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Replication of single-stranded DNA templates by primase–polymerase complexes of the yeast, *Saccharomyces cerevisiae*

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ABSTRACT

A partially purified primase-polymerase complex from the yeast, Saccharomyces cerevisiae, was capable of replicating a single stranded circular phage DNA into a replicative form with high efficiency. The primase-polymerase complex exhibited primase activity and polymerase activity on singly primed circular ssDNA as well as on gapped DNA. In addition, it was able to replicate an unprimed, single-stranded, circular phage DNA through a coupled primase-polymerase action. On Biogel A-0.5m filtration the primase-polymerase activities appeared in the void volume, demonstrating a mass of greater than 500 kilodaltons. Primase and various primase-polymerase complexes synthesized unique primers on single stranded DNA templates and the size distribution of primers was dependent on the structure of the DNA and the nature of the primase-polymerase assembly.

INTRODUCTION

Substantial progress has been made in recent years in the isolation and analysis of the DNA polymerase α and DNA polymerase I in yeast, the major replicating enzyme in eukaryotic chromosomal DNA replication (1-12). In the past, like a variety of eukaryotic DNA polymerases, DNA polymerase I has been isolated utilizing DNA synthesis on gapped DNA templates as an assay. However, this assay is not capable of appraising many in vivo actions of DNA polymerase I, such as rate, processivity, etc. Various forms of DNA polymerase α (or I) have been isolated from a wide variety of eukaryotic cells (3-12). The complexity of structural variations among different preparations may have been, in part, due to the fragility of the multiprotein polymerase assembly, proteolytic degradation, and lack of suitable assays capable of assessing the in vivo actions of a multifunctional polymerase.

Development of new assays and purification schemes by Baril and coworkers (13,14) have resulted in the isolation of DNA polymerase α in a multimeric, multifunctional holoenzyme form from HeLa cells.

We describe here (i) two assays to probe the polymerase and the coupled

primase-polymerase action of yeast primase-polymerase assembly on long single stranded DNA templates, an activity, that is an important prerequisite for a primary chromosomal replicator, (ii) the existence of a high molecular weight holoenzyme-like form of DNA primase-polymerase complex and (iii) the priming by different primase-polymerase complexes on various DNA templates.

MATERIALS AND METHODS

Materials

The sources of most materials used in the present study have been described earlier (15). All ribonucleoside triphosphates were HPLC purified ultrapure grade (99.99% pure) from ICN Biochemicals, and the deoxyribonucleoside triphosphates were from Pharmacia-P.L. Biochemicals. The nucleotides were analyzed by reversed phase HPLC and thin layer chromatography for impurities (<0.1%) and contaminations. The contaminant-free nucleotides were dissolved in sterile HPLC-grade water and neutralized with 4 M NaOH to pH 7.5. Single stranded (ss) M13-ARS1 recombinant phage DNA was constructed by inserting the 838 bp EcoRI/HindIII fragment containing the ARS-1 sequence of yRP7 plasmid in the EcoRI/HindIII digested M13mp18 plasmid DNA. The ssDNA was purified according to published procedure (16). The R199G phage (17) was constructed by inserting the replication origin of bacteriophage G4 in R199 plasmid by Dr. G.N. Godson and obtained from Drs. M. Stayton and A. Kornberg. DNA polymerase I antiserum raised in rabbit was a kind gift of Dr. Lucy M. S. Chang (3).

Methods

Primed DNA Elongation Assay-The R199G template, was hybridized with a synthetic pentadecamer primer (mol ratio DNA:primer = 1:10) and was used as the primed DNA template. The 25 µl reaction volume contained 20 mM Tris-HCl, pH 8.0, 5 mM dithiothreitol (DTT), 5% glycerol, 5 mM $MgCl_2$, 100 µM each of dATP, dGTP, dCTP and ^{3}H-dTTP (100-200 cpm/pmol), 2 mM ATP and 200 pmol (as nucleotide) of primed R199G ssDNA. The reactions were allowed to proceed for 30 minutes at 30°C and terminated by chilling in ice and the DNA was precipitated by adding 1ml of 10% TCA/0.1 M sodium pyrophosphate. The net DNA synthesis was measured by collecting the precipitate on Whatman GF/C filters and counting in a liquid scintillation counter using a toluene based scintillator. One unit of activity is defined as the amount of synthesis

activity that incorporates one nmol of deoxynucleotide per hour at 30°C into TCA insoluble form.

Coupled Primase-Polymerase Assay-In this assay, the 25 µl reaction mixture contained all the components of the "primed DNA elongation assay" except that the primed DNA was omitted and it contained 200 pmol of unprimed R199G DNA and 100 µM each of ATP, GTP, CTP and UTP. Unless otherwise stated, the standard reaction was carried out for 30 minutes at 30°C. The remaining steps of the assay were identical to those of the previous assay.

Activated Calf Thymus DNA and Primase Assays-These assay were carried out as described in an earlier publication (15).

Primer Analysis-Primer synthesis by purified primase, primase-polymerase fraction III, and immunoaffinity purified primase-polymerase complex on poly(dT), poly(dC), and M13-ARS1 DNA was examined. The reaction conditions were essentially the same as described for a standard primase assay except that DNA polymerase I and all deoxynucleotides were omitted and 1000 pmols of ssDNA were added. Labeled and unlabeled ribonucleotides were added to the reaction mixture as follows: 2 nmols, 20 µCi α^{32}P-ATP for poly(dT) template; 2 nmols, 20 µCi α^{32}P-GTP for poly(dC) template; 2 nmols each of 10 µCi α^{32}P-ATP, 10 µCi α^{32}P-GTP, CTP, UTP for all native DNA templates and controls. The reactions were terminated by the addition of 1 µl of 500 mM EDTA. Calf intestinal phosphatase (0.5 units) was added to each sample and the samples were incubated at 37°C for 30 minutes. The primers were successively extracted with phenol/chloroform, chloroform and ether. Ten micrograms yeast tRNA was added and the primers were precipitated with 0.1 volumes of 3M sodium acetate, 2.5 volumes ethanol at -80°C for 30 minutes. The precipitates were collected by centrifugation at 4°C in a microfuge and reprecipitated once. The pellets were dried and resuspended in 5 µl of 95% deionized formamide, 89mM Tris-borate (pH 8.3), 2mM EDTA, 0.1% Xylene cyanol, 0.1% bromophenol blue and then placed in a 100°C bath for three minutes, followed immediately by chilling in an ice-water bath. The samples were loaded on a pre-electrophoresed 20% polyacrylamide (acrylamide:bis = 19:1) gel containing 7M urea and electrophoresed in 89 mM Tris-borate, pH 8.3,& 2 mM EDTA at 2400 V, 50°C in a BioRad Sequi-Gen apparatus. The gels were dried and autoradiographed using Kodak XAR-5 film.

Purification of Primase and the High Molecular Weight (HMW) Primase-Polymerase Complex- Purification DNA primase was carried out according to published procedure (15). The high molecular weight primase-polymerase assembly was purified as described below.

The preparation of yeast extract, ammonium sulfate fractionation of the primase and polymerase activities, and the phosphocellulose chromatography have been described earlier (15). The primase-polymerase activity peak from the phosphocellulose chromatography was precipitated with 0.3 g/ml ammonium sulfate and the precipitate was collected by centrifugation. The precipitate was dissolved in 50 mM Tris-HCl (pH 7.5), 20% glycerol, 1 mM EDTA, 5 mM DTT, 10 μg/ml each of pepstatin A and leupeptin (buffer I) at a protein concentration of 2-10 mg/ml and then dialyzed against 500 volumes of buffer I. The specific activities were as follows : primase, 120 U/mg; polymerase, 60 U/mg. The dialyzate (fraction III) was stored at -80°C, where it remains stable for at least one year.

Gel Filtration Analysis-In order to estimate the mass of the complex, fraction III was analyzed further by gel filtration chromatography on a 0.7 cm X 50 cm column of BioGel A-0.5m. A 0.5ml aliquot of fraction III was loaded on the BioGel column equilibrated with buffer I and was eluted with the same buffer. Forty 0.5 ml fractions were collected. The fractions were assayed for primase and polymerase activities. Purification of primase-polymerase complex in this step was three fold.

Monoclonal Antibody (MAb) Affinity Purification of Primase-Polymerase- Purification of primase-polymerase using an antibody-affinity column provided by Dr. Lucy M.S. Chang was carried out according to published procedure (4) with the following modification. The 3.5 M $MgCl_2$ eluate containing primase-polymerase activities was collected in 0.5 ml fractions in tubes containing 0.5 ml of 2X buffer I and the fractions were mixed immediately. This modification was helpful for stabilizing both primase and polymerase activities. The fractions containing protein were pooled and dialyzed against 400 volumes of buffer I. The specific activities were as follows: primase, 2,400 U/mg; polymerase, 9,500 U/mg. The dialyzate was concentrated to 100-200 μg/ml protein by ultrafiltration and stored at -80°C.

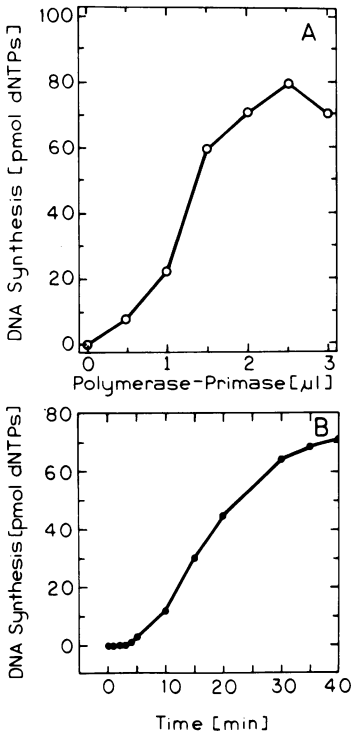

Figure 1 **Replication of Primed DNA by Primase-Polymerase Fraction III.** (A) DNA synthesis on a primed DNA template by primase-polymerase complex fraction III. (B) The time course of replication of primed DNA using 0.6 units of polymerase activity of primase-polymerase fraction III. In both (A) and (B) synthesis was carried out on R199G DNA hybridized with a synthetic pentadecamer primer.

RESULTS

Conversion of Singly Primed Circular ss Phage DNA to Replicative Form by the Primase-Polymerase Complex Fraction III -The DNA synthesis on a singly-primed ss R199G phage DNA by the yeast primase-polymerase fraction III is presented in Figure 1A. The maximal observed dNTP incorporation into TCA insoluble form was 79 pmols. With an input of 200 pmols of template DNA this synthesis accounted for approximately 40% replication. The DNA synthesis catalyzed by the primase-polymerase fraction III had an initial lag period of approximately 3.5 minutes. After this initial lag, DNA synthesis proceeded

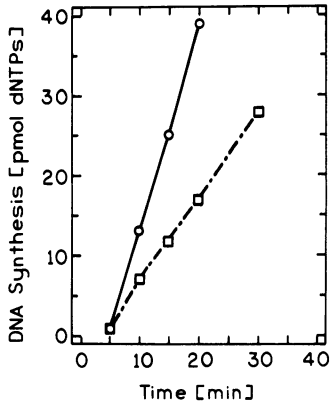

Figure 2 **Enhancement of Replication of Primed DNA by ATP.** The reactions were carried out as described in Materials and Methods except with [○] or without [□] the added 2 mM ATP.

with an apparent linearity for at least 20 to 30 minutes (Figure 1B). The rate of synthesis in this linear phase of DNA synthesis was approximately 200 pmols/hr at 30°C. With only 50% (as determined by replication using DNA polymerase III holoenzyme and single stranded DNA binding protein of E. coli) of the input DNA templates primed with the synthetic primer, the rate of synthesis was approximately 270 nucleotides/min/template and the extent of synthesis is approximately 6320 nucleotides/template/hr. Figure 2 shows the dependence of the elongation reaction on ATP. ATP (2 mM) enhanced the rate and extent of DNA synthesis. However, the effect of ATP was only two fold.

The optimal concentration of Mg^{2+} was approximately 5 mM (Table I). Higher concentration of Mg^{2+} was inhibitory. In the activated calf thymus DNA assay, the optimum Mg^{2+} concentration was 10 mM (data not shown). DNA polymerase II and the mitochondrial DNA polymerase have optimal Mg^{2+} concentrations of 25 mM and 50 mM respectively (9). Aphidicolin, a specific inhibitor of DNA polymerase I and II, had a profound effect on the DNA synthesis on the primed DNA template. An inhibition of 50% was observed with 10μg/ml aphidicolin and the inhibition of the polymerase activity in the activated calf thymus DNA assay was comparable. The polymerase activity on primed DNA template was inhibited strongly by the pol I antiserum (Table I). The half maximal inhibition (50%) of DNA synthesis was achieved with 0.125 μl of antiserum in the standard assays (Tables I&II). Together these data

TABLE I
Characterization of the Primed DNA Elongation Assay

Conditions	DNA Synthesis
Complete	100%
+ α-amanitin (1 mg/ml)	95
- ATP	50
+ aphidicolin (10 μg/ml)	50
+ ammonium sulfate (25mM)	50
+ $MgCl_2$ 0mM	< 2
5mM	100
10mM	60
+ anti pol I serum (0.125 μl)	50
- primed DNA	< 2
- primase-polymerase fraction III	< 2
+ primase-polymerase DEAE sepharose pool	<15
+ immunoaffinity purified primase-polymerase	<10

DNA synthesis activity was measured for 30 minutes in the primed DNA elongation assay as described in the Materials and Methods using 0.6 units of polymerase activity. Activity of 100% equals 79 pmols of dNMP incorporation into TCA insoluble form.

suggest that the active DNA polymerase in the primase-polymerase fraction III was DNA polymerase I rather than DNA polymerase II or mitochondrial DNA polymerase.

The conversion of primed R199G ss to RF form was salt sensitive. A 50% inhibition of DNA synthesis was observed at 25 mM ammonium sulfate. Ammonium sulfate concentration of 160 mM inhibited DNA synthesis completely. In contrast, 50% inhibition of DNA synthesis in activated calf thymus DNA assay was observed at 100 mM ammonium sulfate. However, 160 mM ammonium sulfate inhibited DNA synthesis completely in both assays.

DNA Synthesis by Primase-Polymerase Complex Fraction III on Unprimed ssDNA Template-In the presence of ribonucleotides (100 μM each) and ATP (2 mM), the primase-polymerase complex in fraction III carried out efficient replication

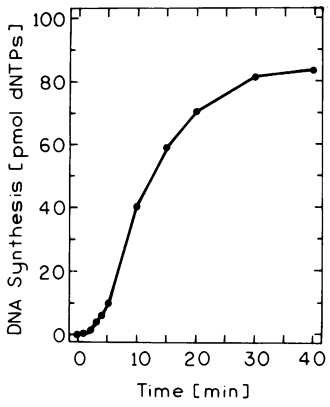

Figure 3 Replication of Unprimed DNA by Primase-Polymerase Fraction III. The reaction was carried out in a manner similar to that for Figure 1 except that unprimed R199G was used as the DNA template.

TABLE II
Requirements of Coupled Primase-Polymerase Reaction

Conditions	DNA Synthesis
Complete	100%
+ aphidicolin (10 μg/ml)	56
+ ammonium sulfate (25 mM)	52
+ anti pol I serum (0.125 μl)	48
+ α-amanitin (1 mg/ml)	100
- GTP, CTP, and UTP	30
- ATP, GTP, CTP, and UTP	<10
- DNA	< 2
- primase-polymerase fraction III	< 2
+ yeast DNA primase	2

DNA synthesis activity was measured for 30 minutes in the coupled primase-polymerase assay using 0.6 units of polymerase activity as described in the Materials and Methods. Activity of 100% equals 86 pmols of dNMP incorporation into TCA insoluble form.

of an unprimed ssDNA template. Time course of DNA synthesis on an R199G template is shown in Figure 3. In the complete system, the extent of deoxynucleotide incorporation accounted for a 43% replication of the input DNA. The omission of GTP, CTP and UTP resulted in 70% decrease of deoxynucleotide incorporation (Table II). Omission of all four ribonucleotides resulted in DNA synthesis less than 10% of that observed with the complete system.

The DNA synthesis catalyzed by the primase-polymerase coupled reaction had a lag time of less than 60 seconds. This is in direct contrast to that observed in the elongation of a primed DNA template. The rate of DNA synthesis was, however, linear up to 20 minutes. The rate of DNA synthesis in the linear range was approximately 250 pmol/hr at 30°C, a value that is comparable to that observed with the primed DNA template.

The DNA synthesis by the primase-polymerase complex is most likely a two step process; priming of the template and elongation of the primer. The priming step of the replication was carried out by primase, as opposed to RNA polymerase, as evident from the lack of effect of α-amanitin (1 mg/ml) (Table II).

The DNA synthesis by polymerase-primase complex was sensitive to aphidicolin and the inhibition by aphidicolin was comparable to that observed with gapped DNA and primed DNA template (Table II). The DNA synthesis was inhibited 52% with 0.125 μl pol I antiserum (Table II). The coupled reaction was as sensitive to ammonium sulfate as the elongation of the primed DNA alone (Table II), suggesting that both the primed DNA elongation assay and the coupled primase-polymerase assay most likely measured the activity of the same form of DNA polymerase.

The Primase-Polymerase Complex with Holoenzyme-like Activity Has a Molecular Weight >5X10^5 Daltons-On Biogel A-0.5m filtration, the following activities eluted in the void volume: (i) DNA primase activity, (ii) DNA polymerase activity on activated calf thymus DNA, (iii) DNA polymerase activity on primed DNA template, and (iv) DNA primase and polymerase activities in the coupled primase-polymerase reaction. This data indicates that all of the above activities were most likely present in a macromolecular complex with a mass of 500 kD or greater. Further chromatography on DEAE sepharose in buffer I using a 0 to 1000 mM KCl gradient resulted in significant loss of

Figure 4 Primer Synthesis by Primase and Primase-Polymerase Complexes. Primers synthesized by 1.0 unit of primase (lane 1), one unit of primase activity of immunoaffinity purified primase-polymerase complex (lane 2), and of primase-polymerase fraction III (lane 3) in 30 minutes at 30°C with 1000 pmol of poly(dT) template.

holoenzyme like activity without any detectable impairment of the gap-filling activity (Table I).

Priming of ssDNA Templates by Primase and Various Primase-Polymerase Complexes-Primase is an integral part of the primase-polymerase complex in a variety of eukaryotic cells. Under certain conditions, however, it can be purified in a state free of polymerase components and their activities (15, 18-20). In order to explore the nature of primer synthesis we have analyzed the primers synthesized by primase and several primase-polymerase complexes on poly(dT), poly(dC), and M13-ARS1 DNA templates.

On poly(dT) template, primase synthesized primers up to 15 nucleotides long (unit length or monomeric) (Figure 4). However, primase-polymerase fraction III and immunoaffinity purified primase-polymerase complex synthesized, in addition to these primers, larger primers, which appeared to be multiples of the 15 nucleotides long monomeric primer (multiunit

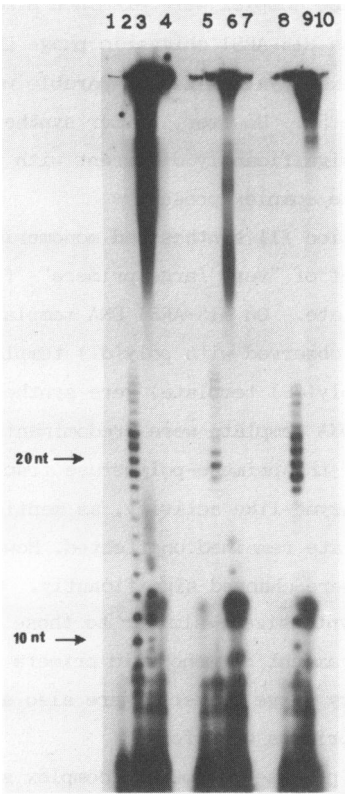

Figure 5 Primer Synthesis by Primase and Primase-Polymerase Complexes on poly(dT), poly(dC), and M13-ARS1 ssDNA Templates. All reactions were carried out for 30 minutes at 30°C with one unit of primase activity on 1000 pmol of input DNA template. Lanes 1-4: primase-polymerase fraction III with: no DNA (lane 1), poly(dT) (lane 2), poly(dC) (lane 3), M13-ARS1 (lane 4). Lanes 5-7: primase-polymerase fraction of DEAE sepharose with: poly(dT) (lane 5), poly(dC) (lane 6), M13-ARS1 (lane 7). Lanes 8-10: immunoaffinity purified primase-polymerase complex with: poly(dT) (lane 8), poly(dC) (lane 9), M13-ARS1 (lane 10).

length or multimeric). Syntheses of similar multimeric primers on poly(dT) templates were also observed by Vishwanath and Baril in HeLa cells (21) and Singh et al. in yeast (22).

We have examined further the multimeric primer synthesis on various ssDNA templates with different primase-polymerase complexes. The primase-polymerase fraction III with holoenzyme like activity, primase-polymerase fraction III after DEAE sepharose chromatography, and immunoaffinity

purified primase-polymerase complex were examined simultaneously using poly(dT), poly(dC), and ss M13-ARS1 chimaeric phage DNA. All of the primase-polymerase complexes synthesized comparable multimeric primers on poly(dT) template (Figure 5). However, primer synthesis on poly(dC) and M13-ARS1 templates were significantly different with respect to the template and the primase-polymerase complex present.

Primase-polymerase fraction III synthesized monomeric primers and in addition synthesized a set of "very large primers" (>50 nucleotides in length) on poly(dC) template. On M13-ARS1 DNA template neither multimeric primers (similar to that observed with poly(dT) template) nor the very large primers (observed with poly(dC) template) were synthesized. The only primers synthesized on M13-ARS1 DNA template were predominantly monomeric in nature.

Further fractionation of the primase-polymerase fraction III on DEAE sepharose impaired holoenzyme-like activity, as mentioned earlier. The priming on poly(dT) template remained unaffected. However, the primers synthesized on poly(dC) were changed significantly. On poly(dC) template monomeric primers were synthesized, similar to those described earlier, but a decrease in the relative amount of monomeric primers was observed. The relative size of the "very large primers" were also attenuated. On M13-ARS1 template only monomeric primers were formed.

Immunoaffinity purified primase-polymerase complex synthesized multimeric primers on poly(dT). However, only monomeric primers were detectably synthesized on poly(dC) and M13-ARS1 templates.

DISCUSSION

The complexity of the replication of the eukaryotic genome and rigid control of cell division make analysis of the mechanism of replication of the eukaryotic genome difficult. Several *in vitro* eukaryotic DNA replication systems have been developed recently (23-25). However, lack of DNA replication mutants in addition to cellular complexities makes the dissection of such *in vitro* systems difficult.

DNA polymerase α of higher eukaryotes and DNA polymerase I of the yeast, *S. cerevisiae*, have been extensively purified in various laboratories and in general are thought to consist of four subunits (3-5). Recent studies in HeLa cells by Baril and coworkers (14) indicated that a multiprotein assembly with complex subunit structure and multiple functions is likely the

cellular form of DNA polymerase α. This multiprotein complex of DNA polymerase α is thus similar in many properties, including a 3' -> 5' exonuclease activity, to DNA polymerase III holoenzyme of E. coli.

In the yeast, S. cerevisiae, our results indicate that DNA polymerase I likely exists in a form that is capable of replicating long single stranded DNA templates similar to that observed by Vishwanath and Baril (21). Primase-polymerase fraction III replicated DNA templates primed with a synthetic oligonucleotide with ~40% of the input DNA replicated in 30 minutes. This synthesis was due to DNA polymerase I as evident from the aphidicolin sensitivity, Mg^{2+} optimum, and pol I antiserum inhibition. The primed DNA replication was stimulated by ATP (Table I, Figure 2). ATP functioned as a stimulator of DNA polymerase I and/or induced primer synthesis by primase in the primase-polymerase complex, thereby increasing the number of primers on the ssDNA template.

Primase-polymerase fraction III also replicated efficiently an unprimed DNA template. This synthesis, however, required a full complement of the ribonucleotides. α-amanitin insensitivity of synthesis indicated priming of the template by the primase component of the primase-polymerase complex. Aphidicolin sensitivity, Mg^{2+} optimum, pol I anti-serum and salt inhibition (Table II) are comparable to that observed with a primed DNA template and the synthesis was likely due to the polymerase I component of the complex.

Highly purified primase-polymerase complex from immunoaffinity purification, with four subunits of 180-140 kD, 78 kD, 58 kD, and 48 kD, appeared to be inactive (less than 10%) on either the primed DNA or unprimed DNA templates, although it displayed strong activity in the activated calf thymus DNA assay (data not shown). Consequently, the high molecular weight form of primase-polymerase complex described here was structurally and functionally different from the immunoaffinity purified primase-polymerase. Further purification resulted in the diminution of activity on single stranded DNA templates without any measurable loss of gap filling activity.

Differences between free primase and the primase in the various primase-polymerase complexes were most prominent in the relative sizes of the primers formed on different templates. On poly(dT) template, free primase synthesized predominantly monomeric primers ("unit-length" as defined by Singh et al., ref. 22) (Figure 4). In contrast to free primase, the primase in the various primase-polymerase complexes examined thus far synthesized

monomeric and multimeric ("multi-unit length") primers (Figures 4 and 5). Primer analysis with DNA primase and primase-polymerase complex of HeLa cells by Vishwanath and Baril (21) also showed that monomeric primers were the predominant species with free primase and multimeric primers were the predominant species only in the primase-polymerase complex. Therefore, variation in the size of the primers synthesized by free primase and primase-polymerase complex is likely common to eukaryotes in general. On poly(dC) template primase-polymerase complex fraction III synthesized monomeric primers. The multimeric primers, observed with poly(dT) template, were not observed. However, a group of "very large primers" (>50 nucleotides) were synthesized. These "very large primers" were synthesized in a poly(dC) template specific manner as no primer synthesis was observed in the absence of DNA template with α^{32}P-GTP. The primers could be degraded by either RNase-free DNase or DNase-free RNase (data not shown). However, these results are not surprising since Cotterill et al. (26) very elegantly showed the incorporation of deoxynucleotides into primers even when using HPLC purified rNTPS, in the complete absence of dNTPs, with Drosophila melanogaster primase-polymerase. The exact mechanism of synthesis of the very large primers is unknown. However Cotterill et al. (26) proposed two mechanisms for primer elongation past the monomeric length and one of these mechanisms may play a role in the synthesis of very large primers. In the immunoaffinity purified primase-polymerase complex a further decrease in the size and the relative amount of the "very large primers" occurred. Thus, primase action is substantially influenced by the various subunits of the primase polymerase assembly. Primase in the primase-polymerase complex synthesized unique primers in a template-specific manner on synthetic DNA templates; free primase and the various primase-polymerase complexes that we have examined thus far synthesized neither the multimeric primers nor the "very large primers" on native DNA templates, such as, M13-ARS1 (Figure 5), øX174, R199G and M13mp18 (data not shown). The only detectable primers formed on any of these native DNA templates were monomeric. Thus, although primase is capable of synthesizing primers of diverse sizes, the primer size is definitely dependent on the DNA sequence and one would predict that the predominant primers formed during chromosomal DNA replication in vivo would be the monomeric variety.

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