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Mammalian cyclin/PCNA (DNA polymerase δ auxiliary protein) stimulates processive DNA synthesis by yeast DNA polymerase III

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ABSTRACT

Human cyclin/PCNA (proliferating cell nuclear antigen) is structurally, functionally, and immunologically homologous to the calf thymus auxiliary protein for DNA polymerase δ. This auxiliary protein has been investigated as a stimulatory factor for the nuclear DNA polymerases from S. cerevisiae. Calf cyclin/PCNA enhances by more than ten-fold the ability of DNA polymerase III to replicate templates with high template/primer ratios, e.g. poly(dA)·oligo(dT) (40:1). The degree of stimulation increases with the template/primer ratio. At a high template/primer ratio, i.e. low primer density, cyclin/PCNA greatly increases processive DNA synthesis by DNA polymerase III. At low template/primer ratios (e.g. poly(dA)·oligo(dT) (2.5:1), where addition of cyclin/PCNA only minimally increases the processivity of DNA polymerase III, a several-fold stimulation of total DNA synthesis is still observed. This indicates that cyclin/PCNA may also increase productive binding of DNA polymerase III to the template-primer and stabilize the template-primer-polymerase complex. The activity of yeast DNA polymerases I and II is not affected by addition of cyclin/PCNA. These results strengthen the hypothesis that yeast DNA polymerase III is functionally analogous to the mammalian DNA polymerase δ.

INTRODUCTION

Proliferating cell nuclear antigen (PCNA) is a 36 kD nuclear protein, antibodies to which are present in some patients with the autoimmune disease systemic lupus erythematosis (1). PCNA is identical to a factor required for efficient in vitro DNA replication of SV40 DNA (2,3), and to cyclin (4,5). Expression of cyclin/PCNA increases in late G1 phase and continues through most of the S phase (6,7,8,9). In addition immunofluorescence studies localize cyclin/PCNA during the S phase to the region of the nucleus where DNA replication likely takes place, whereas non S phase cells produce a diffuse and weak staining pattern (9,10). The genes for the human and rat protein have been cloned and sequenced (11,12). They are highly homologous. Although limited homology to the putative DNA binding regions of E. coli cAMP binding protein and herpes simplex virus Type I DNA binding protein was detected, enzymatic data obtained with the protein from calf suggest otherwise. Calf cyclin/PCNA was originally

isolated as an auxiliary protein for DNA polymerase δ (13,14). The auxiliary protein does not bind to single-stranded or double-stranded DNA, but increases the binding of DNA polymerase δ to the template-primer, presumably through protein-protein interaction (13). Cyclin/PCNA greatly enhances processive DNA synthesis by DNA polymerase δ (14,15). Human and calf cyclin/PCNA are immunologically cross reactive and stimulate calf DNA polymerase δ in an identical fashion (14,16).

This remarkable conservation across species of a protein involved in cell-cycle regulation prompts the question whether a homologous cyclin/PCNA can be found in lower eukaryotes such as yeast. In addition, can one of the yeast DNA polymerases interact with the mammalian cyclin/PCNA? We have recently isolated a new DNA polymerase from yeast, DNA polymerase III, with enzymological properties that resemble DNA polymerases δ from mammalian cells (17,18). These include an efficient proofreading exonuclease, high sensitivity to aphidicolin and a relatively low sensitivity to the dGTP analog N^2-(p-n-butylphenyl)-dGTP (17,18). In this paper I show that DNA polymerase III of S. cerevisiae interacts with calf cyclin/PCNA in a fashion similar to that reported for calf thymus DNA polymerase δ.

MATERIALS AND METHODS

Enzymes

S. cerevisiae DNA polymerase III was purified from the protease deficient strain BJ405 (α, trp1, pep4-3, prb1, prc1) as described (17,19). Fraction VII was used for these studies. DNA polymerases I and II were purified from the same strain through the phosphocellulose and DEAE silica gel high pressure liquid chromatographic steps as described before (17). DNA polymerase I was further purifed by heparin-agarose chromatography, propyl silica gel (Synchrom) HPLC and monoQ (Pharmacia) HPLC. DNA polymerase II was further purified through heparin agarose chromatography and monoQ HPLC. Calf thymus DNA polymerase δ purified through the hydroxy apatite step (Fraction V) and homogeneous cyclin/PCNA from the same tissue were generous gifts from Dr. C.K. Tan (University of Miami) (13,20).

DNA polymerase assays

The 25 µl assays contained 20 mM Tris-HCl, pH 7.8, 4% glycerol, 100 µg/ml of bovine serum albumin, 5 mM dithiothreitol, non-radioactive dNTP's at 80 µM, [^{3}H]dTTP (500 cpm/pmol) at 20 µM, 8 mM Mg-acetate, template-primer and enzyme. Activated calf thymus DNA was added to a final concentration of 125 µg/ml, primed single-stranded mp18 DNA, poly(dA)·$(dT)_{16}$ (40:1) and poly(dA-dT) to 10 µg/ml. Reactions were

incubated at 37°C for 30 min and processed as described (17).

In assays measuring processive DNA synthesis by the DNA polymerases, [^{32}P]dTTP (3,000 cpm/pmol) replaced [^{3}H]dTTP and reaction volumes were lowered to 10 µl. Reaction times were adjusted such that less than 1 pmol of dTMP was incorporated per pmol of primer. Incorporation into acid precipitable radioactivity was between 0.6 and 1.7 pmol/reaction. The products were separated on a 8% polyacrylamide gel in 80 mM Tris-HCl, 80 mM borate, 4 mM EDTA, pH 8.3, containing 7 M urea. The radioactive products were visualized by autoradiography on Kodak XAR film at -70°C with an intensifying screen.

RESULTS

Calf thymus DNA polymerase δ is optimally stimulated by cyclin/PCNA on poly(dA)·oligo(dT) at a high template/primer ratio and a moderate Mg^{2+} concentration (4-8 mM) (13). Similar conditions were used to test the yeast nuclear DNA polymerases for stimulation by cyclin/PCNA. DNA synthesis by DNA polymerase III on poly(dA)·$(dT)_{16}$ (40:1) with 8 mM Mg^{2+} increased about 15-fold at saturating levels of cyclin/PCNA (Figure 1). Half maximal stimulation was at 1.3 µg/ml of cyclin/PCNA. Under the same conditions DNA polymerase δ activity was enhanced about 300-fold with half

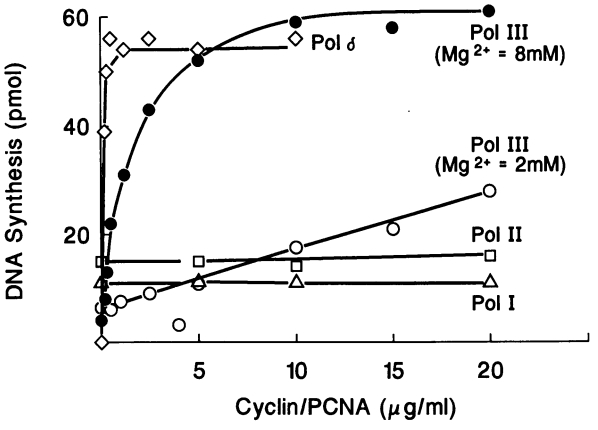

Figure 1. Stimulation of various DNA polymerases by cyclin/PCNA. Standard assays contained 8 mM Mg-acetate poly(dA)·$(dT)_{16}$ (40:1), cyclin/PCNA at the indicated concentrations and 0.15 unit of DNA polymerase I (Δ); 0.1 unit of DNA polymerase II (□); 1 unit of DNA polymerase III (●); 0.75 unit of DNA polymerase III with the Mg^{2+} concentration in the assay lowered to 2 mM (○); 0.4 unit of calf DNA polymerase δ (◇). See Materials and Methods for experimental details.

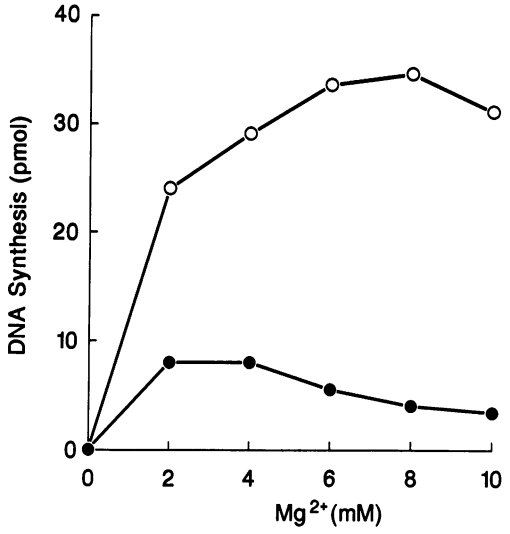

Figure 2. Mg^{2+} dependence of DNA polymerase III activity with or without cyclin/PCNA. Standard assays contained poly(dA)·$(dT)_{16}$ (40:1), Mg-acetate at the indicated concentrations, 1 unit of DNA polymerase III, and no cyclin/PCNA (●) or cyclin/PCNA at 10 µg/ml (○).

maximal stimulation at 0.09 µg/ml of cyclin/PCNA. DNA synthesis by DNA polymerase I or II was not stimulated at any cyclin/PCNA level (Figure 1).

The interaction between DNA polymerase III and cyclin/PCNA is Mg^{2+} dependent. The highest degree of stimulation was observed at 8-10 mM Mg^{2+} (about 13-fold, Figure 2) whereas at 2 mM Mg^{2+} only a 3-fold stimulation was measured. The smaller stimulatory effect at 2 mM Mg^{2+} was at least in part due to a weaker interaction between DNA polymerase III and cyclin/PCNA, because even at the highest level of cyclin/PCNA used no saturation kinetics could yet be observed (Figure 1).

In comparison to gapped templates such as activated calf thymus DNA or poly(dA-dT), DNA polymerase III had a relatively low activity on poly(dA)·oligo(dT) (Table I). The activity diminished gradually as the template/primer ratio was increased, i.e. the primer density decreased (Figure 3). Stimulation by cyclin/PCNA occurred at all template/primer ratios tested, but was most effective at high template/primer ratios. At a ratio of poly(dA)/$(dT)_{16}$ = 2.5, with 10-15 $(dT)_{16}$ primers per $poly(dA)_{400-600}$ template molecule, stimulation by cyclin/PCNA was 5.5-fold (Figure 3). At poly(dA)/$(dT)_{16}$ = 160, with only one out of five $poly(dA)_{400-600}$ template molecules containing a $(dT)_{16}$ primer, stimulation

Table I
Template preferences by the yeast DNA polymerases[a]

Template	Mg^{2+} (mM)	cyclin/PCNA µg/ml	DNA polymerase I	II	III
Activated calf thymus DNA	8	-	100	100	100
	8	10	N.D.[b]	N.D.	100
polyd(dA-dT)	2	-	280	60	110
	2	10	N.D.	N.D.	100
	8	-	100	95	130
	8	10	N.D.	N.D.	160
poly(dA)·$(dT)_{16}$ (40:1)	2	-	210	180	25
	2	10	190	190	90
	8	-	110	460	13
	8	10	120	420	150
Primed SS mp18[c]	8	-	6	9	9
	8	10	6	8	21

[a]Activities for each DNA polymerase in comparison to the activity with activated calf thymus DNA; for details see Materials and Methods.
[b]N.D., not determined.
[c]Single-stranded mp18 DNA was primed with the "universal" sequencing primer (N.E. Biolabs).

by cyclin/PCNA was 38-fold. No stimulation by cyclin/PCNA was observed when activated calf thymus DNA or poly(dA-dT) was used as a template-primer for DNA polymerase III. Replication of primed single-stranded mp18 DNA, however, increased 2-3 fold in the presence of cyclin/PCNA (Table I). Replication of the latter template by DNA polymerases I or II was also not stimulated by addition of cyclin/PCNA.

The low activity of DNA polymerase III on poly(dA)·$(dT)_{16}$ with low primer density could be due to a failure of the enzyme to form a stable DNA-enzyme complex and processively synthesize long stretches of poly(dT)

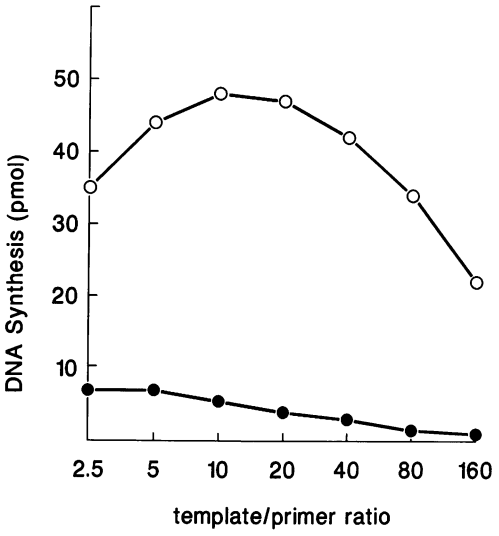

Figure 3. Effect of template-primer ratio on DNA polymerase III activity. Standard assays contained poly(dA)$_{400-600}$ at 10 µg/ml hybridized to (dT)$_{16}$ at the indicated ratio (as A_{260} units) and 1 unit of DNA polymerase III without (●) or with cyclin/PCNA at 10 µg/ml (○).

onto the poly(dA) template. Thus, the processivity of DNA polymerase III was measured under several conditions (Figure 4, Table II). At 2 mM Mg^{2+} the processivity of DNA polymerase III on poly(dA)·(dT)$_{16}$ (40:1) was at a moderate level of 80 (dTMPs incorporated per binding event). Addition of cyclin/PCNA, however, greatly increased processive DNA synthesis to above 400 (the resolution of the polyacrylamide gel), presumably replicating the full-length of the poly(dA) template per binding event (Figure 4, lanes 1,2; Table II). An increase of the Mg^{2+} concentration to 8 mM at the same template/primer ratio decreased processive DNA synthesis in the absence of cyclin/PCNA to 35, but again allowed complete replication of the template in the presence of saturating levels of cyclin/PCNA (Figure 4, lanes 3,5; Table II). At subsaturating levels of cyclin/PCNA, two distinct product size groups were observed, the small size group identical to the size group obtained without cyclin/PCNA and the large size group identical to that obtained with cyclin/PCNA (Figure 4, lanes 4,9). A decrease in the template-primer ratio from 40 to 2.5 was accompanied by a decreased apparent processivity of DNA polymerase III. Addition of cyclin/PCNA to the assay only marginally increased the apparent processivity from 9 to 15 (Figure 4, lanes 6,7; Table II).

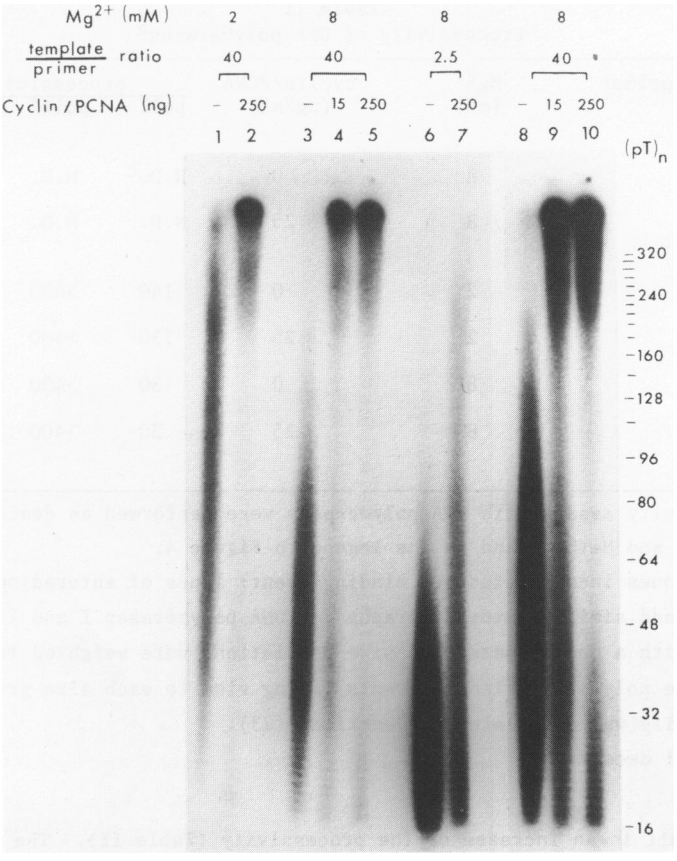

Figure 4. Effect of cyclin/PCNA on the processivity of DNA polymerase III. For details see Materials and Methods. Each 10 µl reaction contained 0.5 unit of DNA polymerase III. Lanes 1-5: the reactions contained 20 µg/ml of poly(dA)·$(dT)_{16}$ (40:1) as template primer, a total of 2 pmol of 3′-termini; lanes 6,7: the reactions contained 20 µg/ml of poly(dA)$(dT)_{16}$ (2.5:1) as template-primer, a total of 32 pmol of 3′-termini; lanes 1,2: the Mg^{2+} concentration in the assay was lowered to 2 mM. Cyclin/PCNA was added at the indicated concentrations. Approximately equal amounts of acid-precipitable radioactivity was loaded in each lane. Lanes 8-10 are a longer autoradiographic exposure of lanes 3-5. The marker lane is a $(pT)_{16}$ ladder, prepared by ligating 5′-[^{32}P]$(pT)_{16}$, hybridized to an equivalent amount of poly(dA), with T_4 polynucleotide ligase. The ladder is resolved up to $(pT)_{320}$.

Processive DNA synthesis by DNA polymerase I on poly(dA)·$(dT)_{16}$ (40:1) was similar to that of DNA polymerase III. At 2 mM Mg^{2+} DNA polymerase I incorporated 140 dTMPs per binding event and at 8 mM Mg^{2+} 30 (Table II, data not shown). With DNA polymerase I, however, addition of cyclin/PCNA

Table II
Processivity of DNA polymerases[a]

template-primer ratio	Mg^{2+} (mM)	cyclin/PCNA (µg/ml)	processivity[b] polI	polII	polIII
2.5	8	0	N.D.[c]	N.D.	9
	8	25	N.D.	N.D.	15
40	2	0	140	>400	80
	2	25	130	>400	>400
	8	0	30	>400	35
	8	25	30	>400	>400

[a]Processivity assays with DNA polymerases were performed as described in Materials and Methods and in the legend to Figure 4.
[b]TMP residues incorporated per binding event; lanes of autoradiographs in Figure 4 and similar autoradiographs for DNA polymerases I and II were scanned with a densitometer and size populations were weighted to quantitate polymerase binding events giving rise to each size group; the processivity was calculated as described (23).
[c]N.D., not determined.

did not result in an increase of the processivity (Table II). The situation is more complex for DNA polymerase II. Both at 2 mM and at 8 mM Mg^{2+}, DNA polymerase II was very processive, incorporating >400 dTMPs per binding event, regardless of whether cyclin/PCNA was present or not (Table II, data not shown).

DISCUSSION

The experiments shown in Figure 4 and Table II clearly indicate that cyclin/PCNA-dependent increase of DNA synthesis by DNA polymerase III can at least in part be explained by an increase of processive DNA synthesis by a DNA polymerase III-cyclin/PCNA complex. Cyclin/PCNA does not exert its effect by mass action, e.g. making the template more accessible for replication by DNA polymerase III. At half-saturating levels of cyclin/PCNA, the products synthesized by DNA polymerase III fall into two distinct size classes, one size class identical to the size class without cyclin/PCNA, and the second size class identical to that obtained with

saturating levels of cyclin/PCNA (Figure 4, lanes 4,9). If cyclin/PCNA worked by a mass action effect, one would expect to measure a single class of intermediate size products. Moreover, no binding of cyclin/PCNA to single- or double-stranded DNA has been observed (13). Further investigations are needed to establish whether the putative DNA binding sites deduced from sequence analysis of the human and rat cyclin/PCNA genes are functional (11,12).

At very high primer densities (i.e. poly(dA)·$(dT)_{16}$ (2.5:1)) the apparent processivity of DNA polymerase III decreases and addition of cyclin/PCNA only slightly increases the average length of products synthesized (Figure 4, lanes 6,7; Table II). These data show that DNA polymerase III does not efficiently carry out a strand displacement reaction, even with cyclin/PCNA present. The 5.5 fold stimulation of total DNA synthesis observed upon addition of cyclin/PCNA cannot be explained by the minimal increase of apparent processivity (Figure 3; Figure 4, lanes 6,7). It is likely that cyclin/PCNA increases productive binding of DNA polymerase III to the template-primer in addition to stabilizing this binding for processive DNA synthesis.

From the data summarized in Table II, it is clear that DNA polymerase I does not interact with cyclin/PCNA. The decrease in processivity upon increasing the Mg^{2+} concentration from 2 mM to 8 mM parallels the decrease in total incorporation and is similar to that observed for DNA polymerase III without cyclin/PCNA (Tables I,II). For DNA polymerase II, no definite statement can be made about its interaction with cyclin/PCNA since it is fully processive at both 2 mM and 8 mM Mg^{2+} (Table II). In fact, poly(dA)·$(dT)_{16}$ is the preferred template for DNA polymerase II, as has been observed by others (Table I) (21,22). Binding of cyclin/PCNA would not be expected to show a large effect and, moreover, could not be measured in this processivity assay. The higher rate of DNA synthesis by DNA polymerase II at 8 mM Mg^{2+} in comparison with 2 mM Mg^{2+} may reflect faster and more productive binding kinetics (Table I).

DNA replication of primed single-stranded phage DNA by any of the three DNA polymerases is very inefficient. Cyclin/PCNA stimulates DNA polymerase III activity 2-3 fold (Table I). The smaller stimulation of DNA polymerase III by cyclin/PCNA on natural DNA versus poly(dA)·oligo(dT) was also observed for DNA polymerase δ (13). The failure of cyclin/PCNA to promote efficient DNA synthesis by DNA polymerase II on the single-stranded phage template suggests that cyclin/PCNA does not interact with DNA polymerase II. DNA polymerase II has not yet been purified to homogeneity

and the enzyme preparation used in this study, although purified through four column steps, was not pure either (21,22). Thus, the possibility exists that a protein related to cyclin/PCNA is present in a complex with DNA polymerase II, obviating the necessity of stimulation by the calf protein. Further purification of DNA polymerase II is necessary to address this question.

In conclusion, the interaction between cyclin/PCNA and DNA polymerase III strengthens our previous assertion that this polymerase is like the mammalian DNA polymerase δ. Cloning and study of the gene and its regulation should provide information of great value to understanding DNA replication in mammalian cells. In addition, stimulation of DNA polymerase III activity on poly(dA)·$(dT)_{16}$ (40:1) should provide a useful assay to detect and purify the yeast analog of cyclin/PCNA. These studies are in progress.

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