Volume 13 Number 8 1985 Nucleic Acids Research

DNA synthesis in yeast cell-free extracts dependent on recombinant DNA plasmids purified from *Escherichia coli*

Ambrose Y.S.Jong* and John F.Scott+

Department of Microbiology, 131 Burrill Hall, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

Received 5 February 1985; Accepted 25 March 1985

ABSTRACT

In our attempts to establish a cell-free DNA replication system for the yeast Saccharomyces cerevisiae, we have observed that recombinant DNA plasmids purified from Escherichia coli by a common procedure (lysozyme-detergent lysis and equilibrium banding in cesium chloride ethidium bromide gradients) often serve as templates for DNA synthesis by elongation enzymes. The templates could be elongated equally well by enzymes present in the yeast cell-free extracts, by the large proteolytic fragment of E. coli DNA polymerase I or by T4 DNA polymerase. The template activity of the purified plasmids was dependent on the presence of heterologous DNA segments in the bacterial vectors. The template activity could be diminished by treatment with alkali. We propose that the ability of recombinant plasmids isolated from bacterial hosts to serve as elongation templates may lead to erroneous conclusions when these plasmids are used as templates for in vitro replication or transcription reactions.

INTRODUCTION

Chromosomal DNA replication in the yeast Saccharomyces cerevisiae is similar to that observed in the cells of higher eukaryotic organisms (1,2). This makes yeast a favorable model organism for the study of eucaryotic DNA replication since it is well suited for detailed genetic and biochemical experimentation. Recently, several investigators have attempted to isolate replication origins using recombinant DNA techniques using yeast as a test organism. Many eucaryotic automously replicating sequence (ARS) elements from yeast (3,4,5) as well as from other organisms (6,7) have been isolated and described. It has been hypothesized that ARS elements, which promote high frequency transformation and extrachromosomal maintenance of recombinant DNA plasmids in yeast, are serving as the initiation sites for DNA replication in plasmid molecules (3). Scott and Brajkovich (8) constructed a novel plasmid called TRPI RI Circle, [more recently named YARp1 (Yeast Acentric Ring plasmid 1)] which consists of ARS1 and a functional TRP1 gene. Further results described by Zakian and Scott (9) and Fangman et al. (10) indicate

that YARp1 has the replicative properties expected for an origin of replication from yeast chromosomal DNA. The analysis of ARS plasmid replication in vitro is one suitable way to explore the nuclear DNA replication mechanism of yeast in more detail.

Most strains of Saccharomyces cerevisiae contain 50-100 copies per cell of a naturally occuring circular DNA plasmid called 2 micron (2 μm) DNA. Control of the replication of 2 μm DNA is also similar to that of chromosomal DNA (11). It exists in high copy number and replicates under nuclear control once per molecule per cell division early in S phase (12). It is stable mitotically and meiotically and has been completely sequenced (13). The 2 μm DNA plasmid is another attractive model template for development of eucaryotic in vitro DNA replication systems.

An early in vitro system, using 2 μm DNA as its template, described by Jazwinski et al. (14) indicated that intact circular product molecules were produced. The results also suggested that the activity stimulating 2 μm DNA replication in vitro might be subject to control by the yeast cell cycle. Scott (15) reported the preferential utilization of a DNA fragment containing ARS1 as template (in comparison with an adjacent yeast chromosomal fragment of similiar size) in extracts prepared as described by Jazwinski and with modifications. However, the system was not specific since bacterial plasmid fragments were also efficiently utilized. Kojo et al. (16) developed another in vitro 2 μm DNA replication system and apparently identified the origins and directions of replication of both native 2 μm DNA and a chimeric plasmid containing the 2 μm DNA. Celniker and Campbell (17) have reported a site-specific in vitro system for initiation of replication on ARS1-containing plasmids. A similar study of DNA replication in vitro in an extract prepared from unfertilized eggs of Xenopus laevis has also been described (18). These results suggest that DNA replication is initiated at a specific site or sites on the eukaryotic DNA segment of chimeric DNA plasmids and usually proceeds bidirectionally.

Recombinant DNA plasmids have become a heavily utilized source of template DNA for both DNA and RNA synthesis in vitro. Their small size, well defined sequences and availability in large quantities in purified form from inexpensive bacterial sources have made them especially tractable. In some applications the templates are used to simply provide highly radioactive hybridization probes produced by nick-translation. In those cases the initiation of DNA synthesis occurs at nicks in the template which either pre-exist or are produced by low levels of non-specific endonuclease treatment.

Thus, low levels of adventitious primer fragments base-paired to the template plasmids or other activation for elongation would be of no concern. However, it has become increasingly popular to use such plasmids as templates in reactions designed to test for initiation of replication or transcription at specific functional sites. In these applications, the presence of adventitious primers or other template activation would clearly present a confusing and disadvantageous situation.

In this paper, we describe various methods for yeast crude extract preparation, either published previously (16) or developed in our laboratory, which we have used to study DNA synthesis in vitro. Apparent ARS-dependent DNA synthesis was observed; however, the synthesis could be mimicked by using the large fragment of E. coli DNA polymerase I or T4 DNA polymerase in place of yeast extract. Our observation of this fact was initiated by an exchange of plasmid DNA templates with Dr. Lawrence Dumas and his colleagues at Northwestern University, who observed that our active templates were able to serve as good templates for E. coli DNA polymerase I in the absence of yeast extract (personal communication). This and further evidence suggests that the bulk of DNA synthesis observed in our yeast crude extracts is, rather than initiation-dependent synthesis, due to elongation of DNA templates activated by the presence of endogeneous base-paired primers or by some other unknown mechanism.

METHODS

Yeast strains used: A364A:mata, ade1,2, ura1, his7, lys2, tyr1, (19); Sc3-[rho°]:matα, trp1, his3-Δ1, ura3-52, gal2, gal10/[cir°], [rho°] (20,21); JSY 12:matα, his3-Δ1, ura3-52, gal2, gal10, trp1/TRP1 RI Circle, [cir°] (9).

Bacterial strains used: HB101:r_K^-, m_K^-, SUPE44, lacZ, leuB6 proA2, B1$^-$, recA13 (22); KM601:Sm^r, recA13 (23); BNN56:trpc9830 (24); LE392:r_K^-, m_K^+, lacY, metB, galK, galT, SUPE, SUPF, trpR (4); HB101/YIp1 (3); HB101/pBR322, (25), KM601/YRp7 monomer (23); BNN56/YRp7-Sc2605 (23); JA221/pJDB219 (26); HB101/pMB9 (22); BNN56/pMT1, (27); LE392/pML2 (28); LE392/Yp412 (23); LE392/Yp412-URA3 (23); LE392/Yp414-URA3 (23).

Enzymes and chemicals: The large fragment of E. coli DNA polymerase I was from New England Nuclear. T4 DNA polymerase was purchased from Bethesda Research Laboratory. Lysozyme was from Sigma. Zymolyase 60,000 was from Kirin Brewery, Japan. All non-radioactive ribo- and deoxyribonucleotides were purchased from P-L Biochemicals. [Methyl-^{3}H] dTTP and [α-^{32}P] dTTP were from New England Nuclear.

Preparation of yeast extract: The yeast crude extracts were prepared according to the method of Kojo et al. (16) and with several modifications described in RESULTS.

Protein assay: Protein concentration was determined by the binding of Coomassie Brilliant Blue G-250 to protein using the method of Bradford (29).

Incubation conditions for in vitro DNA synthesis: The reaction mixture (0.05 ml) contained 35 mM HEPES (pH 7.8), 10 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM ATP, 6.2 x 10^{-2} unit/ml pyruvate kinase, 3 mM phosphoenolpyruvate, 31%

glycerol (v/v), 200 μM each CTP, GTP and UTP, 12.5 μM each dATP, dCTP and dGTP and 10 μM dTTP (labeled with ^3H, 700 cpm/pmol), 20 μg/ml of various covalently closed circular plasmid DNAs and various quantities of active yeast crude extracts.

Assay of DNA synthesis: Reactions were stopped by spotting an aliquot of incubation mixture onto Whatman DE-81 paper (1.5 cm square). Papers were washed in a gently agitated beaker with 0.5 M disodium phosphate (6 times), H_2O (2 times) and 95% ethanol (2 times). [^3H]dTTP was used for the measurement of deoxyribonuleotides incorporated into the DE-81 bound fractions in a Beckman LS 9800 Liquid Scintillation Counter.

Assays using the large fragment of E. coli DNA polymerase I or T4 DNA polymerase: The reaction mixtures containing E. coli DNA polymerase I large fragment were identical to those containing yeast crude extracts. The purified enzyme (0.16 units) was substituted for yeast crude extract in the reaction. The reaction mixture for T4 DNA polymerase (0.05 ml) contained 50 mM Tris-Glycine buffer (pH 8.8), 6 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 6.2 μM EDTA, 0.17 mg/ml bovine serum albumin, 25 μM each of dATP, dCTP, and dGTP, 20 μM dTTP (labeled with ^3H, 700 cpm/pmol), 16.2 mM $(NH_4)_2SO_4$, and 20 μg/ml DNA. T4 DNA polymerase (0.4 units) was added to start the reaction.

Agarose gel electrophoresis and autoradiography: Reaction products labeled by use of [α-^{32}P]dTTP instead of [^3H]dTTP were phenol extracted and ethanol precipitated prior to fractionation on a 1.5% agarose slab gel. Electrophoresis was carried out at 100 V for 5 hr until the marker dye bromocresol purple reached the middle of gel. The gel was dried in a Gel Slab Dryer (Bio-Rad Model 224) and autoradiographed at -70°C with a Dupont Quanta III intensifying screen and Kodak XRP-1 film. Gels to be photographed were stained in 0.5 μg/ml ethidium bromide and photographed using UV transillumination.

Preparation of plasmids: Bacterial cells were grown in TYE broth containing 10 g Difco Tryptone, 5 g Difco Yeast Extract and 8 g NaCl per liter. The cells were usually incubated in the presence of chloramphenicol (0.16 mg/ml) for 12 hr, harvested and frozen at -20°C. Cell lysate was prepared by two methods: In method (I) similar to a procedure in (30), cell pellets were thawed and lysed in a buffer solution containing lysozyme (2 mg/ml), 15% sucrose, 6 mM EDTA, 30 mM Tris-HCl (pH 8.0) on ice for 20 min. A second solution of equal volume containing 1% Triton X-100, 62.5 mM EDTA, 5 mM Tris-HCl (pH 8.0) was added. The lysate was gently inverted (5x) and incubated on ice for 5 min. The cell debris and large DNA were removed by centrifugation. Plasmid DNA was purified from the supernatant by banding twice in a CsCl/ethidium bromide gradient in a Beckman Ti 70 rotor at 40,000 rpm and 17°C for 40 hr. The ethidium bromide was removed by extraction with 2-propanol and the DNA was recovered by dilution (4 fold) with 10 mM Tris-HCl, pH 7.5, 1 mM EDTA followed by precipitation with 2 volumes of 2-propanol. In method (II) plasmid DNA extracts were prepared by a scaled-up version of an alkaline procedure for mini-lysates published previously (31). The covalently closed circular plasmids extracted from 500 ml or 1 liter cultures of E. coli cells were then purified as described for method I.

RESULTS

DNA Synthesis in Yeast Cell-Free Extract Dependent on Template Plasmids Containing Yeast DNA. When plasmids containing a yeast TRP1-ARS1 fragment (YRp7, pMT1) or the 2 μm ARS (pJDB219) were used as templates in a yeast cell-free extract [prepared essentially as described previously (16)],

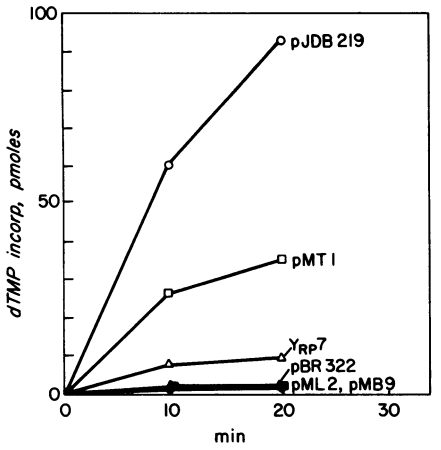

FIGURE 1: A Comparison of Template Activities for DNA Synthesis in a Yeast Cell-free Protein Extract. DNA synthesis activity of the extract from S. cerevisiae A364A (cir+) (0.4 mg protein/ml) with plasmids (20 μg/ml) pJDB219 (PMB9 2 μm ARS+) (○), pMB9 (ARS-) (●), YRp7 (pBR322 ARS1+) (△), pBR322 (ARS-) (▲), pMT1 (PML2 ARS1+) (□) or pML2 (ARS-) (■) was measured in the standard assay for 10 and 20 min at 30°C.

significant DNA synthesis was observed (Figure 1). When the parent vector plasmids containing no yeast sequences [pBR322, pML2 (28), or pMB9, respectively] were used as templates in the same extract, a low background of synthesis was observed usually representing 10-20 fold less incorporation. This indicated that the extract exhibited little nick-translation activity. Incorporation directed by pMT1 and pJDB219 was comparable in extent when corrected for plasmid size. The YRp7 template was less active than pMT1, but still directed 2-3 fold more synthesis than its parent vector pBR322. These results were suggestive of in vitro DNA replication of recombinant plasmids dependent on the presence of either 2 μm ARS or ARS1 of S. cerevisiae, as had been reported by others using comparable systems (14,16,17).

Comparison of yeast cell-free extracts and purified enzymes for in vitro DNA synthesis activity: Detailed examination of the 2 μm ARS and ARS1 dependent reactions with various crude extract preparations and enzymes was carried out (Table I). Both reactions (a) and (b) in Table I exhibited what appeared to be highly ARS dependent DNA synthesis in yeast extracts. Our procedure to prepare yeast crude extracts consisted of cell lysis followed by precipitation of proteins by slow addition of solid ammonium sulfate, with stirring, on ice, to 50% saturation. The precipitated proteins were pelleted by centrifugation and resuspended in ice cold buffer containing 45% saturated ammonium sulfate. After the precipitated proteins were well dispersed, the remaining precipitate was collected by centrifugation. The resulting "backwashed" pellets were found to yield reproducible DNA synthesis activities with low backgrounds of nick-translation [reactions (a) and (b)].

Some modifications of lysis conditions, i.e. with or without detergent

Table I
Comparison of *In vitro* DNA synthesis using DNA templates prepared by Method I in various yeast extracts and using purified enzymes

Reaction:	dTMP incorporated, pmoles (a)	(b)	(c)	(d)	(e)	(f)
Extract Source or Enzyme: / DNA (20 µg/ml)	A364A	Sc3	Sc3 (High Salt)	A364A (DEAE)	Large fragment of *E. coli* DNA polymerase	T4 DNA polymerase
pJDB219 (pMB9 2 µm ARS+) (AI)	92.7	83.3	33.2	33.5	87.9	28.8
pMB9 (ARS-) (AI)	1.2	2.7	1.9	3.6	1.6	1.7
YRp7 (pBR322 ARS1+) (AI)	7.5	8.7	14.2	6.5	9.3	7.4
pBR322 (ARS-) (AI)	1.9	2.9	1.8	2.4	1.3	2.2
pMT1 (pML2 ARS1+) (AI)	33.7	32.0	18.1	10.8	40.2	16.9
pML2 (ARS-) (AI)	1.7	2.5	2.0	4.7	3.4	3.0
YRp7-Sc2605 (YIp1 ARS1+) (AI)	7.0	14.7	9.6	17.5	19.7	—
YIp1 (pBR322 HIS3+ ARS-) AI)	11.1	17.8	14.3	20.6	43.7	—
Without added DNA	0.7	1.1	1.1	1.8	0.7	1.0

All reaction incubations were at 30°C for 20 minutes as described in Methods. Plasmid sources are referenced in Methods. Reactions were stopped by spotting a aliquot on DE-81 paper. All product quantities were normalized to a 0.1 ml reaction volume equivalent. (--) = not determined. All plasmids were purified from *E. coli* which had been amplified with chloramphenicol and were prepared by the triton sucrose method I as described in Methods. They are marked (AI) to distinguish them from plasmids prepared by other methods shown in Table II.

(a) and (b)The yeast crude extracts, from strain A364A (a) or Sc3 (b) were prepared as described in Methods. Extract protein concentration was optimal at 0.65 mg/ml for pJDB219 template.

(c)Crude extracts were made from strain Sc3[rho°][cir°] in a manner essentially identical to (a) except that 390 mM ammonium sulfate was included in the solution which was used to resuspend the spheroplasts for lysis. Extract protein concentration was optimal at 4.0 mg/ml for pJDB219 template.

(d)Yeast crude extracts were prepared by DEAE-cellulose chromatography based on Hiraga *et al.* (18) except that a "Bead Beater" (Biospec Products) was used to disrupt the cells. The reaction mixture contained 35 mM HEPES (pH7.8), 10 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM ATP, 6.2×10^{-2} unit/ml pyruvate kinase, 3 mM phosphoenolpyruvate, 31% glycerol (v/v), bovine serum albumin at 0.2 mg/ml, 0.20 mM each GTP, UTP, and CTP, 31 µM each dATP, dCTP, dGTP and 25 µM dTTP (labeled with ^3H, 700 cpm/pmol), 25 mM KCl, 25 mM NaCl, 20 µg/ml of various plasmid DNAs, and 6.3 mg/ml active fractionated protein.

(e)The reaction mixtures for *E. coli* DNA polymerase I large fragment were identical to those for (a). The large fragment of E. coli polymerase I (0.16 units) was substituted for yeast crude extract in the reactions.

(f)The reaction mixtures for T4 DNA polymerase contained 50 mM Tris-glycine (pH 8.8), 6 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 6.2 µM EDTA, 0.17 mg/ml bovine serum albumin, 25 µM each dATP, dCTP, dGTP, 20 µM dTTP labeled with ^3H (700 cpm/pmol), and 16.2 mM $(NH_4)_2SO_4$. 20 µg/ml DNA was included. 0.4 Unit T4 polymerase was added to initiate the reaction.

and in the presence or absence of 0.1-1.0 M salt have also been tried. For example: with a high salt condition for spheroplast lysis, more protein was precipitated from crude extracts which resulted in less efficient replicative activity, presumably indicating that more inhibitory activity was included in the pellet [reaction (c)]. Furthermore, DEAE-cellulose passage of the extract in a manner similar to that described by others (17,18) did not yield

a better extract, based on total activity and background [reaction (d)].

Surprisingly, the large fragment of DNA polymerase I [reaction (e)] or T4 DNA polymerase [reaction (f)], exhibited the same pattern of activity or lack of activity for particular templates as did each of the yeast extracts. These enzymes both lack 5' to 3' exonuclease activity and are therefore not capable of nick translation. We also have shown that the activities of the active templates were not reduced by passage through a Bio-gel A1.5M column, indicating that the presence of a population of small contaminating fragments free from the plasmid was not a likely cause of activity. The results indicated that the active template preparations contain nascent DNA or RNA primers, probably base-paired to a subset of the supercoiled molecules, or that they are activated for elongation by some other unknown mechanism. Most of our active templates contained either a 2 μm ARS (pJDB219) or ARS1 (YRp7, pMT1). One plasmid (YIp1) which contained a yeast chromosomal HIS3 segment but no functional ARS was found to serve as an elongation template to an intermediate extent (Table I).

Comparison of DNA Templates Prepared by an Alternative Alkaline Lysis Procedure (Method II) and/or Without Amplification by Chloramhenicol: Plasmid DNAs prepared by an alkaline lysis procedure (31), or prepared from cells grown to stationary phase without chloramphenicol amplification by either method, did not exhibit large amounts of the elongation template activity. Overall DNA synthesis with such templates was low: neither chloramphenicol amplified plasmids prepared by Method II nor non-amplified plasmids prepared by Method I or II exhibited any significant DNA synthesis activities (Table II). We also observed that elongation template activity of purified plasmids could be reduced approximately 50% by treatment at pH 12.2 followed by neutralization and precipitation (data not shown). These results support the proposal that the DNA synthesis in yeast crude extracts was due to elongation of pre-primed or activated DNA templates by yeast replicative enzymes. Apparent ARS-dependent activities were observed for all of the various yeast extract preparation methods tried. However, the ratios of DNA synthesis using the large fragment of E. coli polymerase I with various templates were comparable to those observed using the yeast extracts on each set of templates.

Properties of in vitro primer-dependent or activation-dependent DNA synthesis using pMT1 template prepared by Method I: Incubation of pMT1 DNA with a yeast crude extract resulted in incorporation of labeled dTMP into DE-81-bound material. After an initial burst of incorporation, the extent of the

Table II
Comparison of *in vitro* DNA synthesis using unamplified or alkaline-prepared DNA templates in various yeast extracts and using the large proteolytic fragment of *E. coli* DNA polymerase I.

	dTMP incorporated pmoles			
Reaction:	(a)	(c)	(d)	(e)
Extract Source or Enzyme:	A364A	Sc3 (High Salt)	A364A (DEAE)	Large fragment of *E. coli* DNA polymerase I
DNA (20 μg/ml)				
YRp7 (ARS1+) (UI)	2.0	2.0	2.8	3.4
Yp414-URA3 (ARS1-) (UI)	1.6	3.1	3.0	2.8
Yp412-URA3 (ARS1+) (UI)	1.6	2.6	3.7	2.7
YRp7 (ARS1+) (UII)	1.4	2.7	6.0	1.2
Yp414-URA3 (ARS1-) (UII)	1.2	1.9	5.1	1.1
Yp412-URA3 (ARS1+) (UII)	1.2	2.3	4.1	2.1
Trp1 RI Circle (YARp1 ARS1+)	0.9	2.1	2.1	0.6
Without added DNA	0.7	1.1	1.8	0.7
pJDB219 (pMB9 2 μm ARS+) (AII)	2.7		2.0	3.6
pMB9 (ARS-) (AII)	1.8		1.4	2.5
pMT1 (pML2 ARS1+) (AII)	2.0		0.7	1.2
pML2 (ARS-) (AII)	1.7		0.5	0.9
pJDB219 (pMB9 2 μm ARS+) (AI)	36.7		17.8	32.6
Without added DNA	1.5		0.4	0.8

Yeast extracts and reaction conditions were identical to those described in the Table I legend. All of the extracts were stored at -80°C for several weeks between the time the assays in Table I and those above the dotted line in this table were done, and when the assays below the dotted line were done. This storage period resulted in a lower level of activity of the extracts being observed in the later assays. The Yp412-URA3 and Yp414-URA3 plasmids consist of deletion derivatives of YRp7 where one or the other of two portions of the yeast TRP1 ARS1 fragment and a portion of pBR322 have been replaced by a yeast URA3 1.1 kb fragment. Yp412-URA retains ARS1 in functional form while Yp414-URA3 does not. Plasmid DNA templates were prepared from *E. coli* amplified with chloramphenicol by alkaline method II marked (AII), or without amplification by chloramphenicol using triton lysis method I marked (UI) or by alkaline method II marked (UII) as described in Methods. TRP1 RI Circle was prepared from yeast essentially as described previously (9). The pJDB219 template marked (AI) was chloramphenicol amplified and prepared by Method I and was the same as in Table I.

reaction was dependent on the amount of plasmid DNA added. A linear relationship between DNA concentration and dTMP incorporation for a 30 min reaction was observed (data not shown). Similar linear profiles were obtained at various time points, indicating that DNA synthesis was proportional to the concentration of plasmid DNA. Presumably, DNA template was the limiting factor for DNA synthesis in these yeast crude extracts. Titration of protein from yeast crude extract in the reaction was performed. The optimal concentration of yeast protein was between 0.4 and 0.7 mg/ml for plasmid pMT1. A decrease of dTMP incorporation observed with higher concentration of yeast extract protein (>0.7 mg/ml) may be due to a salt effect, *i.e.* a higher concentration of yeast extract resulted in a higher concentration of ammonium sulfate in the reaction.

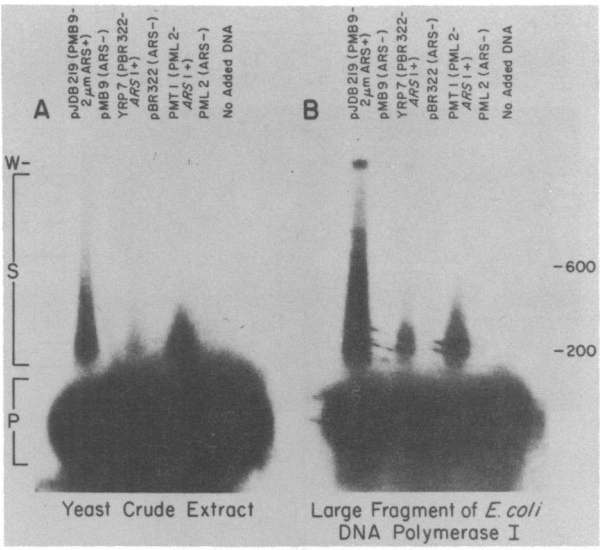

FIGURE 2: DNA Synthesis Products of The Extract from S. cerevisiae A364A (cir+) (Panel A) and the E. coli DNA Polymerase I Large Proteolytic Fragment (Panel B) Reacted With Plasmid Templates or With No Added DNA as Indicated Above Each Lane. The reaction mixtures were phenol extracted following 20 min incubation at 30°C, precipitated by ethanol and applied to 1.5% agarose gels. Agarose gel electrophoresis and autoradiography were as described in Methods. W = position of wells where the samples were loaded. S = small fragment products. P = unincorporated ^{32}P-dTTP. The 200 base pair and 600 base pair indicated positions are based on comparison with un-labeled double stranded ØX174 DNA fragments run on the same gel.

Analysis of in vitro products by agarose gel electrophoresis: The bulk of the labeled products of the reactions using yeast extract or the large fragment of E. coli DNA polymerase I appeared to have similar sizes on neutral agarose gels (Figure 2). The predominant incorporation products with pJDB219, YRp7 and pMT1 templates were small fragments with sizes in the range of 50-600 bases measured in comparison with duplex HaeIII-cut ØX174 DNA fragments. Although larger fragments were visible for pJDB219 template (Figure 2), similar patterns could be observed for both pMT1 and YRp7 after a longer autoradiographic exposure (data not shown). Therefore, predominant incorporation products were not covalently attached to the templates, eliminating the possibility that they were the result of nick-translation or gap filling. The autoradiographic density of bulk incorporation was proportional to the

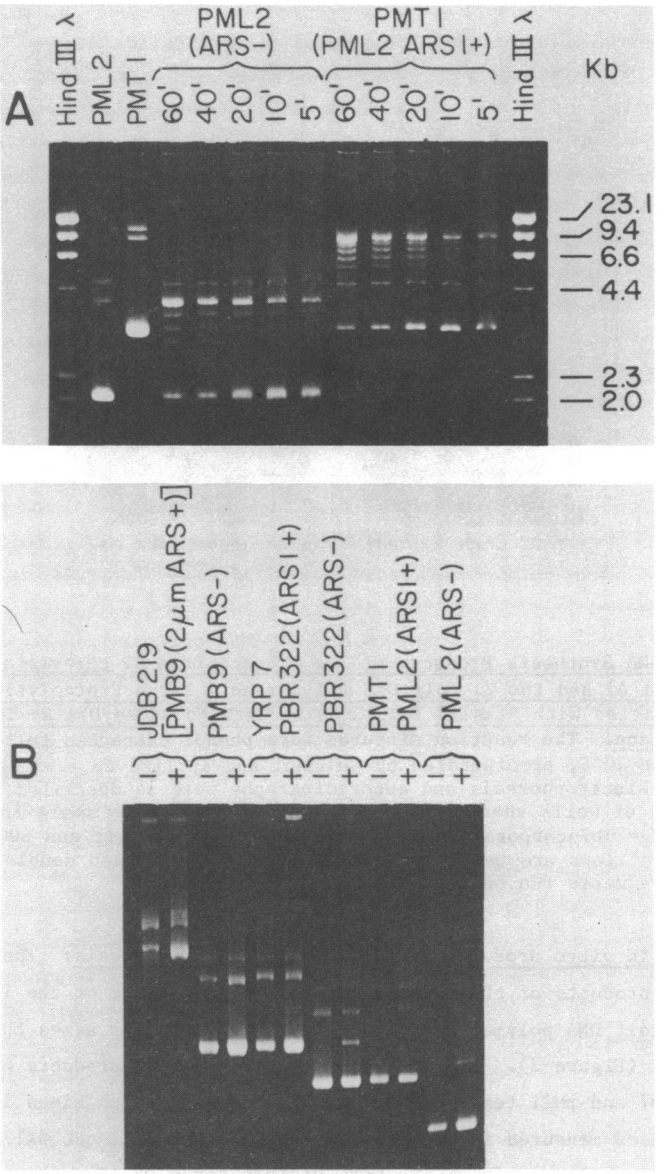

FIGURE 3: Agarose Gel Electrophoretic Analysis of Template DNA Plasmids Before and After Reaction in Yeast Crude Extracts (Panel A) and with the Large Proteolytic Fragment of *E. coli* DNA Polymerase I (Panel B). Template plasmids were as described in Methods and were prepared by Method I. The lanes marked HindIII λ in panel A contained λ bacteriophage DNA cut to completion with HindIII restriction enzyme and served as DNA size markers. The sizes in kilobase pairs are indicated to the right of panel A. Times of reaction in

minutes for each template are shown above lanes in panel A. The lanes marked (-) in panel B were unreacted and those marked (+) were reacted for 20' at 30° C as described in Methods for the Large Proteolytic Fragment of E. coli DNA polymerase I. The gels were 0.7% agarose and were run and photographed as described in Methods.

activity of DNA synthesis shown in Table I, i.e. pJDB219 > pMT1 > YRp7. The autoradiographic density with pMB9, pML2 or pBR322 templates containing only E. coli plasmid DNA was negligible.

Analysis of templates on agarose gels before and after the DNA synthesis reactions verified that the templates were not degraded or nicked to a major extent during the reactions (Figure 3). When DNA synthesis was carried out using crude yeast extract the templates were partially relaxed in a stepwise fashion during the course of the reaction (Figure 3, panel A). This indicated that the bulk of the template DNA remained intact, with little nicking or degradation occuring. The partial relaxation observed was probably due to topoisomerase activity present in the yeast crude extract. In reactions using the large proteolytic fragment of E. coli DNA polymerase I the bulk of the templates were essentially unchanged following a 20 min reaction (Figure 3, panel B). One exception was the observation of a small amount of the pBR322 (ARS-) template being converted to linear form by some unknown means while the bulk remained supercoiled. No such production of linear forms was observed for the other templates. This analysis also demonstrated a lack of any obvious differences in the electrophoretic behavior of plasmids which were active templates for elongation and those which were not, other than differences due to size.

DISCUSSION

We have described DNA synthesis reactions in yeast cell-free extracts dependent on addition of template DNA. The basic profiles of DNA synthesis for various extract preparations relative to DNA templates used were similar to one another (Table I). These data suggest that the proteins essential for DNA elongation synthesis were included in the yeast extract preparations used. The reaction products have been examined by agarose gel electrophoresis and the bulk found not to be covalently attached to the template, indicating that nick-translation and gap filling were negligible. The DNA synthesis observed in our work appeared at first to represent de-novo initiation of DNA replication in vitro due to its supposed dependence on the presence of an ARS in the template DNA. However, our more recent observations make it unlikely that a significant portion of the synthesis observed was dependent

on initiation and may jeopardize the previous conclusions (16,18,32) that ARS-dependent DNA synthetic activities represented initiation of DNA replication.

We have shown that the bulk of the labeled product DNA in our reactions were in the form of small fragments which were not retained by the template even when examined by non-denaturing methods such as agarose gel electrophoresis in pH 8.0 buffers. This is a common observation in eukaryotic in vitro DNA synthesis systems; others have suggested that it may be due to the small average size of discontinuous synthesis products coupled with the absence of a complete fragment joining system in the extracts (25). We have found that the product DNA is homologous to the template by hybridization analysis, but we have no evidence for preferential initiation at any particular site or sites on the supercoiled plasmid templates (data not shown). Our observation that the bulk of incorporation was dependent on the presence of a yeast template segment (2 μm DNA, the ARS1-TRP1 fragment, or the HIS3 fragment), but was not site specific, could be taken to imply that the required sequence was a loading site for the replication initiation apparatus. Alternatively, it may be required for the retention of base-paired oligonucleotides (DNA or RNA) which could provide a primer for synthesis by any DNA polymerase or for some other unknown activtion mechanism. We consider the priming or activation explanation more likely since the synthesis could be accomplished by an E. coli elongation polymerase alone in the absence of yeast extract.

All of our crude yeast extract preparations exhibited extensive DNA synthesis on the 2 μm ARS template pJDB219 prepared by lysozyme-detergent lysis (Method I), but little or no synthesis on the parent vector pMB9 template prepared by the same method. The ARS1 template YRp7 yielded 2 to 4 fold higher incorporation of labeled precursors than its parent vector pBR322 using the same quanity of template. The reaction was very meager in comparison with that observed with pJDB219 template. However, our data reproduces the previously reported observations of Kojo et al. (15), who found that their extracts were very active on the 2 μm ARS template pJDB34 (a close relative of pJDB219), but were only weakly active on YRp7. Interestingly, when we used the ARS1 plasmid pMT1 as template DNA, we found that it was as active as pJDB219, while pML2 template (the vector from which pMT1 was constructed) yielded a low background of incorporation comparable to other ARS-minus templates. In terms of ARS+ versus ARS- template activity in various yeast extract preparations, YIp1 (yeast HIS3 fragment in pBR322) was

an exception in that it exhibited moderate template activity even though it was ARS-. It is possible, but not extensively tested in our work, that the presence of any yeast DNA fragment (ARS+ or ARS-) may correlate with enhanced template activity.

In addition to the presence of a yeast fragment in the plasmid, we found that the method of preparation of the DNA played a critical role in determining presence of elongation template activity in a particular batch of plasmid DNA. Template plasmids prepared by a method which included an alkaline treatment did not yield any significant DNA synthesis even when they contained ARS elements. This suggests that alkaline treatment removed oligonucleotide primers from the plasmids which were retained when the chimeric plasmids were prepared from E. coli by non-disruptive methods or eliminated the activation by some other unknown means. Different yeast crude extracts yielded quantitatively different DNA synthetic activities on individual templates, but the template preparation method and the presence of a contiguous yeast DNA segment were the critical factors in determining high or low activity of the template in the DNA synthesis reactions.

We have also used the large proteolytic fragment of E. coli DNA polymerase I or T4 DNA polymerase as substitutes for yeast crude extracts in the reactions. These enzymes were selected for the comparison since they both lack 5' to 3' exonuclease activity and are therefore not capable of nick-translation. This permitted us to test for the presence of pre-primed or otherwise activated molecules in the template preparations while not being troubled by incorporation at random nicks which were probably present in a small proportion of the plasmids in any preparation. The ability of either of these enzymes to substitute completely for the yeast extract, strongly suggests that the active template preparations contained base-paired primers associated with the plasmid molecules.

The most remarkable aspect of this observation is the correlation of template activity with the presence of a yeast DNA fragment in the E. coli plasmid, for which we have no clear explanation. One reasonable interpretation for the influence of a yeast fragment on the retention of nascent primer strands in the supercoiled molecules is that the E. coli replication apparatus may abort elongation at a site or sites within the foreign insert, especially under the condition of chloramphenicol induced amplification. The result of abortion of the elongation process may be the retention of a base-paired nascent DNA or RNA strand. A likely cause for such abortion events may be the presence of "pause sites" for the E. coli replication apparatus at

sites in the foreign DNA. The sites not normally experienced by the E. coli elongation system might consist of sequence elements present at eukaryotic transcription control or promoter sites, replication origins or other functional sites. While we have observed no site-specificity in our systems, this could provide an alternative interpretation for apparent site-specific initiation observed by others in similar systems by electron microscopy and other methods (16,18), if the primer fragments are retained at preferred sites and serve as starting points for longer product DNA strands in those systems. The alignment of replication bubbles observed in the electron microscope may be site-specific, but in at least some cases may represent preferred abortion sites, rather than true de novo initiation events. A major weakness of this scheme is that leading strand starts should all occur from the ColE1 origin. However, lagging strand starts might occur at several different sites.

The possible presence of primers in the template DNA cannot explain our previous observation of preferential template activity of an ARS1 yeast DNA fragment, since the preference was not observed when the template was labeled by end-filling using the large fragment of E. coli DNA polymerase I (15). Other workers have also observed a low level of apparent site-specific initiation using templates which they verified to have low elongation template activity (17) or which were prepared directly from yeast (14). Thus, it is still possible that a small amount of true initiation may occur in some of the in vitro systems which use naked DNA templates, but this can be easily obscured by the presence of primers in or other activation of the template. Since elongation reactions catalyzed by RNA polymerases can be stimulated by primer termini as well (33,34), primers retained in template plasmids used for in vitro transcription studies may also result in confusion.

In our experience, preparation of the plasmids by alkaline treatment of the lysate yielded recombinant DNA templates having no increased elongation template activity when compared with vector DNA prepared by the same method. Several other preparation methods have also been found to yield templates containing little or no elongation template activity, including Triton X-100 lysis of E. coli cells grown without chloramphenicol amplification. We have also observed that elongation decreased approximately 50% when plasmids exhibiting template activity were treated at pH 12.2 as purified DNA. However, while these preparations contain a much lower amount of template activity, they all exhibit at least some small amount of residual activity

for the elongation polymerases alone.

It is therefore of utmost importance that all researchers who make use of recombinant DNA plasmids as templates for in vitro transcription or replication investigations prepare plasmid DNA using methods which disfavor retention of adventitious primers or other activation. In addition, since many of the reactions in these systems are quite inefficient, frequently utilizing only a few percent of the template molecules, it is necessary to verify that each template preparation does not contain endogenous elongation template activity in detectable amounts, and that the initiation events observed are insensitive to pre-treatments of the templates which would be expected to disrupt primer association or other activation of the template molecules.

ACKNOWLEDGEMENTS

We thank our numerous colleagues in the yeast and bacterial DNA replication field for many helpful conversations and shared items of information. In particular we would like to thank Lawrence Dumas and his colleagues at Northwestern University and Akio Sugino and his colleagues for the free exchange of DNA templates, yeast extracts and information which led to the observations reported here. We also thank Judith Campbell and Michael Jazwinski for sharing information prior to publication; Judith Jaehning, Michael Strathearn and Michael Woontner for critical readings of this manuscript; and Sharon Bartholomew for its careful preparation.

This work was supported by Public Health Service Grants GM27000, GM30602 and GM32072 from the National Institutes of Health and Grant PCM 82-11497 from the National Science Foundation.

+To whom correspondence should be addressed

*Present address: Department of Chemistry, California Institute of Technology, Pasadena, CA 91125, USA

REFERENCES

1. Petes, T. D. (1980) Ann. Rev. Biochem. 49, 845-876.
2. Sheinin, R., Humbert, J. and Pearlman, R. E. (1978) Ann. Rev. Biochem. 47, 277-316.
3. Struhl, K., Stinchcomb, D. T., Scherer, S. and Davis, R. W. (1979) Proc. Natl. Acad. Sci. USA 76, 1035-1039.
4. Hsiao, C. L. and Carbon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 3829-3833.
5. Chan, C. S. M., and Tye. B.-K (1980) Proc. Natl. Acad. Sci. USA 77, 6329-6333.
6. Stinchcomb, D. T., Thomas, M., Kelly, J., Selker, E. and Davis R. W. (1980) Proc. Natl. Acad. Sci. USA 77, 4559-4563.
7. Zakian, V. A. (1981) Proc. Natl. Acad. Sci. USA 78, 3128-3132.
8. Scott, J. F. and Brajkovich, C. M. (1981) ICN-UCLA Symp. Mol. Cell. Biol. 22, 517-527.
9. Zakian, V. A. and Scott, J. F. (1982) Mol. Cell. Biol. 2, 221-232.
10. Fangman, W. L., Hice, R. H. and Chlebowicz-Sledziewska, E., (1983) Cell 32, 831-838.

11. Broach, J. R. (1982) Cell 28, 203-204.
12. Zakian, V. A. Brewer, B. J. and Fangman W. L. (1979) Cell 17, 923-924.
13. Hartley, J. L. and Donelson, J. E. (1980) Nature, 286, 860-865.
14. Jazwinski, S. M. and Edelman, G. M. (1979) Proc. Natl. Acad. Sci. USA 76, 1223-1227.
15. Scott, J. F. (1980) ICN-UCLA Symp. Mol. Cell Biol. 19, 379-388.
16. Kojo, H., Greenberg, B. D. & Sugino, A. (1981) Proc. Natl. Acad. Sci. USA 78, 7261-7265.
17. Celniker, S. E., and Campbell, J. L. (1982) Cell, 31, 201-213.
18. Hiraga, S., Sudo, T., Yoshida, M., Kubota, H. and Meyama, H. (1982) Proc. Natl. Acad. Sci. USA 79, 3697-3701.
20. This strain was constructed by Dr. Bradley C. Hyman (personal communication) in the same manner as described by him for other [rho°] strains (21).
21. Hyman, B. C., Cramer, J. H. and Rownd, R. H. (1982) Proc. Natl. Acad. Sci. USA 79, 1578-1582.
22. Bolivar, F., Rodriquez, R. L., Betlach, M. C., and Boyer, H. W. (1977) Gene 2, 75-93.
23. Kevin McEntee, personal communication.
24. Yanofsky, C., Horn, V., Bonner, M. and Stasiowski, S. (1971) Genetics 69, 409-433.
25. Bolivar, F., Rodriquez, R. L., Greene, P. J., Bettach, M., Heyneker, H. L., Boyer, H. W., Crosa, J. and Falcow, S. (1977) Gene 2, 95-113.
26. Beggs, J. D. (1978) Nature 275, 104-109.
27. Constructed in this laboratory (M. Tondravi and J. Scott, personal communication) by insertion of the TRP1-RI yeast fragment from YRp7 (3) into the EcoRI site of pML2 (28).
28. Lusky, M. and Botchan, M. (1981) Nature 43, 79-81.
29. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254.
30. Radloff, R., Bauer, W. and Vinograd, J. (1967) Proc. Natl. Acad. Sci. USA, 57, 1514-1522.
31. Birnboim, H. C. and Doly, J. (1979) Nucleic Acid Reserch 7, 1513-1522.
32. Plevani, P., Capucci, L., Ginelli, E., Sacchi, N., and Badaracco, G. (1982) Current Genetics 6, 47-54.
33. Yarbrough, L. R. (1982) J. Biol. Chem. 257, 6171-6177.
34. Lavialle, C., Sekura, R., Madden, M-J. and Salzman, N. P. (1982) J. Biol. Chem. 257, 12458-12466.