A novel DNA polymerase induced by *Bacillus subtilis* phage ϕ 29

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ABSTRACT

A novel DNA polymerase induced by *Bacillus subtilis* bacteriophage ϕ 29 has been identified. This polymerase can be separated from host DNA polymerase, by fractionation of extracts prepared from phage infected cells, using phosphocellulose chromatography. The isolated polymerase prefers poly(dA)oligo(dT) as template. The DNA polymerase isolated from the cells infected with a gene 2 temperature sensitive mutant (ts2) showed greater heat-lability than that induced by wild type ϕ 29. The ts2 DNA polymerase was also thermolabile for its activity in the formation of a covalent complex between ϕ 29 terminal protein and dAMP, the initiation step of ϕ 29 DNA replication. These findings indicate that gene 2 is the structural gene for a ϕ 29 DNA polymerase required for the complex formation step of DNA initiation.

INTRODUCTION

Bacillus subtilis phage ϕ 29 has a linear duplex DNA which possesses a 30,000 dalton terminal protein at each 5' terminus (1). Because of the unique structure of the genome, the mechanism of ϕ 29 DNA replication has been an intriguing problem. How does initiation of replication occur? What is the function of the terminal protein? Which DNA polymerase is responsible for the replication? The first clue to the function of the terminal protein was provided by Shih et al (2) who demonstrated that the functional terminal protein forms a complex in vitro specifically with dAMP, the first nucleotide of the 5' end of ϕ 29 DNA (3). This finding was confirmed by Penalva and Salas (4). Recently, we described a cell free system which catalyzes both complex formation and DNA replication of ϕ 29 (5). Using this system, we were able to show that the complex forming activity associated with the functional terminal protein copurified with replication activity for ϕ 29 DNA (6). Furthermore, we showed that the complex between dAMP and the purified terminal protein acts as the primer for succeeding DNA chain elongation (6). Thus, the first step of ϕ 29 replication is the formation of a covalent complex between the free terminal protein and dAMP in the presence

of template DNA containing the terminal protein. The complex then serves as a primer for chain elongation with DNA replication proceeding from both ends of the DNA molecule.

Now, the next question is which DNA polymerase is actually responsible for the ϕ 29 DNA replication? ϕ 29 DNA can replicate normally in the presence of 6-(p-hydroxyphenylazo)-uracil which is a specific inhibitor of host DNA polymerase III (7). Also, this phage is capable of replicating normally in polymerase I deficient *B. subtilis* cells (unpublished observation). Therefore, it is very likely that ϕ 29 encodes its own DNA polymerase, or modifies one of the host DNA polymerases.

In this report, we present the first evidence that ϕ 29 codes for its own DNA polymerase. This polymerase is also shown to be required for the initiation of ϕ 29 DNA replication. A gene 2 temperature-sensitive mutant was found to produce a DNA polymerase of significantly greater heat-lability than that produced by wild type phage. Therefore, gene 2 appears to be the structural gene for the ϕ 29-encoded DNA polymerase.

MATERIALS AND METHODS

Materials

B. subtilis SCR 463 (*polA*, *phe*, *hisB*, *trp*) was used for preparation of cell free extract. Wild type phage and mutants A35 (*ts2*) and F98(*ts2*) have been described (8) and were generously provided by Dr. B. E. Reilly. PolyA(dT)₁₂₋₁₈ (equimolar ratio) was purchased from PL-Biochemicals. Calf thymus activated DNA was prepared according to the method of Gass et al (9). ϕ 29 deprotenized DNA was prepared using proteinase K and SDS as described previously (3). Primed single-stranded DNA of phage M13 (M13 sequencing kit) was purchased from Bethesda Research Laboratories and prepared as recommended. Preparation of phage DNA, DNA-protein, cell free extract, and most reagents and radioactive materials have been described in a previous report (6).

Assay conditions for DNA polymerase

The standard reaction mixture (50 μ l) contains 50mM Tris-HCl (pH7.8), 10 mM MgCl₂, 1mM dithiothreitol, 15 μ M ³H-dTTP (600cpm/pmoles), 5% glycerol, 0.5 mg/ml BSA, polyA(dT)₁₂₋₁₈ 14 μ g/ml and 6 μ l of the polymerase fraction (7 μ g protein). The mixture was incubated for 30 min at 30C. The acid insoluble materials were then measured as previously described (6).

Assay for DNA replication and complex formation

Assay conditions for DNA replication activity, specific for ϕ 29 DNA-protein and complex formation between ϕ 29 terminal protein-dAMP, have been

described (6). For these assays, ϕ 29 DNA-protein was used as template at a concentration of 14 μ g/ml. Six microliter of the active fraction (7 μ g of protein) of phosphocellulose column chromatography was used throughout the experiments.

RESULT

Detection of DNA polymerase activity after infection of ϕ 29

Previous attempts to detect a ϕ 29 induced DNA polymerase using crude extracts were unsuccessful. However, when the crude extracts prepared from phage infected *B. subtilis* (pol A⁻) cells were fractionated using a phosphocellulose column, it was possible to detect a new polymerase activity which is eluted at relatively high salt concentrations (Fig. 1A). On the other hand, when an uninfected cell extract was fractionated, only known host polymerase activities were detected close to the void volume (pol III), and in fraction 28 (pol II) (Fig. 1B) in agreement with results reported previously (9). These results suggest that ϕ 29 specifies its own DNA polymerase or modifies one of the host DNA polymerases. In our previous work (6), we showed that the activity for DNA synthesis specific for ϕ 29 DNA-protein and complex forming activity between dAMP-terminal protein can be copurified. We found that the DNA polymerase activity also corresponds to the same fraction which has complex forming and replication activity (see Fig. 1A, inserted figure and closed circles). Furthermore, synthetic homopolymer used in this study as a template did not support the covalent complex formation between the ϕ 29 terminal protein and dAMP (data not shown). These results suggest the new DNA polymerase described here appears to be specific for ϕ 29 DNA replication.

Gene 2 is the structural gene for the ϕ 29-induced DNA polymerase

In order to identify the structural gene of the DNA polymerase, we fractionated the extracts from cells infected with ϕ 29 temperature sensitive mutants at the permissive temperature using phosphocellulose as described above. Of the ts mutants tested, mutants in gene 2 were found to induce the synthesis of the altered DNA polymerase. The peak fractions containing activity corresponding to the phage DNA polymerase from both wild type and ts2 were compared for their temperature sensitivity. Each fraction was preincubated at 43.5C for the indicated time and then was assayed for DNA polymerase activity. As shown in Fig. 2, the polymerase activity present in phosphocellulose fraction prepared from ts2 infected cells was rapidly lost at 43.5C. On the other hand, the polymerase activity isolated from

Figure 1. Chromatography of phage infected cell extract. Cell free extract prepared from $\phi 29$ infected cells (3L original culture) as described (6) was dialyzed buffer A (20 mM Tris-HCl at pH 7.0, 5mM MgCl_2 , 70mM $(\text{NH}_4)_2\text{SO}_4$, 20% glycerol and 10 mM mercaptoethanol) for 4 hr. The dialysate (10ml) was applied to a phosphocellulose column (40 ml bed volume) equilibrated with the buffer A. After the sample was charged, the column was washed with a buffer A and eluted with a NaCl gradient (0.0-7M) in the same buffer.

(A). Column chromatography of extract prepared from phage infected cells.

○-----○ DNA polymerase activity using poly(dA)oligo(dT) as template.

●-----● DNA replication activity dependent on $\phi 29$ DNA-protein. The inserted figure shows an autoradiograph in fractions 45-53 of an SDS polyacrylamide gel used for detection of complex formation between terminal protein and dAMP as described in (6). (B). Same as A except that an extract prepared from uninfected cells was chromatographed.

wild type infected cells showed no loss of activity under these conditions. Furthermore, addition of the preheated wild type fraction to inactivated ts2 fraction restored the activity (data not shown). These results suggest that gene 2 is the structural gene of the $\phi 29$ -induced DNA polymerase.

Figure 2. Thermoability of the DNA polymerase isolated from ts2 infected cells. Phosphocellulose fraction was prepared from cells infected with ϕ 29 wild type or ts2 mutant at a permissive temperature (33°C), by the procedure described in Fig. 1. Fractions containing phage-induced DNA polymerase were preincubated at 43.5°C for the indicated times, and tested for their activities of DNA polymerase using poly(dA)oligo(dT) as template. The concentration of the template is 14 $\mu\text{g/ml}$. O-----O wild type fraction, ●-----● ts2 fraction. Actual counts of radioactivity at 100% were 8095 cpm (wild type) and 4642 cpm (ts2).

Properties of the ϕ 29 DNA polymerase

The template-primer used in this study was poly(dA)oligo(dT)₁₂₋₁₈. We tested several kinds of template primers as shown in Table I. Of those tested, only poly(dA)oligo(dT) was the preferred template for the activity of the ϕ 29 DNA polymerase. For maximum activity, the polymerase requires Mg^{++} and ATP. The activity was found to be sensitive to high salt and N-Ethylmaleimide.

The polymerase is responsible for the initiation of ϕ 29 DNA replication

An *in vivo* study showed that the gene 2 product is required for ϕ 29 DNA replication (10). As shown in Fig. 1, the DNA polymerase activity assayed using poly(dA)oligo(dT) is eluted from phosphocellulose at the same position as the replication activity specific for ϕ 29 DNA-protein and the complex forming activity. We used this unique feature to test the possibility of whether gene 2 product is also involved in the DNA replication of ϕ 29 *in vitro*. A similar experiment as described in Fig. 2 was carried out using an assay system for ϕ 29 DNA replication. The peak fractions for DNA replication in

TABLE 1.
Properties of the Ø29 induced DNA polymerase

<u>Experiment 1^a.</u>	
Template	Activity (%)
poly(dA)oligo(dT)	100 (7414 cpm)
primed single-stranded DNA	5
native calf thymus DNA	7
heat denatured calf thymus DNA	7
activated calf thymus DNA	6
deproteinized Ø29 DNA	14
<u>Experiment 2^b.</u>	
Complete System	100 (10596 cpm)
no enzyme	2
no DNA	2
no Mg ⁺⁺	2
no ATP	32
with KCl (150 mM)	23
with KCl (350 mM)	3
with NEM (5 mM), no DTT	5

a Reaction mixture contains 4 dNTP. DNA concentration (14ug/ml each)

b Reaction mixture contains dTTP only.

the phosphocellulose column, prepared from either wild type or ts2 infected cell, were preincubated at 43.5C and tested for Ø29 DNA replication activity (Fig. 3). As expected, the fractions in the phosphocellulose column prepared from ts2 infected cells showed significantly higher lability at 43.5C than the fractions prepared from wild type infected cells. This indicates that the DNA polymerase activity of gene 2 product is responsible for DNA replication which is catalyzed by the phosphocellulose fraction in vitro.

To define the step in which the Ø29 DNA polymerase is involved. we further tested the thermolability of this polymerase prepared from ts2 infected cells using an assay system for the initiation of replication. After preincubation of phosphocellulose fraction at 43.5C for times shown in Fig. 4, the fraction was tested for its ability to catalyze complex formations between terminal protein and dAMP. The phosphocellulose fraction prepared from ts2 infected cells showed the same rate of thermolability as shown in the case of replication activity. This proves that Ø29 DNA polymerase is also involved in the initiation of DNA replication.

DISCUSSION

In this report, we have described the isolation of a novel DNA polymerase

Figure 3. Thermolability of the DNA replication activity. Phosphocellulose fractions prepared from wild type and ts2 infected cells as described in Fig.1 were preincubated for the indicated time at 43.5C and tested for their DNA replication activity dependent on ϕ 29 DNA-protein as described previously (6). The concentration of DNA-protein used as template was 14 μ g/ml, O-----O wild type fraction, ●-----● ts2 fraction. Actual counts of radioactivity at 100% were 16,403 cpm (wild type) and 2693 cpm (ts2).

which is encoded by gene 2 of phage ϕ 29. In addition, we provided strong evidence that this DNA polymerase is required for the initiation step of DNA replication.

Our previous attempts to detect the ϕ 29 induced DNA polymerase in the crude extracts prepared from phage infected cells were unsuccessful. This was probably due to the fact that the activity of the phage polymerase is relatively low and that the polymerase prefers synthetic homopolymer as template when assayed *in vitro*. Similar preference has been reported for DNA polymerase induced by Epstein-bar virus (11), by varicellazoster virus (12) and by human cytomegalovirus (13). The ϕ 29 DNA polymerase was eluted at the same position as the replication and complex forming activity in the phosphocellulose column. Therefore, the initiation and elongation of DNA replication seems to be coupled. This could be the reason why the polymerase level is low and the enzyme prefers the synthetic homopolymer as template under the present assay system.

The DNA polymerase fraction in the phosphocellulose chromatography prepared from ts2 infected cells exhibited substantial thermolability compared with the fraction prepared from wild type infected cells (Fig. 2).

Figure 4. Thermolability of the complex forming activity. The same fractions as used in Figs. 2 and 3 were preincubated at 43.5°C for indicated times and tested for their complex forming activities as described previously (6). The concentration of the template is 14 µg/ml. (A). Autoradiographs of SDS polyacrylamide gels. (B). Each band was cut from the gel and counted for their radioactivity. O-----O wild type fraction, ●-----● ts2 fraction. Actual counts at 100% were 10533 cpm (wild type) and 3896 cpm (ts2).

This result indicates that gene 2 is the structural gene for the DNA polymerase. Gene 2 is located just down stream of gene 3 on the Ø29 transcriptional map (14). In our previous paper (6), we showed that complex forming activity associated with the functional terminal protein copurified with DNA replication activity. Moreover, we showed that gene 2 product is associated with the fraction which catalyzes complex formation in the phosphocellulose column. Thus, it is probable that the Ø29 DNA polymerase forms a complex with the terminal protein and catalyzes the initiation step of Ø29 DNA replication.

Stillman et al (15) and Friefeld et al (16) showed that a 140,000 dalton DNA polymerase which forms a tight complex with the adenovirus coded pre-terminal protein is also specified by the adenovirus genome. The structural gene for the Ad-DNA polymerase is located just down stream of the terminal protein gene on the adenovirus transcriptional map (15). In the case of adenovirus, the viral DNA polymerase is also necessary to catalyze the complex formation between the adenoviral pre-terminal protein and dCMP which is considered to be a initiation step for DNA replication (17). Up until now, these two viruses, Ø29 and adenovirus, seem to utilize similar

mechanism of DNA initiation, although ϕ 29 infects bacterial cells whereas adenovirus infects eukaryotic cells. Thus, the protein-priming mechanism for DNA replication appears to encompass the entire biological kingdom.

Recently, Blanco *et al* (18) and Matsumoto *et al* (19) reported that cell free extracts prepared from *sus2* infected cells or *ts2* infected cells at non-permissive conditions cannot catalyze the complex formation between dAMP and the terminal protein. The activity was recovered by adding extract prepared from *sus3* infected cells. However, these experiments using crude extracts do not show that gene 2 protein is directly involved in covalent complex formation, since they were compatible with the possibility that the gene 2 product is responsible for the expression of another gene product which is required directly for catalyzing complex formation. In contrast, we prepared a phosphocellulose fraction from cells infected with a *ts2* mutant at permissive temperature and demonstrated that this fraction is thermolabile for both complex formation and ϕ 29 DNA replication. These results indicate that the gene 2 product is directly involved in the reaction of covalent complex formation. In addition, our results implicate that ϕ 29 replication catalyzed by the phosphocellulose fraction in vitro mimics in vivo replication.

It has been shown previously (20) that ϕ 29 gene 2 produces a protein of about 67,000 daltons. DNA sequence data indicated that there is an open reading frame (OF-7) which could encode a 66,500 dalton protein (14). Since this is the only reading frame capable of encoding a protein larger than 60,000 daltons, we propose that this is the correct reading frame specifying the ϕ 29 DNA polymerase.

In summary, we found a new DNA polymerase induced by phage ϕ 29. This polymerase differs from the host DNA polymerase in several important respects among which are its requirement for a special template when assayed in vitro and its activity in covalent complex formation between the ϕ 29 terminal protein and dAMP. Preliminary data showed that the DNA polymerase activity can be dissociated from complex forming activity using native DNA-cellulose column chromatography. Further characterization of the DNA polymerase and its reconstitution with the complex forming activity is now in progress.

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