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Initiation of Enzymatic DNA Synthesis by Yeast RNA Polymerase I†

P. Plevani‡ and L. M. S. Chang*,§

ABSTRACT: In vitro DNA synthesis by yeast DNA polymerase I can be initiated by partially purified yeast RNA polymerases in the presence or absence of rNTPs. Homogeneous yeast RNA polymerase I initiates DNA synthesis by yeast DNA polymerase I on single-stranded DNA templates only in the presence of all four rNTPs. A protein capable of initiating enzymatic DNA synthesis on single-stranded DNA in the absence of rNTPs has also been separated from partially purified yeast RNA polymerase I fractions. Analyses of the RNA polymerase I initiated replication products of phage fd DNA on alkaline sucrose gradients showed noncovalent linkage between the newly synthesized DNA and the template. Isopycnic analyses of the ribonucleotide initiated fd DNA replication products demonstrated covalent linkage between the initiator RNA and newly synthesized DNA. Results from ^{32}P-transfer experiments confirmed the covalent linkage between RNA and DNA chains and showed the presence of all four ribo- and deoxyribonucleotides at the RNA-DNA junctions. The ribonucleotide found most frequently at the RNA-DNA junction is uridylate and the purine deoxynucleotides occur more frequently than pyrimidine deoxynucleotides.

Purified DNA polymerases from prokaryotic and eukaryotic cells cannot initiate DNA chains de novo (Kornberg & Kornberg, 1974; Bollum, 1974). Analyses of newly synthesized DNA chains in several eukaryotic systems showed the presence of RNA at the 5′ end (Reichard et al., 1974; Waqar & Huberman, 1975a,b; Tseng et al., 1975). It is not known, however, whether all newly synthesized DNA chains are initiated with RNA primers or what enzyme(s) in eukaryotic systems might be involved in synthesis of the primers used in DNA synthesis.

Two DNA polymerases are present in yeast extracts (Wintersberger & Wintersberger, 1970; Helfman, 1973). Studies using synthetic template systems showed that only DNA polymerase I will use oligoribonucleotide as an initiator for the replication of a polydeoxynucleotide (Chang, 1977). In a search for enzymes or proteins capable of initiating DNA synthesis in vitro, we reported that all three partially purified yeast RNA polymerases are capable of initiating DNA polymerase I catalyzed DNA replication in the absence of rNTPs (Plevani & Chang, 1977). The initiation can be enhanced by the addition of all four rNTPs in the in vitro system.

To examine the apparent duality of the partially purified RNA polymerase in the initiation of enzymatic DNA synthesis in the presence and in the absence of rNTPs, it was necessary to continue the purification of the RNA polymerase. We therefore purified yeast RNA polymerase I to homogeneity (Valenzuela et al., 1976) and followed the substrate requirements for initiation activities. This communication describes the separation of rNTP dependent initiation activity of yeast RNA polymerase from a separate component that appears to initiate DNA synthesis with dNTPs alone. The products of yeast RNA polymerase I initiated DNA synthesis and the properties of the RNA polymerase-DNA polymerase coupled reactions are also characterized.

Experimental Procedure

Chemicals Substrates and DNAs. Deoxynucleotide triphosphates (dNTPs)[1] were prepared as previously described (Chang & Bollum, 1971). Commercial sources of other materials used were: rNTPs, [*methyl*-^3H]dTTP, and [2-^{14}C]ATP from Schwarz/Mann; [α-^{32}P]dNTPs from New England Nuclear; Qβ RNA, fd DNA, and $\phi\chi$ 174 DNA from Miles Laboratories; calf thymus DNA and bovine serum albumin from Worthington Biochemical Corp. Phage T_7 DNA was a generous gift from Dr. B. Alberts, University of California at

† From the Department of Biochemistry, University of Connecticut Health Center, Farmington, Connecticut 06032. *Received December 15, 1977.* This investigation was supported by Grant No. CA-17770 and CA-23365, awarded by the National Cancer Institute, Department of Health, Education and Welfare.

‡ On leave from Istituto di Biologia Generale, E.U.L.O., Brescia, Italy.
§ Present address: Department of Biochemistry, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20014.

[1] Abbreviations used for nucleotides are those recommended by the Commission on Biochemical Nomenclature (CBN) of IUPAC-IUB as approved by the Commission of Editors of Biochemical Journals [(1970) *Biochemistry, 9,* 4022]. Other abbreviations used: DTT, dithiothreitol; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetate.

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San Francisco. ^{14}C-labeled fd DNA was prepared as described by Yamamoto & Alberts (1970). The specific activity of ^{14}C-labeled fd DNA was 500 counts per min per μg. *E. coli* DNA was prepared as described by Marmur (1961). Yeast DNA was prepared from yeast nuclei (Bhargava et al., 1972) as described by Marmur (1961). Native DNA was denatured at 500 μg per mL for 10 min in a boiling water bath, followed by rapid cooling in an ice bath. Other chemicals were reagent grade.

Yeast cells. Saccaromyces cerevisiae, strain D 273-10B (PET [ρ^+]) (Sherman, 1965), was grown to late-logarithmic phase, harvested, and stored as previously described (Chang, 1977).

Enzymes. Yeast DNA polymerase I was purified as previously described (Chang, 1977). Yeast RNA polymerase I was purified to homogeneity as described by Valenzuela et al. (1976). Analysis of the yeast homogeneous RNA polymerase I on sodium dodecyl sulfate-acrylamide gel electrophoresis showed subunit structure identical with that reported by Valenzuela et al. (1976).

Separation of Ribonucleotide and Deoxyribonucleotide Initiation Activities. Extract made from 50 g of frozen yeast cells was treated with protamine sulfate (Plevani & Chang, 1977), diluted with equal volume of buffer A (50 mM Tris-Cl buffer at pH 8.0, 1 mM EDTA, 0.5 mM DTT, and 10% glycerol) and loaded onto a 400-mL DE-11 cellulose (Whatman) column previously equilibrated with 0.075 M $(NH_4)_2SO_4$ in buffer A. The column was washed with the equilibration buffer and protein was eluted from the column with 0.3 M $(NH_4)_2SO_4$ in buffer A. Fractions active in initiation of DNA synthesis by DNA polymerase I were pooled and diluted with 3 volumes of buffer A and loaded onto a 200-mL phosphocellulose column (Whatman P-I) previously equilibrated with 0.075 M $(NH_4)_2SO_4$ in buffer A. The phosphocellulose column was washed with the equilibration buffer and protein was eluted from the column with 0.4 M $(NH_4)_2SO_4$ in buffer A. The active fractions were pooled and protein was precipitated at 70% saturation of $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ precipitates were collected by centrifugation, redissolved in 3.0 mL of 0.5 M KCl in buffer A, and fractionated on a 1.5 × 90 cm Sepharose 6B column (Pharmacia) using 0.5 M KCl in buffer A.

Enzyme Assays. DNA polymerase activity was assayed as previously described (Chang, 1977). One DNA polymerase unit is defined as 1 nmol of total nucleotide polymerized into DNA per h at 35 °C, using pancreatic DNase I treated calf thymus DNA as template. The usual reaction mixture for the coupled DNA polymerase-RNA polymerase reaction contained 50 mM Tris-Cl at pH 7.9, 0.5 mM DTT, 8 mM $MgCl_2$, 100 μg per mL of BSA, 0.1 mM each of dCTP, dGTP, dATP, and [*methyl*-^{3}H]dTTP at 120 to 140 counts per min per pmol, 0.1 mM each of CTP, UTP, GTP, and ATP or [2-^{14}]ATP at 30 to 40 counts per min per pmol, and 25 μg per mL of heat-denatured DNA or single-stranded phage DNA. The coupled reactions were generally carried out in small tubes at a final volume of 40 to 150 μL. After addition of all reactants, the tubes were transferred from 4 to 35 °C to start the reaction. Samples (10 to 25 μL) were taken at various times and acid-precipitable material was processed on glass fiber disks and counted in a liquid scintillation counter as previously described (Chang, 1973).

Analyses of the Products of fd DNA and φχ174 DNA Replication on Alkaline Sucrose Gradients. The reactions were carried out as described above and terminated by addition of EDTA, NaCl, and NaOH to give final concentrations of 50 mM, 0.8 M, and 0.3 M, respectively. After incubation at 37 °C for 90 min the samples were dialyzed overnight against 2 L of 1 mM EDTA, 0.8 M NaCl, and 0.1 M NaOH. The samples were then layered onto a linear 5–20% (w/w) sucrose gradient made up in 1 mM EDTA, 0.8 M NaCl, and 0.1 M NaOH. Centrifugation was carried out in a SW 50.1 rotor for 4 h at 45 000 rpm at 4 °C. The gradients were fractionated from the top of the gradient tubes with a Buchler Densi-flow apparatus. The pH of the fractions was adjusted to about 7.5 with 1 M KH_2PO_4 and acid-insoluble radioactivity was determined in each fraction.

Isopycnic Analysis of the Products of fd DNA Replication. Analysis of the products of yeast RNA polymerase I initiated fd DNA replication on formaldehyde:CsCl:Cs_2SO_4 gradients were performed as previously described (Plevani & Chang, 1977).

^{32}P-Transfer Experiments. The reactions (100 μL) were performed as described for the DNA polymerase-RNA polymerase coupled reaction except that the concentration of the rNTPs and [α-^{32}P]dNTPs (500–1100 counts per min per pmol) were 0.05 mM each. $MnCl_2$ was added to a final concentration of 1 mM to increase the amount of RNA synthesized. After 1 h at 35 °C, 0.1 mg of heat-denatured calf thymus DNA was added as carrier and the nucleic acids were precipitated with ice-cold 0.6 M $HClO_4$. The pellet was washed twice with 0.6 M $HClO_4$ and then twice with ethanol. The precipitate was then suspended in 10 mM Tris-HCl (pH 8.4) and precipitated again with 0.6 M $HClO_4$. After a final wash with 0.6 M $HClO_4$ and ethanol, the precipitate was dissolved in 0.3 M NaOH and incubated for 16 h at 37 °C. The isotope transfer analysis was then performed essentially as described by Pigiet et al. (1974).

Results

Separation of Ribonucleotide and Deoxyribonucleotide Initiation of Enzymatic DNA Synthesis. Previous results from this laboratory (Plevani & Chang, 1977) showed that partially purified yeast RNA polymerase I, II, and III can initiate yeast DNA polymerase I catalyzed DNA synthesis on single-stranded DNA templates. The most intriguing aspect of that study was the fact that initiation activity of the partially purified RNA polymerases is not completely dependent upon the presence of rNTPs, although it is stimulated by rNTPs. To determine whether the ribonucleotide independent initiation activity found with the partially purified RNA polymerase is an intrinsic property of yeast RNA polymerases, we have tested homogeneous yeast RNA polymerase I (Valenzuela et al., 1976) for its ability to initiate enzymatic DNA synthesis in the presence and absence of rNTPs. The results from this study showed that the initiation of enzymatic DNA synthesis by RNA polymerase I required the presence of all four rNTPs (Table I). This observation suggests that the deoxyribonucleotide initiation activity previously observed with the partially purified RNA polymerases could be due to the association of protein factor(s) with the enzymes. This factor appears to have been removed from RNA polymerase I during the more extensive purification scheme used in the present work.[2]

The separation of ribonucleotide and deoxyribonucleotide initiation activities was achieved by fractionation of a partially purified yeast extract containing all three RNA polymerases on a Sepharose 6B column in the presence of 0.5 M KCl. Figure 1 demonstrates that activities initiating enzymatic DNA synthesis elute from the Sepharose column in three peaks. Peak

[2] The dNTP initiation activity of the RNA polymerase I preparation was greatly reduced on the sucrose gradient and was completely lost after denatured DNA-cellulose columns.

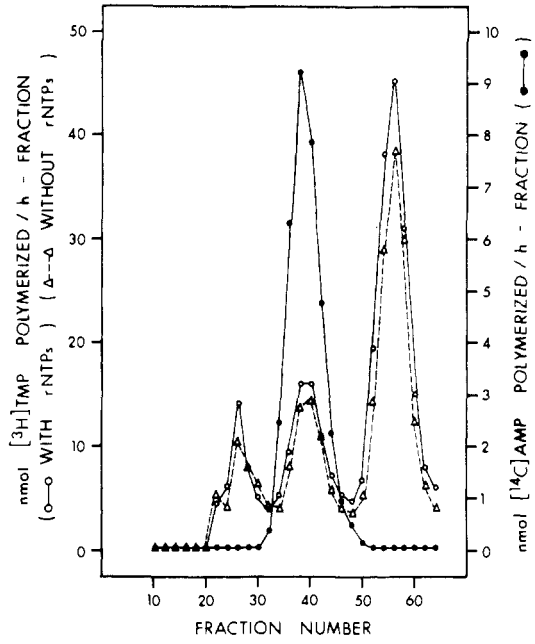

FIGURE 1: Separation of ribonucleotide and deoxyribonucleotide initiation activities on Sepharose 6B column. The partially purified yeast protein fraction was prepared and fractionated on the Sepharose column, and the fractions were assayed in DNA polymerase reactions in the presence or absence of rNTPs as described in Experimental Procedures. The template used was heat-denatured calf thymus at 25 μg per mL and DNA polymerase was at 200 units per mL, and $MnCl_2$ at 1 mM was also included in the reactions when rNTPs were omitted. See Experimental Procedures and text for details.

TABLE I: Requirements for fd DNA Replication in the RNA Polymerase Coupled Reaction.

Reaction conditions	% of DNA synthesized
Complete reaction	100[a]
-yeast DNA polymerase I	<1
-yeast RNA polymerase I	2.7
-fd DNA	<1
-Mg^{2+}	<1
-dCTP	2.5
-dATP	<1
-dGTP	2.3
-ATP	5.8
-GTP	9.2
-CTP	30.0
-UTP	7.2
-ATP and GTP	1.4
-ATP and CTP	2.5
-ATP and UTP	2.3
-GTP and CTP	4.0
-GTP and UTP	3.0
-CTP and UTP	3.8
-ATP, GTP, and CTP	1.9
-ATP, CTP, and UTP	1.2
-GTP, CTP, and UTP	1.1
-ATP, GTP, CTP, and UTP	1.8

[a] One hundred percent corresponds to 219 pmol of [*methyl*-^{3}H]-TMP polymerized per h per reaction. Each mixture contained 0.04 unit of yeast RNA polymerase I and 20 units of yeast DNA polymerase I in a final volume of 40 μL. For complete reaction, see Experimental Procedures.

II corresponds to the RNA polymerase activity. Peaks I and III initiate enzymatic DNA synthesis in the absence of rNTPs. Preliminary characterization studies showed that the initiation activity associated with peak I can be greatly stimulated by Triton X-100 and *Naja naja* venom. An endonuclease activity is also activated in peak I fraction by Triton X-100 treatment. Treatment of peak I fraction with micrococcal nuclease has no effect on the initiation activity, while treatment with trypsin abolished the initiation activity. These results suggest that the initiation activity in peak I is due to a membrane-bound nuclease.

The activity associated with peak III can be completely eliminated by trypsin treatment and is unaltered by micrococcal nuclease treatment. The initiation activity associated with peak III fraction from Sepharose 6B column has now been extensively purified. The molecular weight of the initiation activity is about 37 000 determined by sucrose gradient centrifugation and sodium dodecyl sulfate-acrylamide gel electrophoresis. No exo- or endonucleolytic activities have been detected in the purified peak III protein. Furthermore, the newly synthesized DNA strand in the products of peak III protein initiated enzymatic replication of single-stranded DNA is not covalently linked to the template chain, suggesting de novo synthesis of the product. The mechanism of action of peak III protein in initiation of new DNA chains is under investigation. The experimental results of the dNTP initiation protein are the subject of a future publication.

Properties of the Enzymatic Initiation of DNA Synthesis by Yeast RNA Polymerase I. Since partially purified yeast RNA polymerase I was found to be the most efficient RNA polymerase in initiating enzymatic DNA synthesis in the presence of rNTPs (Plevani & Chang, 1977), the reaction properties of homogeneous yeast RNA polymerase I-yeast DNA polymerase I coupled reactions were investigated in detail. The results are summarized in Table I. The reaction requires an exogenous single-stranded DNA template and a divalent cation. When fd DNA was used as the template, the maximum rate of polymerization of dNTPs were obtained in the presence of magnesium as the divalent cation. $MnCl_2$ at 1 mM catalyzed the polymerization of dNTP in the coupled reaction at about 40% of the rate in 8 mM $MgCl_2$. The amount of ribonucleotide polymerized in the coupled reaction, however, is four- to fivefold higher when manganese is used. In the presence of both magnesium and manganese, deoxynucleotide and ribonucleotide polymerization rates are about 70% and 200%, respectively, of the magnesium catalyzed rates. The presence of salt also affected the rate of DNA synthesis in the coupled reactions. Potassium chloride at 50 mM stimulated the rate of deoxynucleotide polymerization about twofold. Potassium chloride at 100 mM is not stimulatory, and the deoxynucleotide polymerization is inhibited about 50% at 200 mM.

The coupled reaction requires the presence of all four dNTPs and maximum activity was obtained only in the presence of all four rNTPs (Table I). Omission of either ATP, GTP, or UTP reduced DNA synthesis to less than 10% of the control level. In the absence of CTP, however, a significant amount of synthesis (about 30%) can still be demonstrated. Omission of two or more rNTPs in all possible combinations reduced the rate of DNA synthesis to less than 4% of the control level. Yeast RNA polymerase I activity is not inhibited by α-amanitin at low concentration, but is inhibited by high concentrations of α-amanitin (Hager et al., 1976; Schultz & Hall, 1976). The RNA polymers I-DNA polymerase I coupled DNA synthesis is not inhibited by 50 μg per mL of α-amanitin and is inhibited by 80% at 1500 μg per mL of α-amanitin (data not shown) as should be expected.

Initiation of DNA Synthesis on Various Single-Stranded DNAs. When double-stranded DNAs were used as template, no stimulation was observed in the RNA polymerase-DNA

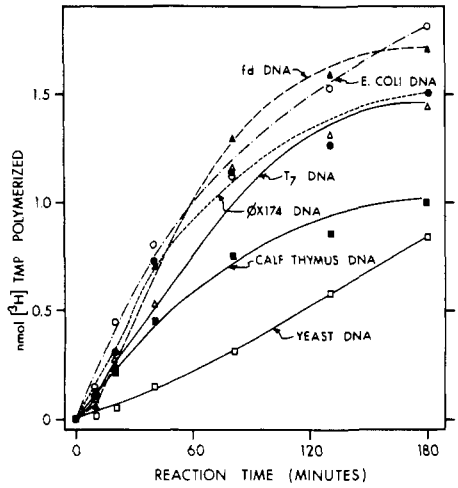

FIGURE 2: Initiation of yeast DNA polymerase I by yeast RNA polymerase I on various single-stranded DNA templates. The coupled reactions (200 μL) were carried out as described in Experimental Procedures. Each reaction contained 80 units of DNA polymerase I, 0.15 unit of RNA polymerase I, and 25 μg/mL of each DNA template. After 3 h of incubation with fd DNA as template, less than 0.10 nmol of [^{3}H]dTMP was incorporated into DNA by yeast DNA polymerase I in the absence of RNA polymerase I.

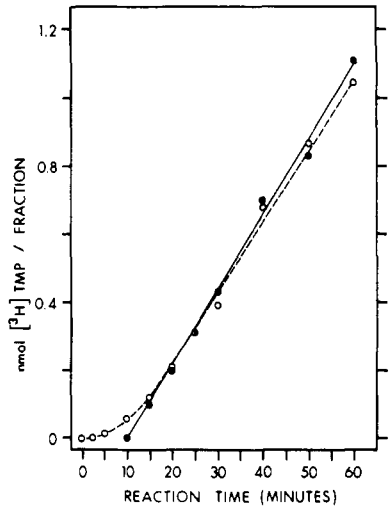

FIGURE 3: Effect of preincubation of template with yeast RNA polymerase I on fd DNA replication by yeast DNA polymerase I. (O—O) The reaction mixture was preincubated at 35 °C and started by simultaneous addition of DNA polymerase I and RNA polymerase I to the complete coupled reaction mixture. (●—●) fd DNA was preincubated with RNA polymerase in complete coupled reaction mixture for 10 min at 35 °C and was started by addition of DNA polymerase. The amounts of enzymes used were the same as described in the legend of Figure 2. See Experimental Procedures for further details.

polymerase coupled reaction. Heat-denatured DNA from different sources and single-stranded circular phage DNAs are suitable templates for the RNA polymerase–DNA polymerase coupled reaction (Figure 2). A lag phase was observed with all the DNAs tested. All reactions proceed linearly for about 90 min and then begin to level off, except when yeast DNA was used as a template. Preincubation of the template and substrates with RNA polymerase I prior to the addition of DNA polymerase eliminates the lag phase, consistent with the idea that RNA polymerase I produces some kind of initiator for the DNA polymerase I (Figure 3). After 3 h of incubation, *E. coli* DNA, fd DNA, $\phi\chi$ 174, phage T_7 DNA, calf thymus DNA, and yeast DNA were replicated to the extent of 85, 81, 71, 65, 43, and 33%, respectively. RNA chains synthesized by RNA polymerase I constitute 5–10% of the newly synthesized product. When ribonucleotide incorporation was monitored in these coupled reactions, no loss in the radioactivity associated with the RNA products was ever observed (data not shown), suggesting the absence of contaminating RNase H activity in our enzyme preparations. For *E. coli* and bacteriophage DNAs, essentially all available template is utilized by the combined action of yeast RNA and DNA polymerases. Figure 4 shows the profile of products of fd DNA replication on a neutral sucrose gradient. All product DNA molecules sediment with the template, showing no displacement of product chains from the template. Since the product chains are small as shown on the alkaline sucrose gradient (Figure 5) over replication of a small region of the template DNA would result in the displacement of products from the template. The results are most consistent with the interpretation that the template DNA sequence is completely utilized and replicated only once in the RNA polymerase–DNA polymerase coupled reaction.

The product of our yeast in vitro DNA replication, however, is not intact double-stranded DNA. In order to generate complete replicative form of the single-stranded DNA template, the RNA must be removed, and the resulting gap and nick repaired by other enzyme activities in the cell.

Analysis of Products of fd DNA Replication on Alkaline Sucrose Gradients. To demonstrate that the products of the

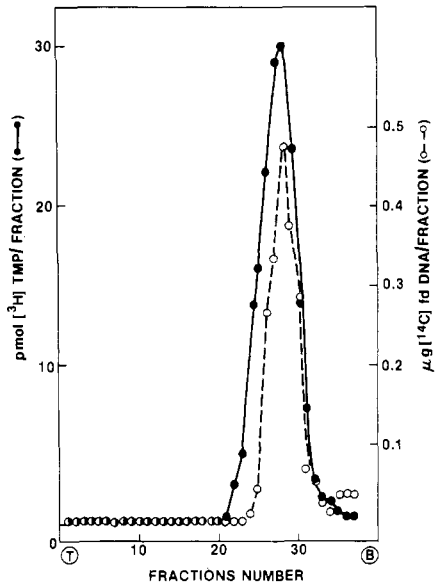

FIGURE 4: Neutral sucrose gradient analysis of the products of RNA polymerase I initiated fd DNA replication. The reaction was carried out as described in the Experimental Procedures except that 50 μg per mL of ^{14}C-labeled fd DNA (500 cpm per μg) was used as template. The reaction was terminated at 12% replication by addition of NaCl and EDTA to 0.8 M and 50 mM, respectively. The products were analyzed on a 5–20% (w/w) sucrose gradient in 0.8 M NaCl, 1 mM EDTA in 20 mM Tris-Cl buffer at pH 7.9. Centrifugation was carried out for 4 h at 45 000 rpm in SW 50.1 Spinco rotor at 4 °C. Analysis of the gradient fractions was as described in the Experimental Procedures.

in vitro replication of fd DNA in the RNA polymerase initiated DNA polymerase reactions are due to de novo synthesis of new chains, we analyzed the products on alkaline sucrose gradients (Figure 5). The products of fd DNA replication were analyzed at early stage of replication, 0.55% replication (Figure 5A). The early products of fd DNA replication were not covalently linked to the template DNA since no ^{3}H radioactivity is associated with the template DNA on the gradient. The complete

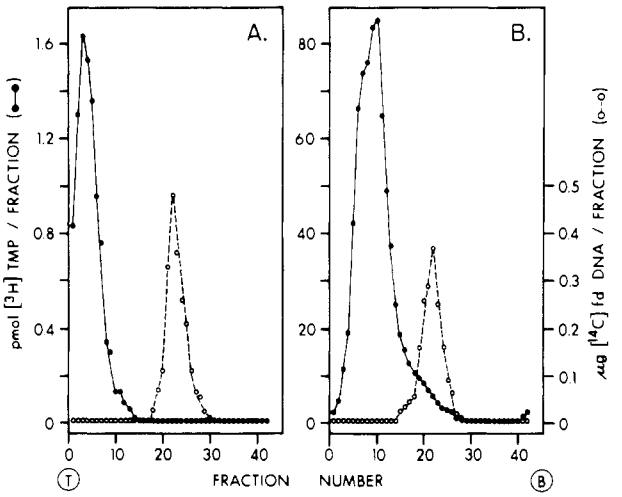

FIGURE 5: Alkaline sucrose gradient analyses of the products of RNA polymerase I initiated fd DNA replication. Reactions were carried out, terminated, and analyzed on alkaline sucrose gradient as described in Experimental Procedures, except that 50 μg/mL of ^{14}C-labeled fd DNA (500 cpm/μg) was used as template. The amounts of RNA polymerase I and DNA polymerase I were the same as described in the legend of Figure 2. Panel A shows the early products of replication and panel B shows the products when the replication was allowed to proceed further. See text for details.

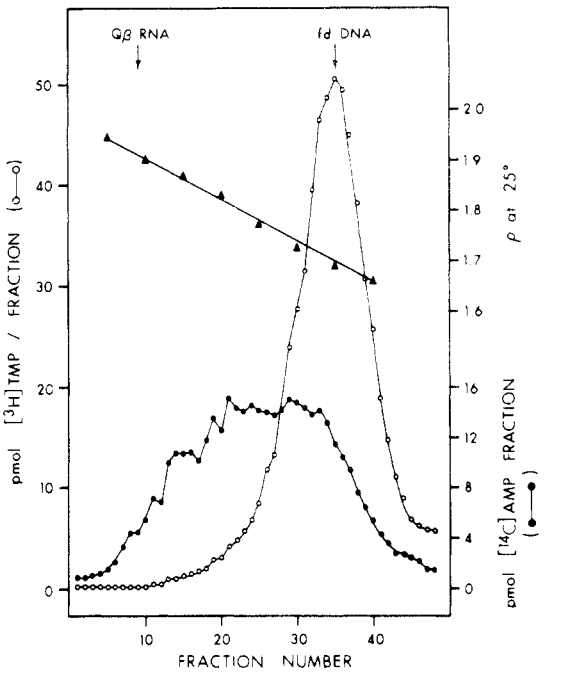

FIGURE 6: Analysis of products of RNA polymerase I initiated fd DNA replication on formaldehyde:CsCl:Cs_2SO_4 gradient. The reactions were carried out as described in Experimental Procedures, except that 1 mM $MnCl_2$ was present in the coupled reaction. The amounts of RNA polymerase I and DNA polymerase I used in the reaction were 0.4 unit and 160 units in a final volume of 200 μL. The replication of fd DNA was allowed to proceed to 25%. Termination of the reaction and analysis of the products were as described (Plevani & Chang, 1977). Radioactivities in each fraction were determined by acid precipitation. The positions of marker nucleic acids, Qβ RNA and fd DNA, were obtained from a separate gradient by optical density measurements at 260 nm. The nucleic acid markers were treated with formaldehyde at 90 °C in the same way as the sample. Fractions 10–18, 19–24, 25–30, and 31–39 of the gradient were pooled and analyzed on alkaline sucrose gradients as described in the text.

separation of product chains with template DNA molecules on alkaline sucrose gradients suggests de novo synthesis of new chains in the RNA polymerase I initiated DNA replication.

The early DNA products of fd DNA replication has a sedimentation coefficient of 2.3 S (Figure 5A). When the replication was allowed to proceed to 58% (Figure 5B), the average size of the products increased to 7.7 S. Similar results were obtained when $\phi\chi$ 174 DNA was used in our in vitro replication system (data not shown). The relatively low molecular weights observed in the products of replication suggest that multiple initiation occurs on the template DNA in the enzymatic system (Chang et al., 1972).

Analysis of Products of fd DNA Replication in Formaldehyde:CsCl:Cs_2SO_4 Gradient. The covalent nature of the RNA primer and DNA product in the RNA polymerase I initiated fd DNA replication was determined by isopycnic analysis of the reaction products on formaldehyde:CsCl:Cs_2SO_4 gradient. The gradient profile is presented in Figure 6. These results show that the bulk of the newly synthesized DNA has a density near that of the template fd DNA. The bulk of the RNA products sedimented to densities lower than that of Qβ RNA marker suggesting covalent linkage of the RNA to DNA. The broad distribution of the RNA on the gradient, however, suggests heterogeneity in the size of both DNA and RNA conjoint products. In order to determine the sizes of fd DNA replication products, formaldehyde:CsCl:Cs_2SO_4 gradient fractions were combined into four pools, and the size of DNA in each individual pool was analyzed by alkaline sucrose gradient centrifugation after overnight incubation in 0.3 N NaOH at 37 °C. The sizes of the RNA initiators were calculated from the dNMP to rNMP (^{3}H/^{14}C) ratios in the original pools. The sedimentation coefficients for the DNA products in the four combined fractions are 2.1 S, 2.9 S, 3.3 S, and 5.0 S corresponding to DNA chain lengths of 14, 35, 52, and 166 nucleotides, for fractions 10 to 18, fractions 19 to 24, fractions 25 to 30, and fractions 31 to 39, respectively (Burgi & Hershey, 1963). The compositions of the fd DNA replication products in the four pools are calculated to be $r(pN)_{\overline{101}}d(pN)_{\overline{14}}$, $r(pN)_{\overline{105}}d(pN)_{\overline{35}}$, $r(pN)_{\overline{52}}d(pN)_{\overline{52}}$, and $r(pN)_{\overline{38}}d(pN)_{\overline{166}}$. The size heterogeneity of the RNA initiators used by DNA polymerase in the replication of fd DNA suggests that the initiation by RNA polymerase and the elongation of RNA initiator by DNA polymerase does not originate at a unique sequence. The isopycnic analysis does show the covalent linkage of the RNA and DNA chains in the products of fd DNA replication.

^{32}P-Transfer Analyses. The covalent linkage of the RNA initiator with DNA product in the in vitro replication of single-stranded circular phage DNAs by yeast enzymes is confirmed by ^{32}P-transfer experiments and the results are shown on Table II. The analysis shows that the sequence at the RNA and DNA junction is not unique since all four deoxyribonucleotides and all four ribonucleotides are found at the junction. The frequency of individual deoxynucleotide occurrences is biased with purine deoxyribonucleotides appearing at more than twice the frequency of pyrimidine deoxyribonucleotides. All four ribonucleotides are found at the junction, but uridylate appeared at least twice as frequently as any other ribonucleotide. The dinucleotide sequence appearing at the highest frequency at the RNA–DNA junction is p(rU)p(dPu). The significance of the base sequence at the RNA–DNA junction in the in vitro DNA replication is not known. Since phage DNAs (nonhomologous) were used in a highly purified yeast enzyme system, it is not reasonable to expect that our system would be able to respond to DNA sequence determined aspects of DNA replication. The high frequency of p(rU)p(dPu) at the RNA–DNA junction probably reflects the specificities of the yeast RNA and DNA polymerases.

TABLE II: ^{32}P Transfer Analyses of Products of fd DNA and $\phi\chi$ 174 DNA Replication.[a]

[α-^{32}P]dNTP substrate	pmol of ^{32}P transferred to ribonucleotides							
	CMP		AMP		GMP		UMP	
	fd	$\phi\chi$ 174	fd	$\phi\chi$ 174	fd	$\phi\chi$ 174	fd	$\phi\chi$ 174
dTTP	1.56	0.60	1.39	0.48	1.17	0.52	3.89	1.37
dGTP	2.01	1.35	2.77	1.53	1.64	1.23	3.32	3.05
dATP	3.57	2.94	3.75	1.77	3.31	1.67	7.82	4.30
dCTP	1.26	0.60	1.18	0.49	1.44	0.91	2.80	1.51

[a] The reactions were carried out as described in Experimental Procedures using 0.15 unit of RNA polymerase I and 60 units of DNA polymerase I for each reaction.

The validity of the results of the ^{32}P-transfer experiment depends on minimal (ideally, zero) internal incorporation of deoxynucleotides in the RNA portion of the chain and minimal internal incorporation of ribonucleotides in the DNA portion of the chain. For the two phage DNAs analyzed in the ^{32}P-transfer experiments, the amount of deoxynucleotide polymerized was 1160 pmol and 1890 pmol for fd DNA and $\phi\chi$ 174 DNA templated reactions, respectively. If all of the ^{32}P-transfer occurred at the RNA–DNA junction, then the average chain lengths of the DNA products (calculated from the ratio of the total amount of deoxynucleotide polymerized to the total amount of ^{32}P transferred) are 27 and 78 for fd DNA and $\phi\chi$ 174 DNA reactions, respectively. This estimate of the size of DNA product is consistent with the results obtained from alkaline sucrose gradient analyses.

More stringent controls for distributed incorporation were carried out by separate determinations of the misincorporation of deoxynucleotides by yeast RNA polymerase I and the misincorporation of ribonucleotides by yeast DNA polymerase I. The conditions used for these control reactions were identical with those of the coupled reactions except that high specific activity [^{32}P]dNTPs were added individually to the RNA polymerase reactions and high specific activity [^{3}H]rNTPs were added individually to the DNA polymerase reactions. The results showed that the ratio of the total dNTP polymerized to the total rNTP polymerized in the RNA polymerase reactions was 5×10^{-4} and that the ratio of rNTP polymerized to the total dNTP polymerized in the DNA polymerase reaction was 1.7×10^{-4}. These control reactions showed the specificities of the yeast RNA polymerase I for rNTPs and yeast DNA polymerase I for dNTPs. They also demonstrated that the transfer of ^{32}P does occur at the junction of the RNA initiator and the DNA product. These experiments show linkage of the RNA and DNA synthesized in the yeast RNA polymerase I–yeast DNA polymerase I coupled reactions.

Discussion

In the past two decades major progress was made on the characterization of DNA polymerases in the eukaryotic systems (Bollum, 1974; Loeb, 1974) highlighting the complexity of DNA replication in complex cells. The advantage of employing the yeast system to investigate this complexity is the relative ease of genetic manipulation in yeast. In earlier communications, extensive purification and characterization of the two major DNA polymerases in yeast (Chang, 1977) and a preliminary study on the abilities of partially purified yeast RNA polymerases to initiate DNA synthesis on single-stranded circular phage DNA (Plevani & Chang, 1977) have been described. Since RNA primers had been detected at the 5′ termini of newly synthesized DNA chains in several eukaryotic cells (Reichard et al., 1974; Waqar & Huberman, 1975a,b, Tseng et al., 1975) and oligoribonucleotides were used as initiators by eukaryotic DNA polymerases (Chang & Bollum, 1972), our results showing that yeast RNA polymerases can produce the primers required by yeast DNA polymerase I in replication of single-standed DNA were not too surprising. The most interesting finding in our earlier study is that the partially purified RNA polymerases can initiate DNA synthesis by yeast DNA polymerase in the absence of rNTPs (Plevani & Chang, 1977).

In order to determine whether the rNTP independent initiation activity is an intrinsic property of yeast RNA polymerases or that the activity is due to a separate protein contaminating or bound to RNA polymerase preparations used, we have examined the properties of homogeneous yeast RNA polymerase I (Valenzuela et al., 1976) in the initiation of DNA synthesis by yeast DNA polymerase I. The results from this study showed that homogeneous yeast RNA polymerase I initiated DNA synthesis is completely dependent on the presence of rNTPs, suggesting that the rNTP independent initiation activity observed in the partially purified RNA polymerase preparations is due to a separate protein(s). In order to establish the existence of a protein capable of initiating new DNA chains in the absence of rNTPs, we were able to fractionate out a low molecular weight protein from partially purified RNA polymerase preparations by the use of high ionic strength on Sepharose 6B column (Figure 1). The purification and properties of this rNTP independent initiation protein is a subject of future publication.

The in vitro DNA synthesis catalyzed by RNA polymerase and DNA polymerase from heterologeous sources had been described by other investigators (Keller, 1972; Spadari & Weissbach, 1975). The results presented in this communication were obtained using enzymes purified from the same organism. In general, the results are in agreement. In fact, when yeast RNA polymerase I is used with calf thymus DNA polymerase-α, calf thymus DNA polymerase-β, chicken embryo DNA polymerase-α, and *E. coli* DNA polymerase I, initiation of DNA synthesis on $\phi\chi$ 174 DNA can be easily demonstrated (unpublished results). Since it is generally thought that RNA polymerases are processive and DNA polymerases catalyze deoxynucleotide polymerization in a distributive manner, it is surprising that DNA polymerase can actually displace RNA polymerase in the RNA polymerase–DNA polymerase coupled reactions. The explanation of this observation is unknown. It is possible that the affinity of RNA polymerase for the 3′-OH of the RNA primer on a single-stranded DNA template is not greatly different from that of the DNA polymerase. Once the DNA polymerase is able to polymerized one or more deoxynucleotides onto the 3′-OH of the RNA primer, the RNA polymerase can no longer use the primer for elongation. It is worthwhile to note that the RNA primer linked to the DNA product is heterogeneous in molecular weight suggesting lack of control in the in vitro system. In the in vivo situation, proteins may be present to facilitate the displacement of RNA polymerase by DNA polymerase during DNA synthesis.

The yeast in vitro DNA synthesizing system is inactive on native DNA (Plevani & Chang, 1977); thus native homologous (yeast) DNA cannot be used. Instead of characterizing the enzyme system with denatured yeast DNA, we choose circular single-stranded phage DNAs as templates. In doing so, the DNA sequence specific aspects of yeast DNA replication cannot be meaningfully analyzed. The analysis of the products of phage DNA replication by the yeast enzymes shows a heterogeneity in the size of the RNA initiators and the size of the DNA products, and an average of low molecular weight for the DNA products. All of these results suggest multiple initiation on the phage DNA template. The presence of all four deoxynucleotides and all four ribonucleotides at the RNA–DNA junction of the replication products suggests that the transfer of the RNA chain by RNA polymerase to DNA polymerase during replication is not DNA sequence specific. The results presented do show that yeast RNA polymerase I can provide yeast DNA polymerase I with RNA initiators for replication of DNA. Yeast RNA polymerases, on the other hand, cannot initiate DNA synthesis catalyzed by yeast DNA polymerase II (Plevani & Chang, 1977). Initiation of DNA synthesis by RNA initiators synthesized by RNA polymerase provides one possible mechanism for de novo synthesis of new chains in DNA replication.

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