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Ribonucleotidyl transferase in preparations of partially purified DNA polymerase α of the sea urchin

Paul W.Morris and Francis M.Racine

Department of Biological Chemistry, University of Illinois Medical Center, Chicago, IL 60612, USA

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ABSTRACT

Three ribonucleotidyl transferase types have been described in the sea urchin: riboadenylate transferase, the DNA dependent RNA polymerases, and a DNA polymerase associated ribonucleotidyl transferase (Biochemistry 15:3106-3113, 1976). In the present work this latter ribonucleotidyl transferase was found to purify with DNA polymerase α through phosphocellulose, DEAE-Sephadex and DNA cellulose and to cosediment at 6.5 S. This ribonucleotidyl transferase was active with Mn^{+2}, but not Mg^{+2}, on calf thymus DNA and poly-(dC). Other synthetic templates elicited DNA polymerase α but no ribonucleotidyl transferase activity. From alkaline hydrolysates of the poly(dC) directed GTP polymerization, we found G_{oh} and G_p in a ratio of 1:16 indicating an average chain length of 17 residues after a 20 min reaction. Co-polymerization of GTP (5 μM) and dGTP (10 μM) yielded a non-random distribution of the ribonucleotide in the deoxyribonucleotide. The properties of this urchin ribonucleotidyl transferase are unlike any previously described eukaryotic transferase and the data is discussed with reference to the known properties of E. coli DNA polymerase I and the primase.

INTRODUCTION

Initiation of DNA polymerization during the replicative process proceeds through a RNA primer in prokaryotic organisms (1) and, most probably, in eukaryotic organisms as well since RNA covalently linked to the 5'-terminus of short DNA fragments has been observed in several eukaryotic systems (2-5). The enzyme responsible for synthesis of this putative RNA primer is not presently identified. In E. coli at least three enzymes are known to catalyze RNA synthesis: 1) DNA dependent RNA polymerase, the rifampicin sensitive polymerase active in priming DNA synthesis on phage M13 DNA (6) and in transcription; 2) the dna G gene product, the 60,000 dalton primase active in priming DNA synthesis on phage G-4 DNA (7-10); and 3) DNA dependent DNA polymerase I, the Mn^{+2} responsive enzyme active in mixed ribo- and deoxyribonucleotide polymerization (11,12). Of these three examples, the first two serve an in vivo function while the latter apparently is a reaction provoked by in vitro conditions.

One of us previously reported an unusual RNA synthetic activity in the sea urchin which co-eluted with DEAE-Sephadex with the DNA-dependent DNA polymerase and was active with Mn^{+2} on a DNA template (13). This enzyme was distinguished from DNA dependent RNA polymerase I, II, and III on the basis of chromatographic properties and inhibition by the deoxyribonucleoside triphosphates. The inhibition of ribonucleotide polymerization by dNTPs is unlike the Mn^{+2} activated E. coli DNA polymerase I which incorporates the NTP in the presence of the remaining three dNTPs (11,12) but is like the primase which synthesizes a shortened RNA-DNA hybrid primer in the presence of the dNTPs (10). An enzymatic activity, reminiscent of the sea urchin activity, has been reported in extracts of chicken reticulocytes (14) and in 3T6 cell nuclei (15). To evaluate this urchin RNA polymerizing activity associated with the DNA polymerase, we report here further purification and characterization. A preliminary report of this work has been presented (16).

MATERIALS AND METHODS

Polymerase assays. The DNA polymerase assays using calf thymus DNA templates were as described in the preceeding manuscript. For assays with synthetic templates the reaction mixture contributed the following final concentrations to the 50 µL assay volume: 6 mM NaF, 48 mM Tris-HCl (pH 7.9 at 30°C), 0.02% 2-mercaptoethanol, 0.6 mM $MnCl_2$, 0.01 mM of the nucleoside triphosphate(s) as specified in the text, ~1400 CPM/pmole of either [^{3}H]-GTP or UTP or ~2800 CPM/pmole of [^{3}H]-ATP (all isotopic compounds from Amersham/Searle) as indicated in the text, and the synthetic template at 0.4 A_{260} units/mL. In the standard assay, 20 µL of enzyme was incubated for 20 min at 30°C; 40 µL aliquots from the reaction were spotted on DE-81 discs (Whatman) and processed for β counting as previously described (13).

For all kinetic analyses to determine K_m or K_i, the assays were scaled up to 150 µL containing 60 µL of enzyme. All concentrations remained unchanged except for the NTP or metal ion which were varied. At 2 min intervals following initiation of the reaction, 30 µL aliquots were spotted onto DE-81 filters and processed. A true initial velocity, rather than just reaction extent, was used in the calculation of all kinetic parameters.

Enzyme Preparations. For the examination of ribonucleotidyl transferase in the DNA polymerase α, two basic preparations have been used. We have routinely used Method I although Method II gave a 6-fold increase in specific activity and was used in the kinetic characterizations.

Method I: 30 mL of embryo extract (prepared as in the preceeding

manuscript) was applied to DEAE-Sephadex (Pharmacia) (3.1 X 40 cm) equilibrated with 0.05 M NaCl in 50 mM Tris-HCl (pH 7.9 at 4°C), 25% (v/v) glycerol, 0.5 mM dithiothreitol (TGD buffer). The DEAE-Sephadex was operated in the ion-filtration mode (13,17) and eluted with 0.5 M NaCl in TGD. The fractions containing DNA polymerase were pooled (~60 mL volume), adjusted to 0.2 M NaCl by slight dilution, and then applied to phosphocellulose (Whatman P11, 2.1 X 4 cm) equilibrated with 0.2 M NaCl in TGD. Following elution with 0.5 M NaCl in TGD the active fractions of DNA polymerase α were pooled (~15 mL), applied to DEAE-Sephadex (3.1 X 40 cm) pre-loaded with a linear gradient constructed of 40 mL each of 0.05 and 0.25 M NaCl in TGD, and eluted with 0.5 M NaCl in TGD (18). DNA polymerase α activity eluting between 0.07 and 0.08 M NaCl (analogous to fractions 30-36, Figure 1a) was pooled and stored in liquid N_2. Overall recovery of DNA polymerase α was 75% (from the initial DEAE column) with a final specific activity of ~150 nmole ^{3}H-TMP/h/mg protein on activated calf thymus DNA template.

Method II: 105 mL of embryo extract was applied to DEAE-Sephadex (5.7 X 43 cm) equilibrated with 0.1 M $(NH_4)_2SO_4$ in TGD containing 5 mM $MgCl_2$ and 0.1 mM EDTA and eluted in the ion-filtration mode (17) with 0.35 M $(NH_4)_2SO_4$. Pooled fractions containing DNA polymerase (150 mL) were absorbed onto Bio-Rex 70 (Bio-Rad, 3.1 X 14 cm) equilibrated with 0.1 M NaCl in TGD and eluted with 0.35 M NaCl in TGD. The pool of DNA polymerase α (33 mL) was diluted 1.5 fold with TGD, applied to phosphocellulose (Whatman P-11, 1.4 X 3.9 cm) equilibrated with 0.2 M NaCl, and eluted with 0.6 M NaCl in TGD. Active fractions (4 mL) were applied to DEAE-Sephadex (1.7 X 32 cm) preloaded with a 22 mL linear gradient of 0.05-0.25 M NaCl in TGD and eluted in the gradient sievorptive mode (18) with 0.5 M NaCl in TGD (Figure 1a). The DNA polymerase α eluting between 0.06-0.09 M NaCl was pooled (9 mL) and adsorbed onto denatured DNA cellulose (0.8 X 2 cm, prepared by procedure of Weissbach and Poonian (19) equilibrated with 0.1 M NaCl and eluted by a linear gradient of 0.1-0.6 M NaCl in TGD (Figure 1b). The 2.8 mL of active fractions were pooled and stored in liquid N_2. The procedure for sucrose density gradient centrifugation was described in the preceeding paper; the purification and recovery at each step is described in RESULTS.

The DNA polymerase β is removed from the α by the phosphocellulose chromatography step. The poly(dC) directed GTP polymerization activity is not readily detected in concentrated samples until a cation exchange chromatography step, either the Bio-Rex 70 or phosphocellulose. For this reason it is difficult to calculate either the units of poly(dC) directed rGTP poly-

merization or its recovery during early stages of purification.

Analysis of ribonucleotide product. For alkaline hydrolysis of the ^{3}H-GMP labeled product 140 µL of Method I enzyme plus 210 µL of the poly(dC) reaction mixture (14,000 CPM/pmole of GTP) were reacted, the polymerization was stopped after 2 or 10 min by the addition of 40 µL of 0.1 M EDTA and applied to G-75 Sephadex gel filtration (0.6 X 25 cm, equilibrated with 0.05 M NaCl in 10 mM Tris-HCl, pH 7.4). The ^{3}H-labeled product and the poly(dC) template eluted in the void volume of the G-75 Sephadex column and were pooled, adjusted to 0.3 N NaOH, hydrolyzed 16 h at 37°C, neutralized, applied to DEAE-Sephadex A25 (0.8 X 11.5 cm, equilibrated with 0.1 M LiCl in 50 mM Tris-HCl, pH 7.4), and eluted with a 90 mL linear gradient of 0.21-0.30 M LiCl followed by a 1 M LiCl wash. Authentic guanosine, GMP, GDP, GTP, and 5'-GtetraP were applied with the sample.

RESULTS

Copurification of ribonucleotidyl transferase with DNA polymerase α. Figure 1 shows the latter stages of a DNA polymerase α purification by Method II (MATERIALS AND METHODS). At the DEAE-Sephadex stage (Figure 1a), the DNA polymerase α chromatographed as three apparently heterogeneous species with the major activity eluting at 0.06-0.08 M NaCl (see also the preceding manuscript). The poly(dC) directed GTP polymerizing activity co-eluted with two DNA polymerase α species at 0.06-0.08 and 0.14-0.20 M NaCl; the poly(dC) dependent activity varied approximately proportional with the DNA polymerase α activity in the low and high ionic strength species. Little, if any, ribonucleotide polymerization was associated with the middle peak of DNA polymerase α either in this experiment, or in other experiments (e.g., Figures 2 and 3, preceeding manuscript) where the middle peak was more distinct. Since these DNA polymerase α patterns were dependent in part upon concentration of the enzyme during loading (high enzyme concentration promoted formation of a single activity peak eluting at 0.06-0.08 M NaCl) and the NTP polymerization was active in two DNA polymerase forms. DEAE-Sephadex was incapable of separating the ribo- and deoxyribo-nucleotidyl transferase activities.

Denatured DNA-cellulose purification of the DEAE-Sephadex enzyme (fractions 29-37, Figure 1a) yielded a single peak of ribonucleotidyl transferase coincident with the DNA polymerase α activity (Figure 1b). Purification of 7 fold (78% activity recovery) and 3 fold (34% activity recovery) was achieved at this step for the dNTP and the NTP polymerization reactions, respectively. Application of an aliquot from the DNA-cellulose pool (fractions 19-25,

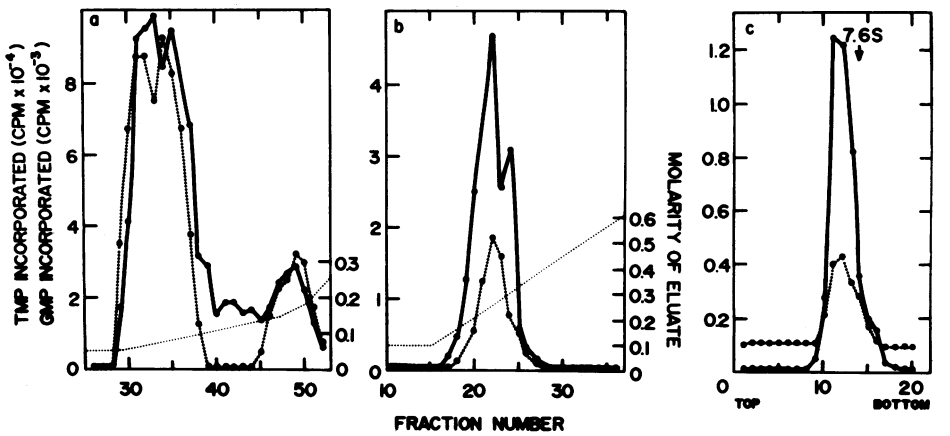

Figure 1. Co-purification of ribonucleotidyl transferase with DNA polymerase α. The DNA polymerase α, purified through phosphocellulose chromatography (Method II), is sequentially applied to DEAE-Sephadex gradient sievorptive elution (1a), DNA-cellulose affinity chromatography (1b), and sucrose density gradient centrifugation (1c). Yeast alcohol dehydrogenase (assayed by the method of Vallee and Hoch (31)) is indicated by the arrow ($S_{20,w}$ = 7.6). Assays for activated DNA directed ^{3}H-TTP + 3dNTP polymerization (open circles) and poly(dC) directed ^{3}H-GTP polymerization (closed circles) were performed as described in the text. The ionic strength in the eluate was determined by conductivity measurements.

Figure 1b) to sucrose density gradient centrifugation yielded a NTP polymerizing activity that cosedimented with the DNA polymerase α (6.5 S, Figure 1c). Recovery of activity was 42% and 47% for the DNA polymerase α and the poly(dC) directed GTP polymerization, respectively. This differential recovery of the two activities was frequently observed and argues against the presumption that the NTP and dNTP polymerizations were catalyzed by identical molecular entities. However, the two activities were indistinguishable on the basis of chromatographic behavior, sedimentation coefficient, resistance to 0.1 mM α-amanitin, and N-ethylmaleimide sensitivity (10 mM completely inhibits both activities at all purification stages). These properties distinguish the two activities from DNA polymerase β which is resistant to N-ethylmaleimide (preceeding manuscript), RNA polymerase II and III which are inhibited by 0.1 mM α-amanitin (20), and all three of the urchin RNA polymerases which sediment at 13-15 S. Therefore, it is unlikely that any of these enzymes catalyze the observed RNA synthesis.

In order to account for the data of Figure 1, one is left with the possibility of either two similar but separate polymerases or a core DNA polymerase α plus some factor able to induce GTP polymerization. Since the poly-

merase α at the DNA cellulose stage was <10% pure (specific activity = 910 nmoles TMP/h/mg, contrasted to literature values of >7500 nmoles TMP/h/mg (21,22)) and showed multiple bands on Na dodecylsulfate gel electrophoresis, neither possibility can be eliminated on the basis of purity. Proteolytic generation of polymerase artifacts does not appear to account for the present ribonucleotide polymerization. The DNA directed NTP polymerization was first found in urchin extracts prepared, fractionated by DEAE-Sephadex, and assayed within a one-hour span (13, and P.W. Morris, unpublished). Quantitatively, similar results were found in large volume extracts fractionated over a more leisurely 12-hour period. Likewise, inclusion of 0.1 M phenylmethanesulfonyl fluoride, 0.1 mM N-α-tosyl-lysyl-chloromethane, or 0.1 M N-tosyl-phenyalanyl-chloromethane, singly or all together, in the extraction buffer did not alter the results.

Template and substrate requirements for ribonucleotide polymerization

DNA polymerase α purified via method I was used to examine the reaction requirements and Table 1 summarizes the results. With respect to calf thymus DNA we found more deoxyribonucleotide polymerization on the DNase I activated DNA with a Mg^{+2} cation than with either the activated DNA-Mn^{+2} or non-activated DNA-Mg^{+2} combinations. This finding agrees well with previously published data (23,24) on eukaryotic DNA polymerase α. These enzyme preparations were active as well on several synthetic templates (e.g., poly(dC), poly(dC)·oligo-$(dG)_{12}$, poly(dA-T), poly(dA)·oligo$(dT)_{10}$, poly(dT)·oligo$(rA)_{10}$, and poly(dC)·poly(dG); Table 1 and additional data). The requirement for a 3'-hydroxyl terminus as a primer of deoxynucleotide elongation was demonstrated by the 20 fold increase in activity due to DNAase I activation of DNA and by the 9 fold increase due to oligo$(dG)_{12}$ priming of the poly(dC). Since no DNA polymerase α is known to be capable of de novo initiation and since poly(dC) weakly self hybridizes at the pH of the assay (25,26), it seems likely that poly(dC) itself supplies the 3'-terminus for elongation.

In contrast to the dNTP polymerization, the NTP polymerization (Table 1) was seen to occur only in the presence of Mn^{+2} on either a DNA or poly(dC) template. We excluded the possibility that this ribonucleotide polymerization, like the terminal riboadenylate polymerase (27), might be Mg^{+2} activated with higher substrate or template concentrations: a 10-fold elevation in GTP or poly(dC) concentration still yielded no detectable polymerization of ^{3}H-GTP. The detection limits prevented quantitation of nucleotide polymerization at <0.1 pmole/assay (<0.05 pmole/assay for ATP); thus we cannot exclude the possibility of a Mg^{+2} dependent NTP polymerization at a rate <1% of the

TABLE 1: Template and Substrate Requirements for the Ribonucleotidyl Transferase of the DNA Polymerase α Preparation.

TEMPLATE	SUBSTRATES	ENZYME ACTIVITY[a]	
		Mg^{+2}	Mn^{+2}
DNA, Activated	^{3}H-TTP+3dNTP	237	43
" "	^{3}H-UTP+3rNTP	<0.1	5.0
" "	^{3}H-GTP+3rNTP	<0.1	4.5
" "	^{3}H-GTP+3dNTP	<0.1	<0.1
DNA, Native	^{3}H-TTP+3dNTP	12	--
" "	^{3}H-UTP+3rNTP	<0.1	5.0
" "	^{3}H-GTP+3rNTP	<0.1	4.9
" "	^{3}H-ATP+3rNTP	<0.1	4.2
" "	^{3}H-UTP+rCTP	--	2.7
Poly(dC)	^{3}H-GTP	<0.1	11
"	^{3}H-dGTP	36	21
"	^{3}H-TTP	--	<0.1
"	^{3}H-ATP	--	<0.05
"	^{3}H-UTP	--	<0.1
$Oligo(dC)_{10}$	^{3}H-GTP	--	<0.1
$Poly(dC)\cdot Oligo(dG)_{12}$[b]	^{3}H-dGTP	312	178
" "	^{3}H-GTP	--	12
Poly(dA)	^{3}H-GTP	--	<0.1
$Poly(dA)\cdot Oligo(dT)_{10}$[b]	^{3}H-TTP	--	241
" "	^{3}H-UTP	--	<0.1
Poly(dT)	^{3}H-GTP	--	<0.1
Poly(dT)·Oligo(rA)[b]	^{3}H-ATP	--	<0.1

[a] Enzyme activity is expressed as pmoles incorporated/20 min assay in either 0.6 mM Mn^{+2} or 5 mM Mg^{+2} with DNA polymerase α as purified by Method 1.

[b] A_{260} ratio of oligomer:polymer = 1:10.

rate seen with Mn^{+2}. The template requirements were equally strict; no templates, other than DNA and poly(dC), elicited the ribonucleotide polymerization (Table 1). Thus we saw neither GTP nor complimentary nucleotide polymerization in response to templates such as poly(dA), poly(dT), or poly(dA-T). The RNA polymerases I, II, and III are active on the latter templates (33,34, and P.W. Morris, unpublished) and are thereby further differentiated from the present ribonucleotidyl transferase. Priming of the homopolymers with complimentary oligomers did not stimulate NTP incorporation. Only in the presence of Mn^{+2} with DNA or poly(dC) have we seen the NTP polymerization, a finding which contrasts strongly with the deoxyribonucleotide polymerization observed with these enzyme preparations on many primed synthetic templates.

Although poly(dC) supported only the GTP polymerization with no detectable polymerization of ATP or UTP, the available evidence (Table 1) shows that the activity is not a non-templated guanylate transferase. The oligomer, oligo(dC)$_{10}$, was incapable of eliciting the GTP reaction. With the DNA template, three of the ^{3}H-NTPs were polymerized equally well (the fourth, CTP, was not tested as a radiolabeled substrate). Due to this difference in NTP incorporation with poly(dC) versus DNA, we infer that base complimentarity was preserved in the ribonucleotide polymerization. This inference is further supported by the observation that deletion of ATP and GTP from the DNA directed reaction resulted in 55% inhibition of ^{3}H-UTP polymerization (Table 1). Previous workers (23,28,29) have found that DNA polymerase α from various sources is only partially dependent upon all four dNTPs for reaction with activated DNA and we found that our urchin polymerase α shows similar requirements for DNA directed dNTP and NTP polymerization. Thus, it is reasonable to conclude that the constituents of the polyribonucleotide product are dictated by base complimentarity with the templating DNA.

A surprising finding is the apparent lack of dependency upon 3'-hydroxyl termini for the ribonucleotide polymerization. This was observed both with DNA and poly(dC) (Table 1) wherein neither DNase I activation nor oligo(dG)$_{12}$ priming, respectively, exerted any stimulatory effect on NTP polymerization. This finding contrasts strongly with the 10-20 fold activity increase seen with the dNTP polymerization reaction and leads to the conclusion that the NTPs may not be elongated from a pre-existant 3'-terminus and that the ribonucleotidyl transferase is functionally quite different from the DNA polymerase α. However, this lack of dependency may be more apparent than real since the 3'-hydroxyl termini preexistant or generated in the DNA or poly(dC) reaction mixture could possibly saturate the primer requirement. If so, the ribonucleotidyl transferase is seen to be saturated at lower 3'-termini concentrations than is the DNA polymerase α.

Since RNA dependent RNA polymerization has been observed in other systems (14), we hydrolyzed the poly(dC) template in alkali (0.2 M NaOH, 35°C, 16 hr) to remove possible RNA contaminants. The alkali-treated poly(dC) was slightly less effective in directing dGTP polymerization (78% of control) whereas it was more active in directing GTP polymerization (2.1 fold). Hence, no dependence on an RNA contaminant was evident and the rGTP polymerization was truly poly(dC) dependent.

Kinetics and inhibition of ribonucleotide polymerization. Substrate concentration dependency of the DNA polymerase α preparations has been deter-

mined by two basic protocols: first, the nucleoside triphosphate was varied while the divalent cation remained constant; and second, the nucleoside triphosphate and the divalent cation were varied in a constant ratio (1:1 and 1:10 mole ratio, NTP:metal ion). The results are summarized in Figure 2 which shows the GTP dependency in the poly(dC) directed reaction with constant 0.6 mM Mn^{+2}. Two distinct kinetic components were seen with K_m values at 11 and 500 μM (Figure 2 and inset). The V_{max} increased some 25-fold between the low and high K_m reaction components. Similar K_m values were found in this reaction when the GTP was maintained in a constant 1:1 or 1:10 mole ratio with the Mn^2 (data not shown). In constract, the dGTP dependency in the poly(dC) reaction was monophasic with a single K_m at 0.57 μM (inset to Figure 2); again there was no difference in the kinetic profile whether Mn^{+2} remained constant (0.6 mM) or was varied with the dGTP. These values should be compared with the K_m for TTP in the activated DNA directed reaction (Mg^{+2} held constant at 6 mM) where we found a biphasic kinetic profile with K_m values of 0.75 and 31 μM (inset to Figure 2). This kinetic complexity means that one will underestimate the total enzyme activity unless high nucleoside triphosphate concentrations are used; thus the purification data presented above gives a specific activity approximate for the low K_m reaction component but much less than for the high K_m reaction component. A low K_m for the ribonucleotide polymerization provides the possibility that this enzyme can be expressed in the cellular milieu.

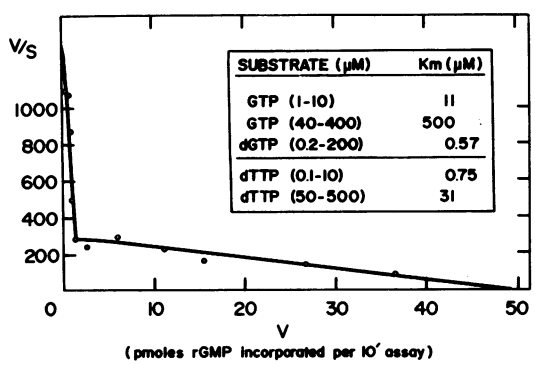

Figure 2. Hofstee plot of the initial velocities for the poly(dC) directed polymerization as a function of [GTP] in constant 0.6 mM Mn^{+2}. The inset summarizes the two K_m values determined from this data as well as the single K_m for dGTP with the poly(dC) (constant 0.6 mM Mn^{+2}) and the two K_m values observed for TTP with activated DNA template (constant 6 mM Mg^{+2}). The concentration range of substrates used in these experiments is given in the inset in parenthesis.

Deoxyribonucleotide inhibition of the NTP polymerization was examined in more detail with dATP in the poly(dC) directed GTP reaction; GTP was used at 5, 6.25, 8.35, and 12.5 μM for the low K_m reaction and at 50, 350, 650, and 1000 μM for the high K_m reaction. A plot of reciprocal velocity versus dATP concentration yielded the straight line with each substrate concentration that is diagnostic for competetive inhibition. The observed K_i was 5 μM (range = 4.5-6 μM) in dATP regardless of the GTP concentration. Any one of the four dNTPs (Table 2) was an effective inhibitor of the poly(dC) directed GTP polymerization; yet neither dATP nor any other dNTP competed with dGTP incorporation. Erroneous polymerization of dATP in response to poly(dC) in the presence of Mn^{+2} was undetectable in our assay system. The DNA directed polymerization of ^{3}H-UTP+3 NTPs was inhibited by dGTP (Table 2) and also by TTP (13). It should be recalled from the previous work (13) that TTP in combination with the remaining 3 dNTPs is significantly more inhibitory than TTP alone. TDP, dGDP, TMP, and dGMP, but not dT or dG, also inhibit the reaction

TABLE 2: Nucleoside Inhibition of the Ribonucleotidyl Transferase

SUBSTRATE	INHIBITOR[a]	%INHIBITION
TEMPLATE: Poly(dC)		
^{3}H-GTP	None[b]	0
	dATP[b]	82
	dGTP	76
	dCTP	76
	TTP	74
	TDP	65
	TMP	23
	dT	0
	dG	0
^{3}H-dGTP	dATP	2
TEMPLATE: Native Calf Thymus DNA		
^{3}H-UTP + 3NTP	None	0
^{3}H-UTP + 3NTP	dGTP[c]	58

[a]The inhibitor was at 50 μM except as noted. Standard assay procedures were used for the poly(dC) and DNA directed reactions.

[b]The dATP inhibition is competitive with GTP when each is varied across a wide range of concentrations ([rGTP] = 5 - 1000 μM; [dATP] = 2.5 - 4000 μM) and the observed K_i = 5 μM.

[c][dGTP] = 100 μM.

(Table 2 and additional data); however, the monophosphate is considerably less potent than the diphosphate. Clearly, base pairing homology is not a requisite for dNTP inhibition of the NTP polymerization and the inhibition cannot arise solely by dNTP competition for inclusion into the ribonucleotide polymer. Also, it may be recalled (13), that the Class I, II, and III RNA polymerases were much less sensitive to dNTP inhibition than the present activity.

Characterization of the product from the poly(dC) reaction. The RNA character of the product from poly(dC) directed GTP polymerization was verified by alkaline hydrolysis. Some 92% of the ^{3}H-labeled hydrolysate fractionated as the mononucleotide while about 6% fractionated as a polydisperse size class intermediate between the mononucleotide and the original polymer as determined by Sephadex G-75 gel filtration. The ^{3}H of the polydisperse fraction was not further characterized. Application of the mononucleotide pool to DEAE-Sephadex ion exchange chromatography showed only two ^{3}H-components which were identified as Gp and G_{oh} by coelution with the authentic compounds. At the end of a 20 min polymerization the ^{3}H-Gp:^{3}H-G_{oh} ratio equaled 16, thus indicating an average chain length of 17 nucleotides. Other nucleotides, particularly pppGp and pGp, were not observable components of the hydrolysate from either a 20 or a 1 min polymerization. There is therefore no evidence for de novo initiation of the GTP polymerization.

When ^{3}H-GTP was polymerized in the presence of 5 μM dGTP, its incorporation was reduced 40-50% and the Gp:dGp mole ratio in the product was 1:4.5 (this latter value was determined from simultaneous assays with ^{3}H separately in either GTP or dGTP). If the Gp were randomly copolymerized with dGP, then the alkaline hydrolysate should contain a predominance of the penta- and hexanucleotide with a 3' terminus of G. Further, the mononucleotide, ^{3}H-Gp, should be a minor component. Our results were contrary to the random copolymerization expectation. The alkaline hydrolysates from copolymerization of ^{3}H-GTP (10 μM) and dGTP (5 μM) were fractionated by ion exchange and the following nucleotide composition found: mononucleotide, ^{3}H-Gp, 72%; dinucleotide, (dG)p(^{3}H-G), 22%; and tri- and tetra-nucleotide, 6%. Higher oligonucleotides were not found. The DNA polymerase α preparation thus does not catalyze random copolymerization of GTP and dGTP but rather shows a distinct preference for blocks of $(Gp)_{n}$. Strict homopolymer formation is not seen since one-fourth of the Gp is 3' to a single dGp.

The structure at the 5' terminus of the oligo(G) product is unknown. As noted above, we do not detect a nucleoside di- or tetra-phosphate after alkaline hydrolysis; additionally, we have not been able to demonstrate label

transfer from $\alpha[^{32}P]$-GTP to dCp. Thus, we can exclude neither de novo initiation nor poly(dC) priming of the oligo(G) product. The apparent independence of GTP polymerization on 3'-hydroxyl terminus concentration (Table 1), in contrast to the strong dependence seen with dGTP, argues against ribonucleotide elongation from a pre-existant 3' terminus. However, it is possible that while the dGTP polymerization is rate limited by low 3'-hydroxyl concentration the GTP polymerization may be rate limited by catalytic inefficiency of the polymerase and therefore not stimulated by the primer. At this time we cannot show whether the 3'-hydroxyl of the poly(dC) participates in the GTP polymerization.

DISCUSSION

RNA undoubtedly participates in initiation of DNA replication in eukaryotes (2-5,15) like it does in prokaryotes. However, the primase catalyzing synthesis of the initiation RNA in eukaryotes is as yet unidentified. It is clear that the priming reaction may be relatively simple, for example, with phage G4 DNA only the DNA binding protein and the E. coli primase plus the substrates are required for synthesis of the 29 residue primer (7,9). On the other hand, the priming reaction may be quite complex, such as with phage ØX-174 DNA where apparently five proteins in addition to the DNA binding protein and the primase are required for the priming reaction (30,31). Elucidation of the enzymatic process in DNA replication in prokaryotes has been promoted by the availability of the less complex systems. A correspondingly simple system in eukaryote DNA replication is not yet described. Partially for reason of the enzymatic complexity in the replication process we have examined the ribonucleotidyl transferase reaction in more crude DNA polymerase preparations from the sea urchins since a moderate fractionation should favor retention of the proteins involved in DNA synthesis. A biological system like the urchin egg may be ideal for examination of the replication apparatus since it has a low DNA content and is pre-loaded with all of the enzymes for replication (13 and references therein).

On the basis of chromatographic behavior, sedimentation coefficient, templating preferences, deoxynucleoside triphosphate inhibition, and α-amanitin resistance, the present activity is quite dissimilar from any of the known DNA dependent RNA polymerase classes; we therefore consider it improbable that a well-known RNA polymerase accounts for our observations. Non-templated ribonucleotidyl transferases also offer no acceptable explanation since the ribonucleotidyl transferase is found to be template dependent. Among the remain-

ing explanations for our observations is the dichotomy presented by E. coli DNA polymerase I and primase; herein lies the crucial question, whether or not our observations reflect in vivo processes or an in vitro induced reaction. Both DNA polymerase I and the primase synthesize RNA or RNA-DNA copolymer under the appropriate conditions, the former in an apparently non-physiological Mn^{+2} induced reaction (11,12) and the latter in a more physiological reaction containing both dNTPs and NTPs with Mg^{+2} (7-10). The DNA polymerase I reaction shows low stringency for the template other than requiring a 3'-hydroxyl terminus for priming and is inactive with UTP. Primase shows considerable stringency for the template in terms of region of the template transcribed and varied helper enzyme requirements for different templates. Further, the primase is inhibited by inclusion of dNTPs with the NTPs in the reaction; the inhibition includes dNMP substitution for NMP in and a shortening of the oligoribonucleotide product.

We note that the present ribonucleotidyl transferase from the urchin shows a degree of template specificity: activity is observed only with poly(dC) and calf thymus DNA. Other synthetic templates, while active in DNA polymerase α catalyzed dNTP polymerization, show no activity for the companion NTP polymerization. Priming of templates, either by DNase I digestion or by complimentary oligonucleotides, has no effect on the ribonucleotidyl transferase. On the other hand, the urchin DNA polymerase α reaction with dNTP and Mg^{+2} is strongly stimulated by template priming. This degree of template stringency exhibited by the ribonucleotidyl transferase component of the DNA polymerase α preparation is difficult to rationalize as equivalent to the Mn^{+2} induced NTP polymerization by E. coli DNA polymerase I. A metal ion induced decrease in reaction specificity of the DNA polymerase α would not be expected to eliminate the ribonucleotidyl transferase activity with such templates as poly(dA)·oligo(dT), especially when the deoxyribonucleotidyl transferase is high with that template. Tests with calf thymus DNA show that the ribonucleotidyl transferase does polymerize UTP as equally well as GTP or ATP; thus, substrate preference does not explain the lack of UTP polymerization with the poly(dA)·oligo(dT).

DNA directed NTP polymerization with the present urchin preparations is inhibited by inclusion of dNTPs in the reaction. With dATP in the poly(dC) directed polymerization of GTP, the inhibition is competetive at a K_i of 5 μM. Base pairing homology is not required for dNTP inhibition of the ^{3}H-NTP polymerization with either the DNA or the poly(dC) directed reaction; a direct competetion between the deoxyribo- and ribo-nucleotides for polymerization

does not explain the inhibition phenomena. In this aspect the present ribonucleotidyl transferase is unlike the E. coli Mn^{+2}-DNA polymerase I reaction but is similar to the non-homologous dNTP inhibition of GTP incorporation by primase (9). In simultaneous incorporation of GTP and dGTP in the poly(dC) directed reaction, approximately 70% of the GMP occurs adjacent to another ribonucleotide and only about 30% is 3' to one to three deoxyribonucleotides. This non-random distribution of the ribonucleotide is more reminiscent of the primase than of the DNA polymerase I reaction.

In summary, we have described some of the enzymatic properties of a DNA polymerase α associated ribonucleotidyl transferase. Although the present activity has similarities with the Mn^{+2}-induced DNA polymerase I reaction, it appears to share more similarities in reaction requirements and product with the E. coli primase. A full evaluation of this sea urchin ribonucleotidyl transferase awaits a more simple replication system where one can demonstrate a priming reaction on single stranded DNA.

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