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Initiation, elongation and pausing of *in vitro* DNA synthesis catalyzed by immunopurified yeast DNA primase: DNA polymerase complex

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Yeast DNA primase and DNA polymerase I can be purified by immunoaffinity chromatography as a multipeptide complex which can then be resolved into its functional components and further reassembled *in vitro*. Isolated DNA primase synthesizes oligonucleotides of a preferred length of 9 – 10 nucleotides and multiples thereof on a poly(dT) template. *In vitro* reconstitution of the DNA primase:DNA polymerase complex allows the synthesis of long DNA chains covalently linked to RNA initiators shorter than those synthesized by DNA primase alone. The SS (single-stranded) circular DNA of phage M13mp9 can also be replicated by the DNA primase:DNA polymerase complex. Priming by DNA primase occurs at multiple sites and the initiators are utilized by the DNA polymerase moiety of the complex, so that almost all the SS template is converted into duplex form. The rate of DNA synthesis catalyzed by isolated yeast DNA polymerase I on the M13mp9 template is not constant and is characterized by distinct pausing sites, which partly correlate with secondary structures on the template DNA. Thus, replication of M13mp9 SS DNA with the native primase:polymerase complex gives rise to a series of DNA chains with significantly uniform termini specified by the primase start sites and the polymerase stop sites.

Key words: DNA synthesis/yeast/DNA primase/DNA polymerase

Introduction

All purified DNA polymerases from prokaryotic and eukaryotic sources are unable to catalyze *de novo* initiation of DNA synthesis. In prokaryotes the initiation reaction is provided by RNA polymerase or by enzymes, called DNA primases, that synthesize RNA primers which can be elongated by the replicative DNA polymerase (Kornberg, 1980). In eukaryotes, initiator RNA molecules have been found at the 5′ end of the nascent DNA chains (Kaufman *et al.*, 1977; Tseng *et al.*, 1979), and the existence of a DNA primase activity was postulated several years ago (Reichard and Eliasson, 1978). Recently, DNA primase activity has been found associated with the replicative DNA polymerase purified from a variety of eukaryotic organisms (Yagura *et al.*, 1982; Conaway and Lehman, 1982; Hubscher, 1983; Wang *et al.*, 1984; Shioda *et al.*, 1982). Our group has been interested in developing an *in vitro* DNA synthesis system from the yeast *Saccharomyces cerevisiae*. This organism is attractive for the study of enzymes involved in DNA replication because it is amenable to the use of powerful techniques of classical genetics and molecular biology. We have purified several proteins involved in DNA metabolism from cells of *S. cerevisiae*, including a DNA primase:DNA polymerase I complex (Plevani *et al.*, 1984). This complex has been isolated recently by an immunoaffinity chromatography procedure that allows the correlation of enzyme activity with polypeptide structure and the physical separation of DNA primase from DNA polymerase (Plevani *et al.*, 1985). The isolated protein species can be reassembled *in vitro* to reconstitute the complex.

The availability of isolated DNA primase and DNA polymerase and the possibility of reconstituting the DNA primase:DNA polymerase complex makes it possible to study separately DNA initiation and DNA elongation reactions, as well as to investigate the products synthesized under coupled enzymatic conditions. Here we report the products synthesized by the isolated enzymes and by the DNA primase: DNA polymerase complex on synthetic and natural DNA templates.

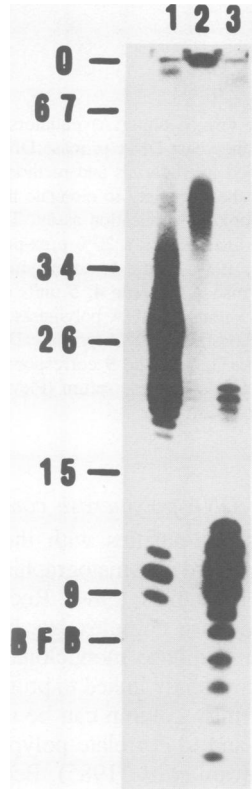

Fig. 1. Analysis of the size of oligo(rA) initiators. Poly(dT) replication reactions were carried out as described in Materials and methods with [α-^{32}P]ATP (500 c.p.m./pmol) as labeled substrate. dATP at 0.1 mM was also added in the reaction mixtures analyzed in **lanes 2** and **3**. After 60 min of incubation, DNA products were isolated by chromatography on Sephadex G-50 columns (Pharmacia) and concentrated by ethanol precipitation. The sample analyzed in **lane 3** was digested with 0.1 mg/ml of pancreatic DNase I for 60 min at 37°C. Samples were denatured and run on a 20% urea-polyacrylamide gel as previously described (Plevani *et al.*, 1984). Size markers were bromophenol blue (BFB) and *Hpa*II-digested [^{32}P]pBR322 DNA. **Lane 1**, 5 units of DNA primase; **lanes 2** and **3**, 5 units of DNA primase plus 5 units of DNA polymerase I.

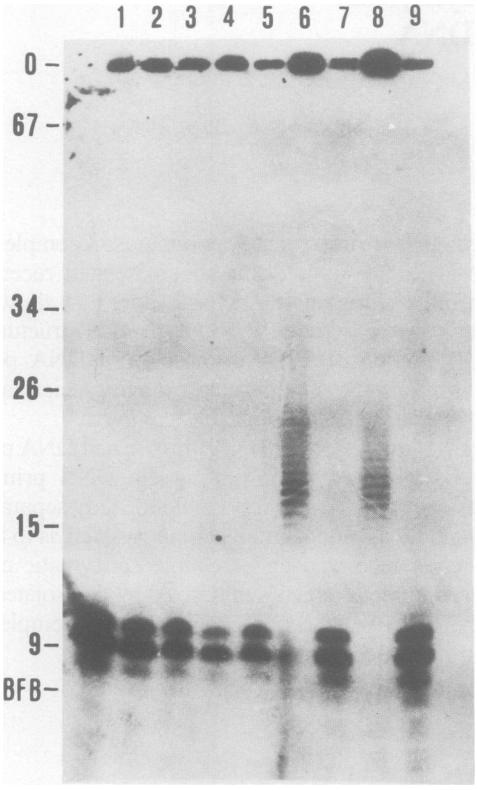

Fig. 2. Utilization of pre-formed oligo(rA) initiators. Labeled oligo(rA) initiators synthesized by the yeast DNA primase:DNA polymerase complex were prepared as described in Materials and methods, and the two yeast enzymes were tested for their capacity to elongate the pre-formed oligo(rA) initiators in a standard poly(dT) replication assay. The products of the elongation reaction were analyzed on a 20% urea-polyacrylamide gel as described (Plevani *et al.*, 1984). **Lane 1**, no enzyme added; **lane 2**, 5 units of DNA primase plus 1 mM ATP; **lane 4**, 5 units of DNA primase plus 0.1 mM dATP; **lane 6**, 5 units of DNA polymerase I plus 0.1 mM dATP; **lane 8**, 5 units of the reconstituted DNA primase:DNA polymerase complex plus 0.1 mM dATP; **lanes 3, 5, 7** and **9** correspond to **lanes 2, 4, 6** and **8**, except that anti-yeast DNA polymerase serum (Plevani *et al.*, 1984) was added to the reaction mixtures.

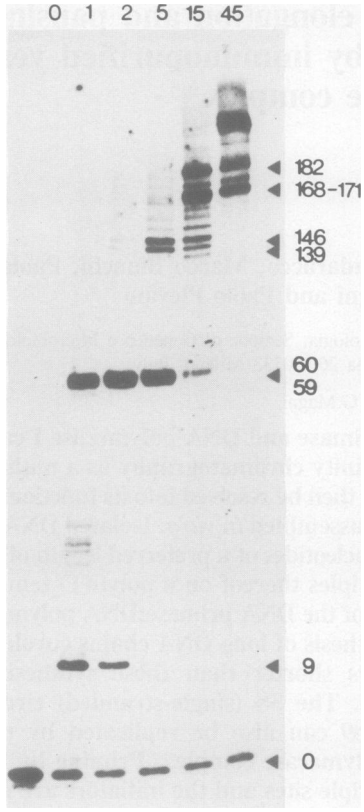

Fig. 3. Pausing sites of DNA polymerase I in primer-initiated SS M13mp9 DNA replication. 5 units of isolated DNA polymerase I were used in a 0.09 ml standard M13mp9 DNA replication mixture containing 7.5 μM of the annealed primer-template prepared as described in Materials and methods. The reaction mixture was incubated at 30°C and, at the indicated times, samples were withdrawn, mixed with an equal volume of formamide and boiled for 3 min. Aliquots of the samples were then applied to a 6.5% urea-polyacrylamide gel and electrophoresed at 50 V/cm.

Results

Yeast DNA primase:DNA polymerase complex

Yeast DNA primase co-purifies with the major yeast DNA polymerase under standard chromatographic procedures (Plevani *et al.*, 1984; Singh and Dumas, 1984). Recently, the yeast DNA primase:DNA polymerase complex has been purified to near homogeneity by using mouse monoclonal antibodies to yeast DNA polymerase I covalently linked to protein A-Sepharose, and the same immunoaffinity column can be used to dissociate the complex physically and to correlate polypeptide structure with enzyme activity (Plevani *et al.*, 1985). Because the yeast DNA primase:DNA polymerase complex can be reconstituted *in vitro* (see Materials and methods) it is possible to study the DNA initiation and elongation reactions separately, or under coupled enzymatic conditions.

Oligoribonucleotide products synthesized by DNA primase and by the DNA primase:DNA polymerase complex

The products synthesized by DNA primase on a poly(dT) DNA template were analyzed by polyacrylamide gel electrophoresis under denaturing conditions and the results are shown in Figure 1. In the absence of DNA polymerase, DNA primase synthesized primarily multiples of 9 – 10 nucleotides with an evident accumulation of products 20 and 30 nucleotides long (Figure 1, lane 1). In the presence of dATP, when DNA polymerase was added to reconstitute the complex, products that failed to enter the gel and mixed ribodeoxyribonucleotides of ~50 residues were detected (Figure 1, lane 2). When these products were digested with DNase I, oligoribonucleotides 8 – 12 residues long were released. These results suggest that DNA primase alone synthesizes oligoribonucleotide chains which vary in length from 10 to 50 nucleotides with an accumulation of products at a periodicity of 10, but when the DNA primase:DNA polymerase complex is reconstituted, mainly the 10-nucleotide initiators are utilized by DNA polymerase. When single-stranded (SS) M13mp9 DNA is used as template, the products synthesized by the isolated DNA primase are more heterogeneous, ranging in size from 40 to 60 nucleotides. Moreover, under coupled DNA polymerase reaction conditions, the labeled initiators released by DNase digestion are detected as a smear of radioactivity corresponding to oligoribonucleotides ranging in size from 10 to 20 residues (data not shown). The dispersive distribution of the oligoribonucleotides synthesized by DNA primase on the M13mp9 DNA molecules probably reflects the sequence heterogeneity of initiators sythesized on a natural DNA template. Since on a poly(dT) template the isolated DNA primase synthesized oligoribonucleotides that accumulated with a periodicity of 10 residues, while the majority of initiator molecules utilized by DNA polymerase are 8 – 12 nucleotides long, we were interested in investigating the possibility that DNA primase could elongate

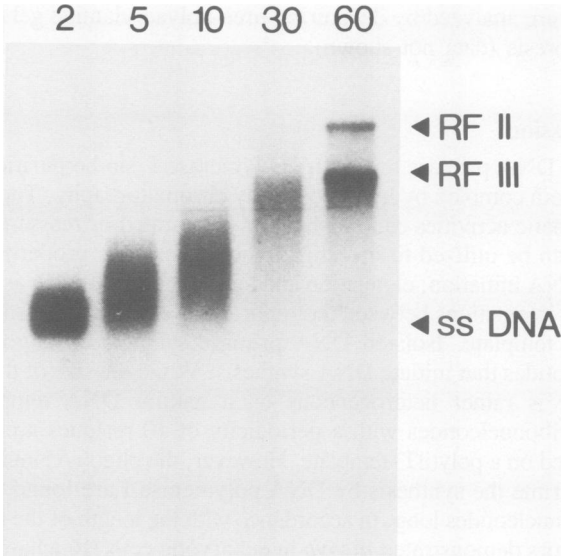

Fig. 4. Time course of M13mp9 DNA replication catalyzed by the yeast DNA primase:DNA polymerase complex. A 0.25 ml M13 DNA replication mixture containing 25 units of the DNA primase:DNA polymerase complex was incubated in the presence of [α-^{32}P]dATP (8000 c.p.m./pmol) as the labeled precursor. After a 2-min pulse, cold dATP was added to 1 mM. At the indicated times, aliquots were withdrawn and the reaction terminated by adding EDTA to 30 mM. DNA products were purified by phenol-chloroform extraction and concentrated by ethanol precipitation. Aliquots of the samples (10 000 c.p.m.) were electrophoresed on a 1.2% agarose gel in Tris-acetate buffer. The gel was subsequently stained with ethidium bromide, dried and autoradiographed. SS, RFI, RFII, RFIII M13mp9 DNA were run in a parallel lane to determine their relative mobility.

to a certain extent pre-formed oligoribonucleotides. The oligo(rA) initiators synthesized by the DNA primase:DNA polymerase complex were isolated as described in Materials and methods, and used to test the capacity of DNA primase and DNA polymerase to elongate the pre-formed oligo(rA) under coupled or uncoupled conditions. As shown in Figure 2, not a single nucleotide was added by the isolated DNA primase, either in the presence of ATP or dATP as substrates (Figure 2, lanes 2 and 4). Rabbit anti-yeast DNA polymerase I serum, added to exclude possible artefacts due to contamination of DNA primase by DNA polymerase, did not change this result (Figure 2, lanes 3 and 5). Conversely, yeast DNA polymerase I can elongate efficiently the pre-formed oligo(rA) initiators, originating molecules ranging from 15 to 30 nucleotides or products that failed to enter the gel, and this elongation reaction was completely inhibited by anti-DNA polymerase I serum (Figure 2, lanes 6 and 7). The products synthesized by the reconstituted DNA primase:DNA polymerase complex are identical to those identified with isolated DNA polymerase (Figure 2, lanes 8 and 9).

These results show that DNA primase can accumulate molecules longer than 8–12 residues only if uncoupled from DNA polymerase, and that oligoribonucleotide synthesis is a processive mechanism, since no elongation of pre-formed initiators is catalyzed by isolated DNA primase.

Replication of primed SS DNA by DNA polymerase I pauses at specific sites

To monitor the progress of DNA replication by DNA polymerase I at the individual nucleotide level, we constructed a specific primer-template substrate as described in Materials and methods. Because of details of the construction of the primer:template substrate, every DNA chain synthesized by DNA polymerase I will contain two labeled dAMP residues at the junction between the primer and the nascent DNA. Fractionation of the nascent chains on a sequencing gel and autoradiography generate a pattern of bands whose intensity is proportional to the abundance of chains of each particular length. Moreover, the length of each chain can be identified by matching its electrophoretic mobility to that of dideoxy-terminated marker chains (Sanger *et al.*, 1978). When we performed this analysis, we detected the accumulation of a specific set of newly synthesized DNA chains as shown in Figure 3. In particular, there are strong pausing sites at nucleotides 9, 59 and 60 (a couple of closely spaced bands), 168–171 (two closely spaced bands), 182 and at sites further downstream that could not be accurately located. Additional pausing sites of a lesser effectiveness were also present. An exhaustive computer search was performed for potential secondary structures in the region of M13mp9 SS DNA between the location of the 17-mer primer and 400 nucleotides downstream of it and revealed (i) at nucleotide 9, a sequence of bases complementary (18 perfect matches) to sequence 304–324 of the template; (ii) at nucleotide 61, a potential hairpin with a stem of 12 bp (11 perfect matches) and a loop of nine bases; (iii) at nucleotides 172 and 183, weaker stem-loop structures whose formation would be favored by the unfolding of alternative structures caused by the replication of the template. These latter correspondences are of questionable significance, however, since other possible stem-loop structures of approximately equal stability which do not appear to affect the rate of polymerization can be found in the template sequence. Despite these partial inconsistencies, it appears that the two earliest and strongest pause sites correspond exactly to the region of strongest secondary structures in the sequences surveyed. The abundance of chains stopping at a particular site first increases and then decreases, indicating that chains in each particular band can be further extended by yeast DNA polymerase I and that no site behaves as an absolute block. Thus, yeast DNA polymerase, without the aid of additional factors (e.g., DNA-binding proteins) can synthesize past double-stranded regions of up to 19 bp. On the other hand, it can be estimated that, while the mean time lapse required for the addition of a single nucleoside monophosphate to the growing chain is ~1 s or less, pause sites can stall the polymerase for up to several minutes (by densitometric tracing we calculated that DNA polymerase stalls at the first pause site with a mean time of 40 s and at the second site with a mean time of 3.5 min).

Replication of single-stranded M13mp9 DNA

When M13mp9 SS DNA was used as template, 60–80% of the input DNA was replicated by the DNA primase:DNA polymerase complex after 60 min of incubation at 35°C in the presence of the four rNTPs. When the labeled DNA products were analyzed by agarose gel electrophoresis, all the radioactivity was found to be associated with DNA molecules migrating as RFII and RFIII (double-stranded replicative forms). The newly synthesized DNA was not covalently linked to the template, because all nascent DNA chains could be dissociated from the template when the gel was run in a second dimension under alkaline conditions (Plevani *et al.*, 1984, and data not shown). Moreover, labeled products migrating as replicative forms, could also be visualized by using [γ-^{32}P]ATP as precursor so that nascent molecules would be labeled only at the 5′ end (data not shown). We followed the time-course of the replication of SS M13mp9 DNA by incubating the template with the DNA primase:DNA polymerase complex and labeled precursor dNTPs for 2 min, and then adding a chase of cold dNTPs (Figure 4). After the 2-min pulse the radioactivity migrated close to the position of the SS DNA template. A smear of labeled products was detected after

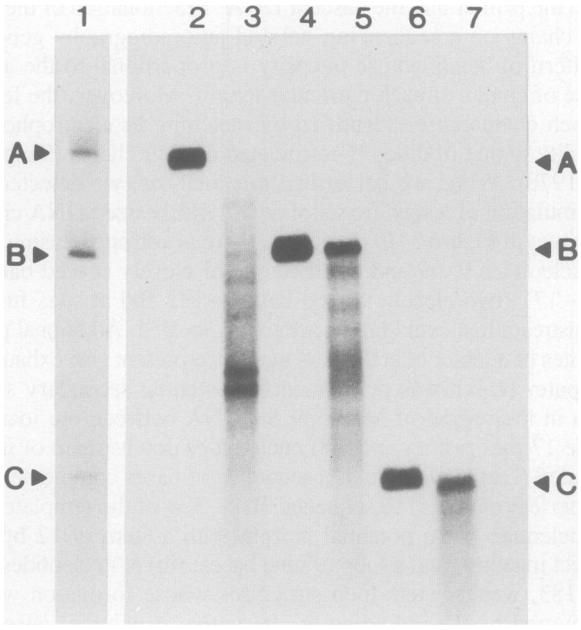

Fig. 5. S1 nuclease digestion of M13mp9 DNA products synthesized by the DNA primase:DNA polymerase complex. Labeled M13mp9 replication forms were prepared and isolated as described in the legend to Figure 4, except that [α-^{32}P]dATP was not chased with cold dATP. After addition of 3 μg M13mp9 RFI as carrier, the DNA products were digested with 5 units of restriction endonuclease *Nci*I as specified by the manufacturer. The DNA fragments were separated by electrophoresis on a 1.5% agarose gel and recovered separately from the gel by overnight diffusion in Tris-EDTA buffer. The DNA was then concentrated, digested with 5 units of S1 nuclease as described by Maniatis *et al.* (1982) and analyzed by gel electrophoresis on a 1.5% agarose gel and autoradiography. **Lane 1**, *Nci*I digested M13mp9 DNA products; **lane 2**, repurified fragment of ~4.3 kb; **lane 4**, repurified fragment of ~2.3 kb; **lane 6**, repurified fragment of ~0.6 kb; **lanes 3, 5** and **7** correspond to the fragments analyzed in **lanes 2, 4** and **6**, respectively, after S1 nuclease digestion.

5–10 min of chase, while radioactive bands migrating as M13mp9 RFII and RFIII appeared after 30 min and accounted for almost all the DNA products after 60 min of incubation. The appearance of products corresponding to M13mp9 RFIII DNA molecules is due to the presence of SS linear DNA template in our M13mp9 DNA preparation, and this result suggests that both linear and circular SS DNA can be used as template by the yeast protein complex. To verify whether the DNA products migrating as double-stranded DNA molecules were indeed fully replicated or contained unreplicated regions, we digested the replicated DNA with restriction enzyme *Nci*I and endonuclease S1. As shown in Figure 5, three labeled fragments of ~4.3 kb, 2.3 kb and 0.6 kb were detected after *Nci*I digestion of the labeled products, as expected from the restriction of RF M13mp9 DNA. We then re-isolated the *Nci*I fragments from the gel and analyzed them for the presence of SS DNA regions by S1 digestion. None of the fragments was completely unaffected by S1 digestion, indicating that at least a portion of the molecules contained SS regions. In particular, the longest *Nci*I fragment contained one or more SS gaps, since almost none of the species of 4.3 kb was left after S1 digestion. However, the radioactivity remaining after the digestion was detected partly as a smear, and partly as discrete bands of shorter length. The finding of discrete bands indicates that both the initiation and termination events are not totally random, and this hypothesis is further supported by the evidence of discrete DNA bands when the products of the reaction were analyzed by denaturing urea-polyacrylamide gel electrophoresis (data not shown).

Discussion

Yeast DNA primase and DNA polymerase I can be purified as a protein complex by immunoaffinity chromatography. The two enzymatic activities can be physically separated or reassembled and can be utilized to investigate the mechanistic properties of the DNA initiation, elongation and termination reactions, as well as the interactions between the replicative yeast enzymes and the DNA template. Isolated DNA primase synthesized oligoribonucleotides that initiate DNA synthesis. While the size of the initiator is rather heterogeneous on a natural DNA template, oligoribonucleotides with a periodicity of 10 residues are synthesized on a poly(dT) template. However, the oligo(rA) initiators that prime the synthesis by DNA polymerase I are found to be ~10 nucleotides long, in accordance with the length of the RNA initiators demonstrated *in vivo* in eukaryotic cells (Reichard and Eliasson, 1978; Kitani *et al.*, 1984). It is possible that the DNA polymerase can displace the DNA primase from the template and dNTPs polymerization can take over on the available 3′-OH end of the initiator RNA. Alternatively, the two protein species of the complex may not dissociate from the template, but produce a steric rearrangement, so that the 3′-OH terminus is rendered available for dNTPs polymerization by DNA polymerase when initiator RNA molecules ~10 nucleotides long have been synthesized. Moreover, oligoribonucleotide synthesis by isolated DNA primase is a processive mechanism, because in the absence of DNA polymerase we failed to detect addition of rNTPs or dNTPs at the individual nucleotide level.

Yeast DNA polymerase I can partly replicate M13mp9 DNA template initiated with a single 17-nucleotide primer. Several pausing sites where DNA polymerase will stall have been identified, but none of them behaves as an absolute block of DNA synthesis. While the strongest pausing sites correlate with putative secondary structures on the template DNA, the relationship of other arrest sites to secondary structures was not so obvious (see also Weaver and DePamphilis, 1982). The overall rate of *in vitro* DNA synthesis on viral DNAs is rather slow, so that additional DNA polymerase co-factors may be necessary to obtain physiological rates of synthesis.

The yeast DNA primase:DNA polymerase complex efficiently replicates SS M13mp9 DNA, so that the large majority of the template is converted into RFII and RFIII replicative forms. Similar results have been obtained recently with a DNA primase: DNA polymerase complex from higher eukaryotes (Riedel *et al.*, 1982; Grosse and Krauss, 1984). The finding of complete duplex DNA molecules is likely to be correlated with multiple initiation events catalyzed by DNA primase. In fact, we found that the DNA products migrating as RFII and RFIII are not fully double-stranded, because gaps of various lengths can be identified by S1 nuclease digestion of the unreplicated SS DNA regions. However, because S1 digestion of the DNA products synthesized by the DNA primase:DNA polymerase complex generates discrete DNA bands we conclude that the initiation and termination events are rather specific on a M13mp9 DNA template. Because it has also been shown that mouse DNA primase initiates SV40 *in vitro* DNA replication at a specific site (Tseng and Ahlem, 1984), it will be interesting to investigate the specificity of *in vitro* DNA synthesis catalyzed by the yeast protein complex on templates containing homologous DNA sequences (Kearsey,

1984) that may be involved in the control of DNA replication.

Materials and methods

Reagents and substrates

Ribonucleoside and deoxyribonucleoside triphosphates were purchased by Sigma; [methyl-^{3}H]TTP, [8-^{3}H]dATP, [α-^{32}P]ATP and [α-^{32}P]dATP from Amersham; M13 single-stranded primer (17 bases), the Klenow fragment of *Escherichia coli* DNA polymerase I, *Nci*I restriction endonuclease and S1 nuclease were purchased from New England Biolabs; poly(dT) of chain length from 1000 to 8000 residues was synthesized with terminal deoxynucleotidyl transferase as previously described (Plevani *et al.*, 1984); SS and RF M13mp9 DNAs were prepared as previously described (Plevani *et al.*, 1984). Other chemicals were reagent grade.

Enzymes

The purification of the yeast DNA primase:DNA polymerase complex and the physical separation of the two activities will be detailed elsewhere (Plevani *et al.*, 1985). Briefly, a clarified yeast extract prepared from 200 g of mid-log *Saccharomyces cerevisiae* strain D273-10B [PET(ϱ^+)] as previously described (Plevani *et al.*, 1984) was directly loaded onto a 2.5 ml column containing 7 mg mouse monoclonal IgG against yeast DNA polymerase I covalently linked to protein A-Sepharose (Pharmacia). After extensive washing the yeast DNA primase:DNA polymerase can be eluted with 3.5 M $MgCl_2$, buffered with 50 mM Tris-HCl at pH 8.0. DNA primase can be dissociated from DNA polymerase after re-binding of the complex to the same immunoaffinity column and elution with a $MgCl_2$ gradient from 0 to 1 M. The isolated DNA primase and DNA polymerase physically reassociate in a low ionic strength buffer, since the protein complex can be re-bound to the mouse monoclonal column.

Enzyme assays

DNA polymerase activity was assayed with DNase-treated calf thymus DNA as described (Plevani *et al.*, 1984). Poly(dT) or M13 DNA replication by the yeast DNA primase:DNA polymerase complex also have been described (Plevani *et al.*, 1984). 1 unit of DNA polymerase and 1 unit of DNA primase correspond to 1 nmol of TMP and AMP incorporated per h under the respective specific assay conditions.

Preparation of the annealed M13 primer-template substrate

M13mp9 SS circular DNA (0.65 mM) and the 17-nucleotide long primer (3 μM) were mixed in 0.06 ml 10 mM Tris-HCl at pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, heated to 65°C and allowed to cool down slowly to room temperature. The primer was then extended by adding dGTP to 0.02 mM, [α-^{32}P]-dATP to 0.3 μM (sp. act. 3.2 mCi/mmol) and 7.5 units of Klenow fragment in a final volume of 0.075 ml. After 15 min of incubation at 20°C, cold dATP was added to 0.02 mM and incubation continued for an additional 10 min. After stopping the reactions with 10 mM EDTA, the primer-template was purified by phenol-chloroform extraction and applied to a 2.5 ml Bio-Gel-A1.5 m (Bio-Rad) column equilibrated in 200 mM Tris-HCl at pH 8.0, 1 mM EDTA. Radioactive fractions eluting in the void volume were pooled and the length of the putative 20-mer primer annealed to the SS M13mp9 DNA was checked by comparing its electrophoretic mobility in denaturing polyacrylamide gels with that of dideoxy-terminated DNA chains prepared using the same primer-template (Sanger *et al.*, 1978).

Isolation of oligo(rA) molecules synthesized by DNA primase

A poly(dT) replication reaction (0.18 ml) was carried out as described with 100 units of DNA primase and 100 units of DNA polymerase in the presence of dATP and [α-^{32}P]ATP (Plevani *et al.*, 1984). After 60 min of incubation, the reaction was stopped by addition of EDTA and SDS to 20 mM and 1%, respectively, and the products were separated from unincorporated nucleotides by chromatography on a 5 ml Sephadex G-50 column (Pharmacia). Afer ethanol concentration, the oligoribonucleotide initiators were released from *in vitro* synthesized DNA products by digestion at 37°C for 30 min with 1.5 units of pancreatic DNase I. DNase was inactivated and oligo(rA) initiators re-annealed to the poly(dT) (10 μg/ml) by heating the mixture at 80°C for 10 min and cooling down slowly to room temperature.

Acknowledgements

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