by probing with rabbit antiserum against DNA polymerase II. As shown in Fig. 6B, subunits A and B coeluted with the DNA polymerase II activity. However, the 34-kDa (C) and 30-kDa (C') polypeptides found in DNA polymerase II from wild-type cells (12 and Fig. 6C) were not detected. Note that the membrane filter of Fig. 6B was developed twice longer than that of Fig. 6C in order to make sure absence of C and C' polypeptides. Thus, the catalytic subunit A of DNA polymerase II (>200 kDa polypeptide) forms a stable complex with subunit B in the absence of both the 34- and 30-kDa polypeptides, while the 29 kDa polypeptide seemed to be dissociated from the complex (Fig. 6B). The control experiment with wild-type cells (Fig. 6C) showed that, while the 29-kDa polypeptide was copurified with the polypeptides corresponding to subunits A, B and C on a MonoS column, the amount of the 30-kDa polypeptide (C') was considerably less than that of the most purified DNA polymerase II (12). Thus, it is highly possible that the 30-kDa polypeptide is a degradation product of the 34-kDa subunit C. The result strongly suggests that the 29 kDa polypeptide is a subunit of DNA polymerase II (subunit D).

Table 1. Spontaneous reversion rates of dpb3Δ mutant cells

<table>
<thead>
<tr>
<th>Parent strain</th>
<th>Revertible Marker</th>
<th>Spontaneous Reversion Rate (×10⁶)</th>
<th>DPB3</th>
<th>dpb3Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMY50-1B</td>
<td>lys1-1</td>
<td>0.28 ± 0.18</td>
<td>5.7 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>AMY50-1B</td>
<td>ade2-1</td>
<td>1.8 ± 0.1</td>
<td>4.7 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>XS803-2C</td>
<td>his1-7</td>
<td>4.5 ± 0.8</td>
<td>9.8 ± 2.2</td>
<td></td>
</tr>
</tbody>
</table>

Numbers are the mean ± standard deviation of three determinations.
each) in MonoS were analyzed by Western blotting as (B) except for the filter result was very reproducible.

MonoQ and MonoS columns as described in (A) and the active fractions (20 nmoles of DNA polymerase II is shown on the right. The strong signal migrated approximately 116.5 kDa is a non-specific band reacted with rabbit antiserum. (C) The DNA polymerase II varied considerably from preparation to preparation and varied at the stage of the purification, the polypeptide is likely a degradation product of the 34-kDa polypeptide (subunit C). On the other hand, the 29-kDa polypeptide (subunit D) copurified with the subunits A, B and C throughout the purification. Thus, the subunit structure of the DNA polymerase II is A, B, C and D.

A possible nucleoside triphosphate-binding consensus sequence is identified in the DPB3 gene. However, there was no detectable NTPase, or DNA (or RNA) helicase activity in the nearly homogeneous DNA polymerase II (12). Thus, it is less likely that the DPB3 polypeptide is either a NTPase or helicase, although it is still possible that other DNA polymerase II subunits mask the activity. Alternatively, the DPB3 polypeptide may bind to NTP to promote the DNA polymerase II formation in vivo. Availability of each DNA polymerase II subunit gene (4,13), and these polypeptides are absent from DNA polymerase II from dpb3Δ mutant cells (Fig. 6B), strongly suggesting that both polypeptides are the product of DPB3. The observed molecular mass of each polypeptide was significantly greater than predicted from the nucleotide sequence (Fig. 3). These polypeptides, therefore, might migrate abnormally in SDS-polyacrylamide gel electrophoresis, with the 30-kDa polypeptide (C') possibly being a proteolytic product of the 34-kDa polypeptide (subunit C). Alternatively, they might be modified post-translationally and the difference in migration may reflect the extent and/or the type of modification. Since the amount of the 30-kDa polypeptide in the DNA polymerase II varied considerably from preparation to preparation and varied at the stage of the purification, the polypeptide is likely a degradation product of the 34-kDa subunit C. On the other hand, the 29-kDa polypeptide (subunit D) copurified with the subunits A, B and C throughout the purification. Thus, the subunit structure of the DNA polymerase II is A, B, C and D.

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The abundance of the DPB3 transcript fluctuated during the cell cycle and peaked at the same time as CDC9 (Fig. 5). The 5' upstream region of DPB3 contains the 5' ACGCGT3'-related sequence, ACGCGA (Fig. 3). It has been strongly suggested that the sequence ACGCGT (the recognition sequence of restriction enzyme MluI) has an important role in the coordinate expression during the cell cycle of genes involved in DNA replication (15, 16). Included within this group are the genes for all four subunits of DNA polymerase I (6,19,35; and Hinkle, D., personal communication), DNA polymerase III catalytic subunit (CDC2) and yeast PCNA (POL30 (36), and the three subunits of DNA polymerase II (13; this study and Araki et al., in preparation),
suggesting that expression of the genes for nuclear DNA polymerases and their associated subunits are coordinately regulated during the cell cycle and turned on prior to the initiation of chromosomal DNA replication.

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