In vitro replication of bacteriophage PRD1 DNA. Characterization of the protein-primed initiation site

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ABSTRACT

Bacteriophage PRD1 replicates its DNA by means of a protein-primed replication mechanism. Using single-stranded oligonucleotide templates carrying the sequence corresponding to the 25 first bases of the 3' end of PRD1 DNA, and Mg²⁺ as the activating metal ion of the phage DNA polymerase, we show that the fourth base from the 3' end of the template directs, by base complementarity, the dNMP to be linked to the phage terminal protein (TP) in the initiation reaction. This result suggests that phage PRD1 maintains its 3' end DNA sequences via a sliding-back mechanism. The single-stranded DNA templates could not be replicated by the PRD1 DNA polymerase, much in contrast to the natural TP-DNA. Nevertheless, the analysis of the transition products obtained with TP-DNA and origin-containing oligonucleotides suggests that sliding-back occurs stepwise, the fourth base being the directing position during the entire process.

INTRODUCTION

Bacteriophage PRD1 is a membrane-containing virus belonging to a family of closely related viruses, including PR3, PR4, PR5, PR72, and L17 (1–6), able to infect a variety of Gram-negative bacteria, including *Escherichia coli* (6, 7). The phage genome is comprised of a single, linear dsDNA molecule 14,925 bp long (8) which has 110 bp long inverted terminal repeat sequences (9), and a terminal protein (TP) covalently linked to each 5' end (10, 11). For a detailed review on the PRD1 system, see (12).

The replication of the PRD1 genome follows the basic principles of a protein-primed mechanism (reviewed in 13). This replication mechanism has been extensively described in both adenovirus (14) and *Bacillus subtilis* phage ϕ 29, the latter being the best understood protein-primed replication system of prokaryotic origin (13, 15). Previous work on the replication of bacteriophage PRD1 DNA led to the purification of both TP and phage DNA polymerase (16, 17), as well as to the isolation of the phage genome (18), and resulted in a minimal *in vitro* replication system. Phage PRD1 DNA replication starts with the formation of a covalent bond between a molecule of TP and the 5' terminal nucleotide, dGMP, in a reaction catalyzed by the

phage DNA polymerase (11, 16). This initiation reaction is stimulated by ammonium ions, that probably favor the formation of a TP-DNA polymerase complex (16). Subsequent to initiation, elongation of the initiation complex by the same DNA polymerase takes place, in a processive manner, resulting in the formation of full-length daughter DNA molecules (17, 19). The PRD1 DNA polymerase possesses, in addition to both TP-primed initiation and DNA polymerization activities, 3'-5' exonucleolytic activity (17, 19). PRD1 DNA polymerase can be activated not only by Mg²⁺, but also by Mn²⁺ (19). The latter metal ion significantly stimulated the initiation reaction when compared to Mg²⁺, although it had a negative effect on the overall DNA replication (19). Furthermore, in the presence of Mn²⁺, the DNA polymerase is able, in the absence of DNA, to covalently link any of the four dNMPs to the TP (19).

It has been recently shown that ϕ 29 DNA polymerase can use short oligonucleotides as templates and that they can be replicated by a protein-priming mechanism (20). The results of these studies clearly indicated that protein-primed ϕ 29 DNA replication does not start at the first base of the genome, but that the second position from the 3' end of the template is employed for the initial base pairing and formation of the corresponding TP-dAMP initiation complex. Altogether, these observations led to the proposal of a sliding-back mechanism for the transition from protein-primed initiation to normal DNA elongation based on the presence of a repetitive sequence at the DNA terminus, that could be a common feature of protein-primed replication systems (20). In the case of bacteriophage PRD1, the template DNA contains four C residues at both termini (9). A previous mutational analysis of this end sequence indicated that changes within the sequence repetition, particularly in the fourth position, had a deleterious effect on the initiation reaction, whereas changes at the first position had no effect (21). An approximately 20 bp long minimal origin of replication has been reported for phage PRD1 DNA, and several bases scattered along this origin have been shown to be of importance for replication to occur (21, 22).

In the present study we have further characterized PRD1 DNA replication *in vitro* using synthetic oligonucleotides. A mutational analysis of the terminal four template nucleotides allowed us to conclude that the first dNMP to be covalently linked to the TP is the one complementary to the fourth position on the DNA

template. This, in turn, is suggestive of a sliding-back mechanism for the maintenance of the DNA end sequences during the replication of phage PRD1.

MATERIALS AND METHODS

Nucleotides, proteins and DNA templates

Unlabeled nucleotides were purchased from Pharmacia PL-Biochemicals. Phage T4 polynucleotide kinase and DNA polymerase were obtained from New England Biolabs. (α - 32 P)dNTPs (400 Ci/mmol) and (γ - 32 P)ATP (3000 Ci/mmol) were from Amersham. Bacteriophage PRD1 TP and DNA polymerase were purified as previously described (16, 17). In the case of TP, the last gradient centrifugation step described in (16) was substituted by gel filtration through a HiLoad Superdex 75 column (Pharmacia) in 50 mM Tris-HCl, pH 7.5, containing 500 mM NaCl, at a flow rate of 10 ml/h.

The phage genome (TP-DNA) was isolated as reported (18). Single-stranded oligonucleotides were synthesized according to (23). The 25-mer oligonucleotide with the sequence of the template strand of the PRD1 origin is designated to as ori(25)t, and that corresponding to the sequence of the displaced strand is designated to as ori(25)d. The sequence of the ori(25)t 25-mer is 3'-CCCCTATGCACGGGGAGGGGTGGAT-5'. The double-stranded oligonucleotide ori(25) was obtained by heating at 65°C and slow cooling to room temperature an equimolar mixture of both ori(25)t and ori(25)d. Point substitutions in the ori(25)t sequence are indicated by a three-symbol code where the first letter corresponds to the nucleotide to be changed, the number indicates its position from the 3' end, and the second letter corresponds to the nucleotide introduced (for instance, C1T designates the oligonucleotide in which the first base C has been changed to T). The oligonucleotide ori(25)t was labeled at the 5' end (10000 cpm/0.37 pmol) with T4 polynucleotide kinase and purified as described (24). The 29-mer oligonucleotides corresponding to the sequence of the bacteriophage ϕ 29 right origin, oriR(29)t and oriR(29)d, have been previously described (20). Poly (dC)₂₀₀ was purchased from Pharmacia PL Biochemicals.

Initiation complex formation

The replication initiation assay was carried out in samples containing, in a volume of 25 μ l, 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol (DTT), 5% glycerol, 0.1 mg/ml bovine serum albumin (BSA), 20 mM ammonium sulphate, 0.25 μ M (α - 32 P)dNTP (2.5 μ Ci), 500 ng of either TP-DNA or the indicated oligonucleotide as template, 18 ng and 100 ng of purified DNA polymerase and TP, respectively, and 5 mM MgCl₂. When indicated, 1 mM MnCl₂ was added instead of MgCl₂. After incubation at 30°C for 10 min, the reaction was stopped by addition of EDTA and SDS to 10 mM and 0.1%, respectively. The samples were then filtered through Sephadex G-50 spun columns in the presence of 0.1% SDS, and the excluded volumes analyzed by SDS-PAGE in 10% polyacrylamide gels, and subsequent autoradiography. Quantitation was done by excising from the gel the radioactive band corresponding to the initiation complex and measuring the Cerenkov radiation, and by densitometry of the autoradiographs in a 300A computer densitometer (Molecular Dynamics).

Replication assay

The replication assay was performed as described for the formation of the initiation complex but with the indicated

Figure 1. Initiation reaction carried out with several DNA templates. The templates employed, TP-DNA, ori(25)t, and the double-stranded ori(25), are indicated on the left. On the top, the metal ion used as activator of PRD1 DNA polymerase is indicated; A, C, G, and T, designate the dNTP used in each reaction.

unlabeled dNTPs (20 μ M) in addition to (α - 32 P)dGTP. Samples were incubated at 30°C for 10 min and processed as described for the initiation assay. Analysis was done by SDS-PAGE in thin 12% polyacrylamide gels (360×280×0.5 mm), and autoradiography.

3'-5' exonuclease assay on ssDNA. TP protection assay

The incubation mixture contained, in a volume of 12.5 μ l, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 5% glycerol, 0.1 mg/ml BSA, 20 mM ammonium sulphate, 1.5 ng of 5' labeled ori(25)t oligonucleotide as substrate, 18 ng of DNA polymerase, TP as indicated, and 10 mM MgCl₂. After incubation at 30°C for 10 min, the reaction was stopped by addition of 3 μ l of sequencing gel loading buffer. Control samples contained 0.3 units of T4 DNA polymerase instead of PRD1 DNA polymerase. The samples were analyzed by electrophoresis in 20% polyacrylamide gels in the presence of 8 M urea, followed by autoradiography.

RESULTS

Initiation with ssDNA templates

TP-primed initiation of replication of ssDNA was tested with a 25 base-long oligonucleotide with the sequence corresponding to the phage PRD1 origin template-strand (ori(25)t). As shown in Fig. 1a, this oligonucleotide was able to support the formation of the TP-dGMP initiation complex to an extent similar (87%) to that obtained with the TP-DNA genome. However, it should be noticed that both DNAs were present in saturating amounts and that there was a large molar excess (about 1000-fold) of the oligonucleotide compared to the phage genome. The double-stranded oligonucleotide ori(25), hybrid of ori(25)t and ori(25)d, was an inefficient template for initiation, the reaction being only about 10% of that obtained with ori(25)t (Fig. 1a), suggesting that dsDNA opening is a pre-requisite for protein-primed initiation.

The template specificity of the protein-primed initiation reaction with ssDNA was tested using different oligonucleotides. When the oligonucleotide ori(25)d, complementary to ori(25)t, was used as template, the amount of TP-dGMP formed was about 15% of that produced with ori(25)t (not shown). The use of poly(dC) decreased the extent of the reaction to about 1% of that obtained with ori(25)t (not shown). No reaction could be detected with

Figure 2. Effect of PRD1 TP on the 3'-5' exonucleolytic activity of PRD1 DNA polymerase on ori(25)t. The position corresponding to the DNA template (lane 1) is indicated on the left. (2), reaction mixture containing PRD1 DNA polymerase (18 ng); (3) and (4), reaction mixtures containing, in addition to 18 ng of PRD1 DNA polymerase, 20 ng and 100 ng of PRD1 TP, respectively; (5), reaction mixture containing 0.3 units of T4 DNA polymerase; (6), sample containing T4 DNA polymerase (0.3 units) and 100 ng of PRD1 TP. The amount of ori(25)t was 1.5 ng in all cases.

the single-stranded 29-mer oligonucleotides oriR(29)t and oriR(29)d, corresponding to the sequences of the right replication origin of bacteriophage ϕ 29 (Méndez et al., 1992). These results indicate that, in the case of phage PRD1, protein-primed initiation with ssDNA is sequence specific.

It has been previously shown that, in addition to Mg^{2+} , the PRD1 DNA polymerase can be also activated by Mn^{2+} , without a remarkable effect on the nucleotide specificity of the initiation reaction carried out with TP-DNA template (19; see also Fig. 1a and b). When each of the four different (α - ^{32}P)dNTPs was used as substrate for the initiation reaction carried out with either ori(25)t or double-stranded ori(25) as template and Mg^{2+} as activating cation, the degree of nucleotide specificity was comparable to that observed with TP-DNA, although the formation of some TP-dTMP (about 5% of the amount of TP-dGMP formed) was observed in the case of ori(25)t (Fig. 1a). However, when Mn^{2+} was used as the activating metal ion, a high degree of unspecificity could be observed in reactions carried out with ori(25)t as template (Fig. 1b). Under these conditions, the relative amounts of TP-dAMP, TP-dCMP, and TP-dTMP formed were approximately 17%, 3%, and 160%, respectively, the amount of TP-dGMP. With the same metal ion and double-stranded ori(25) as template, the formation of TP-dAMP and TP-dCMP was not detected, whereas the complex TP-dTMP was formed in an amount corresponding to about 30% that of TP-dGMP. Thus, with oligonucleotide templates, the use of Mn^{2+} as activating ion had a deleterious effect on nucleotide specificity.

The single-stranded template is not degraded by the 3'-5' exonuclease activity of PRD1 DNA polymerase

PRD1 DNA polymerase has a 3'-5' exonuclease activity on ssDNA that, as in many other DNA polymerases, is most probably used in the correction of misincorporated nucleotides (17, 19). To test whether PRD1 DNA polymerase could degrade the single-stranded template, its exonuclease activity was assayed,

Name	3'-sequence	Substrate				Substrate usage (%)				Activity (%)
		A	C	G	T	A	C	G	T	
ori(25)t	CCCC--					-	-	100	7	100
C1T	TCCC--					-	-	100	2	41
C2A	CACC--					-	-	100	57	6
C2G	CGCC--					-	-	100	68	6
C2T	CTCC--					-	-	100	21	11
C3T	CCTC--					-	-	100	3	52
C4A	CCCA--					-	-	-	100	42
C4G	CCCG--					-	100	-	-	29
C4T	CCCT--					100	-	14	1	16

Figure 3. Analysis of the protein-primed initiation site of phage PRD1 DNA. The different oligonucleotide templates employed, and their corresponding 3' end sequences are listed on the left. The initiation reactions carried out with each of the four dNTPs (A, C, G, and T), and the corresponding quantitation, done by densitometry, are shown in the central panels. The column on the right indicates the activity of the reaction obtained with the correct dNTP relative to that obtained with ori(25)t and dGTP (100%). In this case, 100% corresponds to 2.5 fmoles of TP-dGMP formed.

both in the absence and in the presence of PRD1 TP, using the 5' ^{32}P -labeled ori(25)t oligonucleotide. As seen in Fig. 2, in the absence of TP, the DNA polymerase of phage PRD1 was able to degrade the oligonucleotide to a considerable extent in a processive manner. However, the presence of TP was able to protect ori(25)t to an extent of about 70%. It should be noted that under the conditions employed in the initiation assay (500 ng of DNA substrate), degradation of ori(25)t was not detected (not shown). The protection by the TP is due to a specific protein-protein interaction, as clearly shown by the fact that the PRD1 TP could not protect ori(25)t from being degraded by the phage T4 DNA polymerase (Fig. 2).

Initiation is directed by the fourth nucleotide at the 3' end of the template

A mutational analysis of the PRD1 origin of replication was carried out with single-stranded oligonucleotides. Single base changes were introduced in either of the first four positions from the 3' end of ori(25)t. The oligonucleotides obtained are listed in Fig. 3 and were assayed as templates for TP-primed initiation with each of the four (α - ^{32}P)dNTPs. As can be seen in Fig. 3, alteration of the first (C1T) or third (C3T) position did not produce any change in the nucleotide specificity of the initiation reaction, (α - ^{32}P)dGTP being the dNTP incorporated. However, changes in the fourth position (C4A, C4G, and C4T) had a drastic effect on the reaction. In these cases the incorporated (α - ^{32}P)dNTP was the one complementary to the nucleotide present in the fourth position of the template DNA. These results clearly show that initiation is directed by the fourth nucleotide from the 3' end of the template. Mutations in the second position (C2A, C2G, and C2T) resulted in all cases in a very inefficient TP-dGMP formation, suggesting an involvement of the second base in the positioning/formation of the initiation complex.

Figure 4. Analysis of the transition products obtained with different DNA templates. The areas corresponding to initiation plus transition, and elongation are shown separately. (a), initiation and elongation experiments carried out with TP-DNA and ori(25)t; (b), initiation reactions done with the mutant oligonucleotides C1T, C2T, and C3T. The reaction substrate was either 20 μ M dGTP (dG) or the four dNTPs (4dN), each at a concentration of 20 μ M. The position of the transition products corresponding to a molecule of PRD1 TP bound to one (TP-(dG)₁), two (TP-(dG)₂), or three (TP-(dG)₃) dGMP molecules are indicated with bars.

Replication of ssDNA

The fact that the initiation reaction in PRD1 occurs at the position complementary to the fourth base of the template and that the first four bases of the template are Cs is suggestive of a sliding-back mechanism to maintain the integrity of the ori sequence during the replication of the phage genome, as it occurs in the case of phage ϕ 29 (20). As it can be seen in Fig. 4a, with TP-DNA as template, the two first elongation steps corresponding to the transition from the use of a protein as primer (TP-dGMP) to the use of DNA could be observed in the presence of dGTP, 0.5 μ M being the minimal dGTP concentration at which transition could be detected (not shown). Taking into account that the 3' terminal sequence of the template has 4 consecutive Cs, the absence of a band corresponding to the expected product TP-(dGMP)₄ is probably due to the interference of the strong 3'-5' exonuclease activity of PRD1 DNA polymerase in this limited elongation assay. However, extensive elongation took place in the presence of the four dNTPs (Fig. 4a). In the case of ori(25)t, however, no replication could be detected, and transition beyond the second position was never observed, even at high (20 μ M) dGTP concentration. Identical results were obtained using the double-stranded oligonucleotide ori(25) (results not shown). The addition of ATP or the phage PRD1 early proteins P12 or P19 (25, 26) did not have any effect on the replication of the oligonucleotide (not shown). In view of these results, we analyzed whether a change in either of the first three bases of ori(25)t had an effect on the transition pattern showed by the oligonucleotide. As shown in Fig. 4b, identical results as those observed with ori(25)t were obtained with oligonucleotides with changes at the first (C1T) and second (C2T) positions. However, the change of the third base (C3T) resulted in the formation of only the initiation band TP-dGMP.

DISCUSSION

Protein-primed DNA replication must involve 1) the recognition and unwinding of dsDNA ends (replication origins), 2) the formation at each end of an initiation complex between the TP

and the first dNMP, and 3) DNA synthesis with concomitant strand-displacement. Earlier studies done with adenovirus indicated that dsDNA might not be an essential requirement for origin recognition during protein-primed replication since ssDNA derived from several plasmids and also some single-stranded oligonucleotides were active templates for initiation (27–30). Protein-primed DNA replication of PRD1 ssDNA ori sequences has been reported (21, 22). The results of these experiments suggested a putative origin of replication comprising the first 20 nucleotides at the 3' end of PRD1 DNA since several base substitutions within this sequence affected DNA replication, albeit to a different extent. More recently, it has been shown that, in the case of phage ϕ 29, linear ssDNA is an active template and can be replicated by the phage DNA polymerase using TP as primer (20). A mutational analysis carried out with single-stranded oligonucleotide templates with altered ϕ 29 ori sequences allowed to conclude that the initiation reaction is directed by the second nucleotide from the 3' end of the template. Since a special translocation step must be needed to recover the terminal nucleotide, a sliding-back mechanism relying on a terminal base repetition in the template that could account for this translocation was proposed (20). Interestingly enough, the genomes of viruses of different origins (i.e., ϕ 29, PRD1, Cp-1, adenovirus), as well as those of linear plasmids, that possess terminal proteins and replicate their DNAs by a protein-primed mechanism, have sequence repetitions at their ends (see 13 for original references). Thus, it has been suggested that the sliding-back mechanism could be a common feature of protein-primed replication (20).

In the work described here we show that the single-stranded oligonucleotide ori(25)t, carrying the template sequence (25 nucleotides) corresponding to the 3' end of PRD1 DNA, is able to support the formation of the TP-dGMP initiation complex. Under the conditions employed in this study, the reaction with ori(25)t was nearly as efficient as that obtained with PRD1 TP-DNA. The observed reaction with ssDNA showed a number of interesting features. First, there was a high degree of template specificity. The use of an unrelated template, such as poly(dC), or templates with sequences corresponding to the origin of replication of phage ϕ 29 resulted in a practically undetectable reaction. Our results are also in agreement with the previous observations indicating that mutations in several positions along the 25 first residues of the 3' end of PRD1 DNA abolished protein-primed replication (21). However, it should be borne in mind that the latter results were obtained with a 239 bp-long DNA fragment corresponding to the PRD1 left terminal sequence, and full replication of this duplex DNA was measured. Thus, in addition to differences in the nature of the DNA employed, the requirements for elongation and initiation may be different.

It has been shown that, in addition to Mg^{2+} , Mn^{2+} can support and even stimulate the formation of the TP-dGMP initiation complex using the natural PRD1 TP-DNA as template, without any major effect on the nucleotide specificity of the reaction (19). However, the use of Mn^{2+} with ori(25)t had a clear deleterious effect on the initiation reaction, increasing the degree of nucleotide unspecificity. At the present time, we do not know if this effect is due to misincorporations of several nucleotides at a given position or represents an impairment of proper initiation site recognition. Thus, with ssDNA templates, Mg^{2+} was used as metal ion. The possibility that the 3'-5' exonucleolytic activity of PRD1 DNA polymerase was generating heterogeneous 3' template sequences was ruled out. Our results show that the PRD1 TP was able to protect the single-stranded

oligonucleotide from being degraded by the DNA polymerase. This protection, also observed in the $\phi 29$ system (31), is apparently the result of the formation of the TP-DNA polymerase complex. The effect of the TP is specific since it cannot prevent the oligonucleotide from being degraded by a non-related DNA polymerase such as the T4 enzyme. Furthermore, the high template specificity indicates that it is very unlikely that the different DNA molecules that would be produced by the exonucleolytic activity of free PRD1 DNA polymerase could be efficient templates.

Our mutational analysis shows that, in the case of phage PRD1, initiation occurs at the position complementary to the fourth base from the 3' end of the template. Changes in the first three bases did not affect the base specificity of the initiation reaction, although differentially affected its efficiency. In particular, changes at the second position resulted in a strong decrease of the formation of the TP-dGMP complex. It is possible that changes at this position indirectly affect the positioning of the director base relative to the DNA polymerase dNTP binding site, reducing the catalytic efficiency and/or the accuracy of the initiation reaction. Of the changes made at the fourth position, C4A was the most efficient one. Interestingly, some of the changes made at this fourth position resulted in higher accuracy of incorporation of the complementary dNTP as compared with the template sequence ori(25)t (see Fig. 3), indicating that certain mispairings occur with preference to others.

Our results lead to the hypothesis that PRD1 may maintain the integrity of its DNA ends via a sliding-back mechanism for the transition from protein-primed initiation to elongation, as illustrated in Fig. 5. The fact that the fourth C residue directs the initiation reaction implies that sliding-back to the first position would occur either in a single jump or stepwise, driven by further dCMP additions, and may require more stringent conditions than in the case of $\phi 29$. The stepwise dCMP addition seems more plausible in view of the fact that only the mutation C3T, modifying the terminal redundancy necessary for sliding-back, had an effect on the transition pattern observed with ori(25)t. This stepwise manner would occur by a polymerization event taking place after each sliding-back step, and implies that the fourth C residue is the director base during the replication of the 3'-CCCC repeat. In the adenovirus genome, in which the terminal sequence of the template strand has the more complex reiteration 3'-GTAGTA, it has been demonstrated that changes in the first G residue did not affect the formation of the pTP-dCMP complex, whereas additional substitution of the G residue at position four drastically inhibited this reaction. These data led to the previous proposal that the G-residue at position four of adenovirus DNA would act as the template nucleotide during the formation of the pTP-dCMP complex (20). However, unlike the PRD1 strategy, the sliding-back mechanism in adenovirus could not occur stepwise after single nucleotide extensions of the initiation complex. Recently, it has been reported the existence of a kinetic barrier to further elongate the adenoviral pTP-CAT product, that is released by high dCTP concentration (32). These results would agree with a rate-limiting sliding-back step in which this pTp-CAT product, initiated from the fourth template residue, slides back in a single jump to be paired with the first three bases of the template, thus regenerating the adenovirus DNA ends.

In the $\phi 29$ system, it has been shown that an intact terminal repetition is necessary to obtain full replication of the template oligonucleotide (20). However, in the case of PRD1, we were not able to obtain complete replication of the oligonucleotide

Figure 5. Alternative pathways for the recovery of the 3' end sequences of phage PRD1. The proposed sliding-back mechanism can occur either in a single jump (path a) or stepwise (path b). The thin arrows indicate sliding-back steps, whereas thick arrows correspond to polymerization events. The director base in each polymerization event is outlined in each case. TP is represented by a circle.

ori(25)t. Since TP-DNA could be elongated *in vitro* and the transition step could be also detected, it is possible that in PRD1 the presence of the parental TP molecule is essential for an efficient sliding-back from such an internal position. It is conceivable that the TP holds in place the initiation complex through TP-TP interactions if, as expected, the sliding-back translocation disassembles the TP-DNA polymerase complex. At this point it is important to mention that the studies previously done with the PRD1 system (21, 22) indicated that replication of both a single-stranded oligonucleotide and of the 239 bp-long terminal DNA fragment can occur. Since the assay conditions of these earlier studies were similar to those employed in the work reported here, the discrepancy must arise from the fact that we are using a system with purified DNA polymerase and TP, whereas the work previously described (21, 22) relies on the use of a partially purified protein mixture of these two phage proteins derived from an *E. coli* extract. Thus, it is plausible that the cell extract may contain some unknown (host) factor that could help in the process of DNA replication.

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