A primase activity which permits DNA synthesis by yeast DNA polymerase I on a single-stranded circular øX174 or M13 DNA or on poly(dT)ₙ, has been extensively purified by fractionation of a yeast enzyme extract which supports in vitro replication of the yeast 2-µm plasmid DNA (Kojo, H., Greenbaum, B. D., and Sugino, A. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 7261-7265). Most of this DNA primase activity was separated from DNA polymerase activity, although a small amount remained associated with DNA polymerase I. The primase, active as a monomer, has a molecular weight of about 60,000. The primase synthesizes oligoribonucleotides of discrete size, mainly eight or nine nucleotides, in the presence of single-stranded template DNA and ribonucleoside 5'-triphosphates; it utilizes deoxyribonucleoside 5'-triphosphates as substrate with 10-fold lower efficiency. Product size, chromatographic properties, α-amanitin resistance, and molecular weight of the primase activity distinguish it from RNA polymerases I, II, and III. The DNA products synthesized by both primase and DNA polymerase I on a single-stranded DNA template were 200–500 nucleotides long and covalently linked to oligoribonucleotides at their 5'-ends. Addition of yeast single-stranded DNA-binding protein (Arendes, J., Kim, K. C., and Sugino, A. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 673–677) stimulated the DNA synthesis 2-3-fold.

The yeast Saccharomyces cerevisiae has at least two distinct DNA polymerases, I and II (1, 2). DNA polymerase I is a true DNA replicase (3), whereas DNA polymerase II is believed to be a repair enzyme (2). As with all other known DNA polymerases (4), neither yeast polymerase initiates DNA synthesis on a single-stranded circular DNA. Since yeast chromosomal replication is discontinuous and proceeds bidirectionally with the synthesis of Okazaki fragments (5), these are presumably initiated by RNA primers. Therefore, a DNA primase activity similar to the Escherichia coli dnaG product is believed to exist in yeast and other eukaryotes (6).

An in vitro 2-µm yeast plasmid DNA replication system has recently been developed and shown to mimic in vivo replication (7–9). Biochemical fractionation of this activity was undertaken to identify and purify yeast DNA replication proteins (10). Since the in vitro DNA replication is discontinuous, it was expected that yeast DNA primase could be identified among the activities of the extract. In the course of this study an activity has been purified which is absolutely required for a full reconstitution of the in vitro replication. This activity was separable from both DNA and RNA polymerase activities. It polymerized oligoriboro- or oligodeoxyribonucleotides on a single-stranded circular DNA, and such oligomers were utilized as primers by yeast DNA polymerase I as well as by other DNA polymerases tested. In this paper, we describe the purification and characterization of this yeast DNA primase activity. Similar activities have recently been identified in several eukaryotic systems (11–16); most of these activities were found to be associated with DNA polymerase α or α-like DNA polymerase (12–15). In yeast, a similar primase activity can be detected in the DNA polymerase I preparation (17, 18). However, its association with the polymerase seems to be weaker than in other systems.

EXPERIMENTAL PROCEDURES

Materials

Chemicals

[methyl-3H]dTTP, [α-32P]dNTPs, [α-32P]GTP, [α-32P]UTP, and [γ-32P]ATP were purchased from Amersham Corp. [3H]dATP was from New England Nuclear. α-Amanitin was a gift of Dr. Robert A. Voelker of this institute. Aphidicolin was provided by Dr. A. H. Todd of Imperial Chemical Industries, Inc., London, England. All unlabeled dNTPs and rNTPs were purchased from P-L Biochemicals and used without purification. DEAE-Sephadex A-25, heparin-Sepharose CL-6B, and Sephacryl S-200 were from Pharmacia Fine Chemicals. DEAE-cellulose (DE32) and phosphocellulose (P-11) were from Whatman.

Nucleic Acids

Calf thymus DNA was purchased from Worthington. Activated calf thymus DNA was prepared as previously described (3) øX174 viral single-stranded DNA was from Bethesda Research Laboratories and M13mp7 viral single-stranded DNA was prepared as described (19). Poly(dT)ₙ was from P-L Biochemicals. P1smid pDB36 DNA, which consists of pMB9 and yeast 2-µm plasmid form A DNA (20), was prepared as previously described (8).

Enzymes

Yeast DNA polymerases I and II were purified from S. cerevisiae A364A as previously described (2), with slight modification (1). Bacteriophage T4 DNA polymerase was purified from E. coli infected by T4 and CM82 (gene 45) as published (21). Bacteriophage N4 DNA polymerase was the same as published (22). Yeast single-stranded

A. Sugino, unpublished data.
DNA binding protein (ySSB) was the same as previously described (23). All restriction endonucleases used in this study were from Bethesda Research Laboratories.

Methods

DNA Polymerase Assays

Reaction mixtures (0.1 ml) were 50 mM Tris-Cl (pH 8.0), 10 mM MgCl₂, 2 mM dithiothreitol, 10 μg of bovine serum albumin, 5 μg of activated calf thymus DNA, 10% glycerol, 100 μM each of four deoxyribonucleoside 5'-triphosphates (dATP being either H- or 32P-labeled, 400 cpm/pmol), and the enzyme. The reaction was carried out at 37 °C and stopped by the addition of 2 ml of 5% trichloroacetic acid and 1% sodium dodecyl sulfate. After 10 min at 0 °C, the DNA was collected by filtration on Whatman GF/C filter discs. The filters were washed three times with cold 5% trichloroacetic acid, once with 3 ml of ethanol, dried, and counted in an LKB liquid scintillation counter. The unit of yeast DNA polymerase activity was defined as described for DNA polymerase assays.

DNA Primase Assays

Poly(dT)₉, Template—The reaction mixtures (0.1 ml) contained 50 mM Tris-Cl (pH 8.0), 0.2 mM dithiothreitol, 10 μg of bovine serum albumin, 10 mM MgCl₂, 10% glycerol, 2 mM ATP, 50 μM dTTP and dATP (dATP being either H- or 32P-labeled, 400 cpm/pmol), 1 μg of poly(dT)₉, 0.7 unit of yeast DNA polymerase I or T4 DNA polymerase, and DNA primase. One unit of DNA primase activity was defined as that which permitted 1 nmol of DNA synthesis in 30 min at 37 °C. Under the primase assay conditions, no significant DNA synthesis was observed without addition of primase, even in the presence of 2 units of DNA polymerase. Upon addition of primase, DNA synthesis was proportional to the amount of enzyme added and linear with time for more than 60 min.

DNA Topoisomerase Assay

Conversion of 0.5 μg of X174 viral single-stranded DNA to the covalently closed relaxed circular form was monitored by electrophoresis in 1% agarose gels.

Purification of DNA Primase

Wet cell pellets of either S. cerevisiae A364A (MATα ade1 ade2 trpl cir+) (2.5 x 10¹¹ cells) or 20B-12 were suspended in 400 ml of 10% sucrose, 50 mM Tris-Cl (pH 7.5), 10 mM EDTA, and frozen in liquid nitrogen. Crude extract was made as published before (8) except that 0.5 M NaCl was included in the lysis buffer. Ammonium sulfate precipitates were suspended in 250 ml of buffer A and dialyzed against the same buffer for 8 h at 0 °C (Fraction I). The dialyzed sample was applied to a single-stranded DNA cellulose column (4.8 x 15 cm) equilibrated with buffer A; the column was washed with 500 ml of buffer A, and the enzyme was eluted with 500 ml of 0.5 M NaCl buffer A. The activity was found in the 0.5 M NaCl elute, dialyzed against buffer A, applied to a DEAE-Sephadex A-25 column (34 x 20 cm) equilibrated with buffer A, and eluted with 1500 ml of a linear gradient of 0.5 to 1 M NaCl containing buffer A. Primase activity was eluted at 0.1 M NaCl. Active fractions were pooled, dialyzed against buffer A, and applied to a heparin-Sepharose column (1 x 2 cm). The primase activity was eluted from heparin-Sepharose with 100 ml of 0.1-1.5 M NaCl linear gradient in buffer A.

The active fraction was dialyzed against buffer A for 3 h and applied to a 2-ml hydroxylapatite column equilibrated with buffer A. The activity was eluted with 20 ml of a 0-0.4 M potassium phosphate buffer (pH 6.8) linear gradient in 10% glycerol, 10 mM 2-mercaptoethanol, 10 mM dithiothreitol and 0.05% sodium azide, containing buffer A. Primase activity was eluted at 0.15 M potassium phosphate, pooled, and concentrated by dialyzing against buffer containing 30% polyethylene glycol 6000 (Fraction VI). The sample was finally dialyzed against 0.15 M NaCl buffer A for 2 h at 0 °C, and 0.5 ml was applied on a 20-ml Sephacryl S-200 column equilibrated with buffer A/0.15 M NaCl. The activity was eluted with the same buffer (Fraction VII).

Gel Electrophoresis

Electrophoresis in 5 or 20% polyacrylamide slab gels (35 x 45 x 0.025 cm) containing 7 M urea was performed in 20 ml Tris-borate (pH 8.3), 2.5 mM EDTA buffer (27). RNA-DNA reaction products were analyzed with 1% sodium dodecyl sulfate, 10 mM Tris-Cl, and precipitated with ethanol after addition of 5 μg of yeast tRNA as carrier and 0.3 M sodium acetate. The pellets were suspended in 10 μl of 80% formamide, 5% glycerol, 0.05% bromphenol blue, 0.05% xylene cyanol, heated at 95 °C for 2 min, and quickly chilled

\footnote{1}The abbreviations used are: ySSB, yeast single-stranded DNA binding protein; RF, replicative form.

\footnote{2}Nucleic acid concentration is expressed as total nucleotide concentration.

\footnote{3}This inactive mixture was employed as a receptor for the complementation assay. The reaction mixture (0.1 ml) for complementation assay contained 0.5 μg of pJDB36 DNA (consisting of pMB9 and form A 2-μm plasmid DNA), receptor mixture, 50 μM each of the four dNTPs, 200 μM each CTP, GTP, and UTP, and 2 mM ATP, 50 mM Tris-Cl (pH 8.0), 10 mM MgCl₂, 2 mM dithiothreitol, 20% glycerol, and 100 μg/ml bovine serum albumin. The reaction was performed at 30 °C for 60 min. One unit of complementation activity was defined as that activity which supports 1 nmoI of pJDB36 DNA synthesis at 30 °C in 1 h.

Nuclease Assays

Double-stranded DNase activity was assayed by conversion of 0.5 μg of X174 RI to RF II (nicked circular) and RF III (double-stranded linear) DNA. This conversion was detected by electrophoresis in 1% agarose gels. Single-stranded DNase was measured by conversion of 0.5 μg of X174 viral circular single-stranded DNA to the linear single-stranded DNA. These DNAs were separated from each other by electrophoresis in 1% agarose gels. RNase activity was measured by the conversion of H-labeled poly(A), (50 cpm/pmol) to acid-soluble form. RNase H activity was measured as previously described (24).

\footnote{4}F. E. Wilson and A. Sugino, unpublished observation.
with ice water before loading on the gel. For protein analysis, 10 and 11% polyacrylamide gels containing 0.1% sodium dodecyl sulfate were used (25). Proteins were stained as described by the supplier of the silver stain kit (Bio-Rad).

Nearest Neighbor and Terminal Structure Analyses
DNA synthesis was carried out as described above except that [α-32P]dNTP or [γ-32P]ATP was used as radioactive substrate. After 60 min at 30 °C, the reaction mixture was adjusted to 10 mM EDTA and then applied to a 5-ml column of Sephadex G-100 (fine) equilibrated with 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl. The DNA in the excluded volume was collected, precipitated with ethanol along with 60 μg of yeast tRNA as carrier, dissolved in 0.1 ml of 0.3 M NaOH, and incubated for 15 min at 37 °C. After neutralization with 1 N HCl and 50 mM Tris-HCl (pH 7.5), the sample was diluted 10-fold with 7 M urea, 30 mM Tris-HCl (pH 7.5), 1 mM EDTA, and applied on a DEAE-cellulose column (0.5 × 15 cm) with pancreatic RNase I-digested yeast tRNA as a marker as previously published (26). The column was washed with 10 ml of the same buffer and the oligonucleotides were eluted with 40 ml of a 0-0.5 M NaCl linear gradient in the same buffer. Mononucleoside monophosphate (Np) fractions for nearest neighbor analysis, or the radioactive activity located between (Np)3 and (Np)4 for the terminal structure analysis, were pooled and further analyzed and identified by Dowex AG 1-X2 or polyethyleneimine chromatography as published (28, 29).

RESULTS
Identification and Purification of DNA Primase Activity from Yeast—During fractionation of the activity which catalyzed 2-μm plasmid DNA replication (10), an activity (P3 in Fig. 1) which is required for full reconstitution of the in vitro DNA replication was separated from the main DNA polymerase activity (P3 and P4 in Fig. 1). A typical reconstitution experiment is shown in Fig. 2. Further characterization of this P3 fraction showed that it permits DNA synthesis catalyzed by highly purified yeast DNA polymerase I, but not by DNA polymerase II, on E. coli plasmid pMB9 (20) or E. coli single-stranded DNA and RF I DNA used to assay rNTPs or ATP. Since this activity is characteristic of DNA primase (11-16), we designated this activity a yeast DNA primase and further purified it by both a complementation assay using yeast extrachromosomal DNA as a template.

No double-stranded or single-stranded DNase, RNase, or DNA topoisomerase activities were detected in the most highly purified DNA primase preparations. Absence of DNase and DNA topoisomerase was determined by failure to observe any change in the electrophoretic properties of the pX174 single-stranded DNA and RF I DNA used to assay these activities following incubation with 1 unit of primase at 30 °C for 60 min. In the RNase assay, less than 5 pmol of radioactive poly(A), became acid-soluble upon incubation at 30 °C for 60 min with 2 units of the enzyme. In the RNase H assay, less than 3 pmol of RNA were released from a [3H] RNA-DNA hybrid (specific activity of 450 cpm/pmol) after a 60-min incubation at 30 °C with 0.2 unit of enzyme.

When fractions of the Sephacryl S-200 gel filtration column were analyzed by 11% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, a predominant band (about 80% of the protein in the gel) with a molecular weight of about 60,000 could be detected, which coincides with the primase activity (Fig. 4, A and B). Additional minor bands could be seen. The molecular weight of the primase activity was also estimated to be about 60,000 by the gel filtration (Fig 4A). These size estimations are in good agreement, assuming the major polypeptide band at 60,000 in the sodium dodecyl sulfate-polyacrylamide gel to be the DNA primase, and suggest that yeast DNA primase is active as a monomer. Further evidence that primase activity is associated with a purified yeast DNA polymerase I.

A summary of the purification is shown in Table I. Both complementing activity and primase activity copurified throughout the fractionation shown in Table I, and it is very likely that the activities are the same protein. Unlike some other eukaryotic primases (12-19), most of the yeast DNA primase activity can be separated from DNA polymerase activities, as shown in Fig. 3. However, a small amount of the activity still co-eluted with the DNA polymerase (Fig. 3C-b). This might suggest that association of DNA primase with DNA polymerase in yeast is weaker than in other eukaryotes.

No double-stranded or single-stranded DNase, RNase, RNase H, or DNA topoisomerase activities were detected in the most highly purified DNA primase preparations. Absence of DNase and DNA topoisomerase was determined by failure to observe any change in the electrophoretic properties of the pX174 single-stranded DNA and RF I DNA used to assay these activities following incubation with 1 unit of primase at 30 °C for 60 min. In the RNase assay, less than 5 pmol of radioactive poly(A), became acid-soluble upon incubation at 30 °C for 60 min with 2 units of the enzyme. In the RNase H assay, less than 3 pmol of RNA were released from a [3H] RNA-DNA hybrid (specific activity of 450 cpm/pmol) after a 60-min incubation at 30 °C with 0.2 unit of enzyme.

When fractions of the Sephacryl S-200 gel filtration column were analyzed by 11% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, a predominant band (about 80% of the protein in the gel) with a molecular weight of about 60,000 could be detected, which coincides with the primase activity (Fig. 4, A and B). Additional minor bands could be seen. The molecular weight of the primase activity was also estimated to be about 60,000 by the gel filtration (Fig 4A). These size estimations are in good agreement, assuming the major polypeptide band at 60,000 in the sodium dodecyl sulfate-polyacrylamide gel to be the DNA primase, and suggest that yeast DNA primase is active as a monomer. Further evidence that primase activity is associated with a
Purification of Yeast DNA Primase

TABLE I
Summary of purification of DNA primase from yeast

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Step</th>
<th>Total volume</th>
<th>Total protein</th>
<th>Primase activity</th>
<th>Complementation assay</th>
<th>Ratio of primase to complementation activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ml</td>
<td>mg</td>
<td>Total units/mg</td>
<td>Total units/mg</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Crude extract</td>
<td>250</td>
<td>18,200</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>II</td>
<td>Single-stranded</td>
<td>150</td>
<td>250</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>III</td>
<td>DEAE-Sephadex</td>
<td>200</td>
<td>200</td>
<td>5,500</td>
<td>200</td>
<td>1.10</td>
</tr>
<tr>
<td>IV</td>
<td>Phosphocellulose</td>
<td>80</td>
<td>2.5</td>
<td>1,600</td>
<td>2,500</td>
<td>1.02</td>
</tr>
<tr>
<td>V</td>
<td>Heparin-Sepharose</td>
<td>20</td>
<td>0.5</td>
<td>1,400</td>
<td>2,800</td>
<td>—</td>
</tr>
<tr>
<td>VI</td>
<td>Hydroxyapatate</td>
<td>5</td>
<td>0.15</td>
<td>820</td>
<td>4,700</td>
<td>1.44</td>
</tr>
<tr>
<td>VII</td>
<td>Sephacyl S-200g</td>
<td>1</td>
<td>0.08</td>
<td>30</td>
<td>4,700</td>
<td>—</td>
</tr>
</tbody>
</table>

Indicates that enzyme activity was not measured, mainly due to contamination by large amounts of nucleases and inhibitors.

A portion of the hydroxyapatite fraction was used for this purification step; therefore, the yield of enzyme activity was corrected.

FIG. 3. Purification of a yeast DNA primase activity. Crude extract from 500 g of S. cerevisiae 20B-12 was made as previously described (8), and DNA primase activity assayed on poly(dT), template was purified as described under “Experimental Procedures.” A, DEAE-Sephadex A-5 column chromatogram; B, phosphocellulose column chromatogram of pooled activity from DEAE-Sephadex column; C-a, heparin-Sepharose chromatogram of DNA primase activity after phosphocellulose; C-b, heparin-Sepharose column chromatogram of DNA polymerase activity after phosphocellulose.

In the figure, DNA primase-rich preparations contain more of a polypeptide with a molecular weight of 60,000 than relatively primase-free preparations of DNA polymerase I.

DNA Primase Synthesizes Oligoribonucleotides or Mixed Primers—Yeast DNA polymerase I, which has some primase activity (see Fig 3), was very inefficient in utilizing single-stranded φX174 viral DNA as a template. However, in the presence of the purified enzyme, there is a great increase in DNA synthesis (Fig. 5). This stimulation of DNA synthesis requires ATP. Omission of any one of the other ribonucleoside 5’-triphosphates reduced DNA synthesis by 20%, and omission of all three reduced the DNA synthesis by 40% (Table II). Although the ribonucleoside 5’-triphosphate requirement for this DNA synthesis was not very strict, it is comparable to that observed for mouse DNA primase activity (30). Lowering the concentration of dNTPs (<5 μM) increased the requirement for ribonucleoside 5’-triphosphates considerably. (Total DNA synthesis is decreased to 64%, and omission of the three rNTPs reduces this synthesis by 75%.)

Since the primase activity requires ribonucleoside 5’-triphosphates to stimulate DNA synthesis on single-stranded templates, it appears that the primase synthesizes oligoribonucleotides and that these serve as primers for the reaction catalyzed by yeast DNA polymerase I. Yeast DNA primase stimulates ATP-dependent DNA synthesis catalyzed not only by yeast DNA polymerase I, but also by calf thymus DNA polymerase α, by prokaryotic polymerases such as those of bacteriophage T4, N4 (22), the large fragment of E. coli DNA polymerase I (which is deficient in 5′→3′ exonuclease activity), and by E. coli DNA polymerase III holoenzyme (Table III).

To test the proposal that oligoribonucleotides which are produced by the primase, 32P-labeled products of the primase reaction were analyzed by polyacrylamide gel electrophoresis. As shown in Fig. 6A, the products made by DNA primase on a φX174 single-stranded DNA template are short ribonucleotides. The major products are estimated to be nine nucleotides long. (They migrate at the position of a DNA sequence marker which is 10 nucleotides long, but the primase products have 5’-triphosphate termini, whereas the DNA sequence marker does not.) Incubation of the reaction products with 0.5 N NaOH resulted in disappearance of the oligomers (data not shown), providing further evidence that the products synthesized by primase are oligoribonucleotides. Fig. 6B is an autoradiogram of the products made by the primase in the presence of only dNTPs. Synthesis of oligonucleotides

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Footnotes:

a Indicates that enzyme activity was not measured, mainly due to contamination by large amounts of nucleases and inhibitors.

b A portion of the hydroxyapatite fraction was used for this purification step; therefore, the yield of enzyme activity was corrected.

c Due to the concentration of the enzymes, total activities after heparin-Sepharose column chromatography seemed to increase.
FIG. 4. Sephacryl S-200 gel filtration and polyacrylamide gel electrophoresis of yeast DNA primase in the presence of sodium dodecyl sulfate. A. 25 units of fraction VI of DNA primase preparation (0.5 ml) were applied to a 20-ml Sephacryl S-200 column equilibrated with 0.15 M NaCl/buffer A and were eluted with the same buffer. DNA primase activity was measured using poly(dT), and yeast DNA polymerase I. \( V_0 \), the excluded volume, and \( V_t \), the included volume, are indicated. In the upper panel, the column was calibrated with ferritin (Fer), catalase (Cat), bovine serum albumin (BSA), and chymotrypsinogen (Chy). \( B \), the indicated fractions of Sephacryl S-200 (0.25 ml) were precipitated with 10% trichloroacetic acid and applied on 11% polyacrylamide gel in the presence of sodium dodecyl sulfate. After electrophoresis, the gel was stained with silver and dried. \( Fr \), the fraction number of Sephacryl S-200 column, and \( Mr \), the molecular weight markers, are indicated. C, denaturing polyacrylamide gel electrophoresis of yeast DNA polymerase I preparations which are DNA primase-rich (lane a) and relatively DNA primase-free (lane b). The peak of DNA polymerase I activity in Fig. 3C-b (lane b) (8 units, 0.5 \( \mu \)g of protein) and a similar fraction which maintained substantial DNA primase activity (lane a) (5 units, 0.3 \( \mu \)g of protein) were applied on a 10% polyacrylamide gel in the presence of sodium dodecyl sulfate and electrophoresed as in B. Open arrows indicate polypeptide bands which differ in the two DNA polymerase I preparations.
Fig. 5. Stimulation of yeast DNA polymerase I activity is rNTP-dependent. The complete reaction mixture (0.5 ml) contained 0.7 unit of yeast DNA polymerase I, 0.1 unit of primase, 200 μM each dNTP, 50 μM each dNTP (dTTP was labeled with 32P), and 0.05-ml aliquots were withdrawn, and acid-insoluble radioactivity was measured in the complete reaction mixture (○) and in the absence of primase (△), ATP (△), or DNA polymerase (□).

TABLE II

<table>
<thead>
<tr>
<th>Omission or addition</th>
<th>DNA synthesis</th>
<th>Per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol</td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>153</td>
<td>100</td>
</tr>
<tr>
<td>−ATP</td>
<td>4</td>
<td>2.6</td>
</tr>
<tr>
<td>−CTP</td>
<td>120</td>
<td>78.4</td>
</tr>
<tr>
<td>−GTP</td>
<td>132</td>
<td>86.2</td>
</tr>
<tr>
<td>−UTP</td>
<td>135</td>
<td>88.2</td>
</tr>
<tr>
<td>−CTP, GTP</td>
<td>98</td>
<td>64.0</td>
</tr>
<tr>
<td>−CTP, UTP</td>
<td>103</td>
<td>67.3</td>
</tr>
<tr>
<td>−CTP, GTP, UTP</td>
<td>89</td>
<td>58.2</td>
</tr>
<tr>
<td>−ATP, CTP, GTP, UTP</td>
<td>3</td>
<td>2.0</td>
</tr>
<tr>
<td>−DNA polymerase I</td>
<td>4</td>
<td>2.6</td>
</tr>
<tr>
<td>−Primase</td>
<td>24</td>
<td>15.7</td>
</tr>
<tr>
<td>+α-Amanitin (500 μg/ml)</td>
<td>160</td>
<td>105.0</td>
</tr>
<tr>
<td>+α-Amanitin (1 mg/ml)</td>
<td>154</td>
<td>100.5</td>
</tr>
</tbody>
</table>

occur, demonstrating that primase can use dNTPs as a 32P transfer substrate, although the oligomers made are shorter and the reaction is less efficient. Partial sensitivity of these oligomers to alkaline digestion may indicate a minor contamination of the reaction with rNTPs (lanes c and d). Nonetheless, the presence of 4- and 5-mers, even after alkaline digestion, indicates that the yeast DNA primase can synthesize oligodeoxynucleotides. When the 32P-labeled products of reactions, including both primase plus yeast DNA polymerase I and rNTPs as well as dNTPs, were analyzed by polyacrylamide gel electrophoresis, oligoribonucleotides were no longer seen, and the reaction products were found to be of higher molecular weight (data not shown), indicating that the oligoribonucleotides made by primase are efficiently utilized by yeast DNA polymerase I and attached to a large molecular weight DNA.

That this oligoribonucleotide-synthesizing activity is distinct from previously described yeast RNA polymerases (26) is indicated by the insensitivity of the ATP-dependent DNA synthesis to high concentrations of α-amanitin; concentrations up to 1 mg/ml were tested (Table II). Although in most eukaryotes RNA polymerases II and III are α-amanitin-sensitive and RNA polymerase I is insensitive, in yeast it is RNA polymerases I and II which are sensitive and RNA polymerase III which is not (26). Therefore, the α-amanitin resistance of the primase activity distinguishes it from yeast RNA polymerases I and II.

Covalent Linkage between Oligoribonucleotides and DNA Chains—Polyacrylamide gel data indicated covalent linkage between the oligoribonucleotides made by the primase and the DNA chain made by DNA polymerase I. To confirm this, 32P transfer experiments have been done using [α-32P]dNTPs. The products made by both the primase and DNA polymerase I in the presence of both ribo- and deoxyribonucleoside 5'-triphosphates on ϕX174 single-stranded DNA as template were purified, digested with alkali, and neutralized. The mononucleotides were isolated by DBAE-cellulose column chromatography and then separated on a Dowex AG 1-X2 column to identify radioactive 2'-(3')-nMPs. The results of the 32P transfer experiments are summarized in Table IV.

Although the transfer of 32P from deoxyribonucleotide to purine ribonucleotides (GMP and AMP) was 2-3 times higher than to pyrimidine nucleotides (UMP and CMP), no specific junction sequences were revealed, in agreement with results of similar analysis in other eukaryotes. The total frequency of 32P transfer from dNMP to RNMP was 0.96–0.56%, or once per every 200–400 nucleotides. This is consistent with the proposal that every DNA molecule has an RNA primer at its 3′ end.
Purification of Yeast DNA Primase

DNA Polymerase I—Recently we have purified a single-stranded DNA-specific binding protein (ySSB) that stimulates the activity of yeast DNA polymerase I on φX174 single-stranded DNA primed with DNA fragments (23). Therefore, we were interested to know whether the RNA-primed DNA synthesis reaction catalyzed by yeast DNA polymerase I is also stimulated by ySSB. As shown in Fig. 7, ySSB stimulated the RNA-primed DNA synthesis at least 2-fold, particularly after long incubation. Interestingly, the reaction catalyzed by the DNA polymerase I containing a small amount of DNA primase activity is stimulated by ySSB to the same extent as is the complete system. However, the overall efficiency of this reaction was still poor.

An autoradiogram of the products of DNA synthesis on φX174 single-stranded DNA in the presence of various combinations of the purified yeast DNA replication proteins is shown in Fig. 8. Production of the principal reaction product, RF II DNA, is increased when ySSB is added to the RNA-primed DNA synthesis reaction, in agreement with the data of Fig. 7.

As in prokaryotic systems (31), oligonucleotide synthesis by primase was inhibited by addition of ySSB; only 10–20% of the activity remained (data not shown). For this reason, stimulation of RNA-primed DNA synthesis by ySSB is believed to occur primarily by interaction with single-stranded DNA to destabilize secondary structures in the DNA, and not by an interaction with DNA polymerase I.

DISCUSSION

An activity which primes DNA synthesis catalyzed by yeast DNA polymerase I and a number of other DNA polymerases on a single-stranded circular DNA or poly(dT), has been purified by using both an in vitro 2-μm DNA replication complementation assay and a typical DNA primase assay (12, 13). We designated it yeast DNA primase because this activity makes oligoribonucleotides in the presence of rNTPs and template DNA. The oligonucleotides synthesized by this primase are of discrete size, mainly eight or nine nucleotides; they are ribo- or deoxyribonucleotides or mixed oligomers (when synthesized in the presence of both ribonucleotides and deoxyribonucleotides as substrates) and these oligonucleotides are used as primers by yeast DNA polymerase I and other DNA polymerases tested. This is consistent with the notion that RNA primer size in eukaryotes is mainly 8–10 nucleotides (6), although direct in vivo data are missing in yeast.

Plevani and Chang (32) have shown that the products of typical RNA polymerases I, II, and III serve as primers for yeast DNA polymerase I on a single-stranded circular DNA template. However, the primase activity described in this paper differs from these RNA polymerases in product size, chromatographic behavior, α-amanitin resistance, and molecular weight. The main products of RNA polymerases I, II, and III are longer and more heterogeneous than the small oligonucleotides synthesized by the primase activity described here (26, 32). The primase activity was eluted from DEAE-Sephadex at a lower ammonium sulfate concentration than the RNA polymerase activities (32). The α-amanitin insensitivity of the primase reaction indicates that it is distinct from RNA polymerases I and II (26). Finally, in yeast RNA polymerase I, II, and III preparations, no polypeptides with M, of 60,000–65,000 were detected (26).

A small amount of primase activity was detected in the preparation of yeast DNA polymerase I shown in Fig. 3. Moreover, the molecular weight of free DNA primase purified in this study is almost identical to one of the polypeptides

Fig. 6. Analysis of the products of DNA primase using φX174 single-stranded DNA template. The products made by incubating DNA primase at 30 °C for 30 min with φX174 single-stranded DNA in the presence of rNTPs (one of the rNTPs was [γ-32P]ATP) (A) or dNTPs (one being [α-32P]dATP) (B) were analyzed on a 7 M urea-20% polyacrylamide gel (27). Autoradiograms were exposed for 8 h (A) or 48 h (B) at −80 °C. + and − indicate addition and omission of rNTPs, dNTPs, or α-amanitin. A, lanes a–e, and B, lanes a–d, contained DNA primase; B, lanes e and f, contained DNA polymerase I of yeast and 100 μg/ml aphidicolin. Lanes c and d of B were subjected to alkaline digestion in 0.3 M NaOH for 15 h at 37 °C before electrophoresis.

5′-end, since the size of the products made by the primase and DNA polymerase I on φX174 single-stranded DNA is 300–500 nucleotides, as determined by alkaline sucrose density gradient sedimentation (data not shown).

ySSB Stimulates the Reaction Catalyzed by the Primase and
Purification of Yeast DNA Primase

The reaction mixtures (0.1 ml) contained 0.7 unit of DNA polymerase I, 0.1 unit of DNA primase, 2 mM ATP, 200 µM other rNTPs, and 50 µM each dNTP (one of the dNTPs was α-32P-labeled with a specific activity of about 5000 cpm/pmol). The reaction mixtures were incubated for 30 min at 30 °C, and the reactions were terminated by the addition of 10 mM EDTA. After alkaline digestion of the samples, total radioactivity was measured as before (8) using 0.02 volume sample.

Table IV

<table>
<thead>
<tr>
<th>α-32P-labeled substrates</th>
<th>Total radioactivity in DNA (cpm)</th>
<th>2'(3')-rAMP (cpm)</th>
<th>2'(3')-rCMP (cpm)</th>
<th>2'(3')-rGMP (cpm)</th>
<th>2'(3')-rUMP (cpm)</th>
<th>Total radioactivity recovered in rNMPs (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dATP</td>
<td>450,000</td>
<td>955</td>
<td>387</td>
<td>616</td>
<td>542</td>
<td>2500 (0.00556)</td>
</tr>
<tr>
<td>dCTP</td>
<td>473,000</td>
<td>452</td>
<td>219</td>
<td>308</td>
<td>261</td>
<td>1248 (0.00262)</td>
</tr>
<tr>
<td>dGTP</td>
<td>448,000</td>
<td>718</td>
<td>398</td>
<td>510</td>
<td>200</td>
<td>1826 (0.00407)</td>
</tr>
<tr>
<td>dTTP</td>
<td>480,000</td>
<td>503</td>
<td>253</td>
<td>359</td>
<td>235</td>
<td>1350 (0.00281)</td>
</tr>
<tr>
<td>Total</td>
<td>1,850,000</td>
<td>2628</td>
<td>1257</td>
<td>1793</td>
<td>1238</td>
<td>6916 (0.0037)</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate the ratio between each [32P]2'(3')-rNMP recovered and total radioactivity incorporated into DNA.

Fig. 7. ySSB stimulates the reaction catalyzed by DNA polymerase I and DNA primase. The reaction mixture (0.25 ml) contained 1.4 units of DNA polymerase I, 0.5 unit of DNA primase, φX174 single-stranded DNA, 200 µM each rNTP, 2 mM ATP, 50 µM each dNTP (dTTP was α-32P-labeled), and 2 µg of ySSB. The reaction mixture was incubated at 30 °C. A 50-µl aliquot was withdrawn at 30-min intervals, and acid-insoluble radioactivity was assayed as before (8). –SSB, –primase, and –polymerase indicate omission of the respective components from the reaction mixture.

observed in DNA primase-rich preparations of DNA polymerase I (Fig. 4C). This strongly supports the idea that free DNA primase is associated in vivo with yeast DNA polymerase I. This finding of both DNA polymerase-free and polymerase-associated yeast primase activities has been recently reported by others (17, 18), who characterized the polymerase-associated activity.

Previous studies of yeast DNA polymerase I showed no indication of an associated primase activity (2, 32). Recent analysis of the polypeptide composition of purified yeast DNA polymerase preparations indicated that there are at most two polypeptides of molecular weight of 140,000 and 110,000 (23, 33), and that yeast DNA polymerase I probably consists of only the M, = 140,000 polypeptide since there were indications that the M, = 110,000 polypeptide was derived from the larger one by proteolysis (33). These purified preparations of DNA polymerase contain no polypeptides corresponding in size to the primase described here. In agreement with these results,
we find that after extensive purification, the primase activity can be removed from DNA polymerase I. We interpret the ability to obtain polymerase-associated primase activity as well as both activities free of each other to indicate an in vivo association of the two activities, although such an association might be weaker than in other eukaryotes where primase activity was found to be tightly associated with DNA polymerase α or an α-like DNA polymerase (12–16). Nonetheless, other eukaryotic primase activities have been separated from DNA polymerase activities. Mouse primase can be separated from DNA polymerase activity by column chromatography (34), while separation of Drosophila primase from polymerase activity required incubation with urea (35). Interestingly, at least one of the polypeptides found in each of these extensively purified DNA primases has $M_\text{r} = 50,000–60,000$ (34, 35, and this study).

As the size of in vivo RNA primers in eukaryotes is discrete, and the dinucleotides at the junction between RNA primer and DNA chain are not unique (6), it has been proposed that DNA primase does not recognize specific DNA sequences to initiate and terminate RNA primer synthesis, but that the tertiary structure of DNA as organized by nucleosomes determines the initiation and termination of RNA primer synthesis by primase (6, 36). However, recently there have been indications that some primases may initiate RNA synthesis at specific sites on the template (37, 38). When a small single-stranded intestine DNA fragment was used as template, reaction products of nonrandom size were detected, suggesting that yeast DNA primase initiates primers with some degree of specificity. Upon examination of the DNA sequence surrounding those initiation sites, the only sequence similarity detected among the primase gene. Among the temperature-sensitive cell division cycle (cdc) mutants of yeast are DNA synthesis mutants, e.g. cdc2 and cdc8 (39, 40). Examination of DNA primase activity of cdc mutants 27, 7, 8, 9, 2, and 6 revealed no temperature sensitivity of any of these extracts in vitro, suggesting that none of these mutants bears a mutation in the DNA primase gene. By analogy to prokaryotic systems, it is expected that many DNA replication proteins should be required for yeast chromosomal and 2-μm plasmid DNA replication. Therefore, more DNA replication genes need to be identified in yeast. Recently, in fact, many new DNA replication mutants have been isolated (41, 42). Since a typical DNA primase assay using as template either poly(dT), or single-stranded viral DNA cannot be performed using crude extracts, our in vitro complementation assay using either 2-μm or ARS plasmid DNA as template should be particularly useful for identifying DNA primase mutants in these collections.

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REFERENCES