Determination of the 5'-Terminal Nucleotide of ϕ X174 DNA in RF II in the Late Phase of Infection

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Received April 16, 1971

Elucidation of the mechanism of DNA replication is one of the fascinating problems at present. With bacteriophage ϕ X174 system, it is our interest to know what is the mechanism for producing single-stranded circular DNA. When phage ϕ X174 infects with host *E. coli* cells, viral DNA injected is transformed into double-stranded form, or RF.¹⁾ Synthesis of progeny ϕ X 174 DNA starts within 10 to 15 min after infection. At this late phase of infection, ϕ X174 NDA is exclusively synthesized using complementary strand as template.^{2,3)}

In this stage RF II is the intermediate molecule in synthesizing ϕ X174 DNA, in which viral strand is in a linear form.

Determination of the 5'-end of this linear ϕ X174 DNA is considered to give an information on the mechanism of replication of ϕ X174 DNA. Sinsheimer *et al.*⁴⁾ reported the nucleotide sequence at the 5'-terminal of the linear viral DNA in RF II as CCG-. We also, independently, determined the 5'-end nucleotide of linear ϕ X174 DNA to be deoxycytidylic acid. The result is in accordance with the reported data.⁴⁾

γ -³²P-ATP was prepared by the procedure of Glynn and Chappell.⁵⁾ The specific activity was about 1×10^8 cpm/ μ mole ATP. Polynucleotide kinase was prepared by a slight modification of the procedure of Richardson.⁶⁾ The sonicate of *E. coli* B cells infected with bacteriophage T₄ was treated with streptomycin and autolyzed for 1 hr at 37°C. To the autolysate was added saturated solution of ammonium sulfate, and the precipitate produced at 8~33% saturation was collected and dissolved in 0.01 M potassium phosphate buffer, pH 7.5, containing 0.01 M 2-mercaptoethanol. The enzyme was further purified by DEAE-cellulose column chromatography and then by phosphocellulose column chromatography. The enzyme had the specific activity of 6500 units/mg protein and did not have DNase activity.

E. coli C_N was grown to a cell concentration of 1×10^9 /ml in TPG-CA medium (amino acid mixture was replaced by casamino acids),¹⁾ and the cells were infected with ϕ X174 *am* 3 at a multiplicity of 5 at 37°C. Thirty minutes after infection, cells were collected and lysed with lysozyme-EDTA. DNA synthesis of the host was suppressed by treating cells with mitomycin C⁷⁾ before phage infection. DNA

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was extracted from the lysate with phenol and subjected to a sedimentation through neutral linear gradient of 5 to 20% sucrose buffered with 0.03 M Tris-HCl containing 0.5 M NaCl and 0.01 M EDTA, pH 7.5. Fractions containing RF II were collected and RF II was precipitated with ethanol. The precipitate was dissolved in 0.5 M NaCl-0.03 M Tris-HCl buffer-0.001 M EDTA, pH 7.5, and the sedimentation through neutral sucrose gradients was repeated twice. RF II was then denatured with alkali and sedimented through an alkaline sucrose gradient (pH 12.5) in order to separate linear ϕ X174 DNA from the closed complementary strand. The fractions corresponding to linear ϕ X174 DNA were collected, and the DNA solution was treated with alkaline phosphatase (30 μ g) after dialysis against 0.001 M EDTA-0.1 M Tris-HCl buffer, pH 8.0, and was concentrated to 1 ml. The 5'-termini of the linear strands were then phosphorylated as described by Wu and Kaiser.⁸⁾ The reaction mixture contained 60 m μ M γ -³²P-ATP, 75 units polynucleotide kinase, 12 mM MgCl₂, 10 mM 2-thioglycol and 1.25 ml of DNA solution (OD_{260m μ} =0.60) in 0.07 M Tris-HCl buffer, pH 7.6. The reaction mixture was incubated for 30 min at 37°C, and 35 units of the enzyme were added again. Incubation was continued for additional 40 min and the reaction was stopped by adding EDTA to 20 mM. The mixture was dialyzed against 0.1 M NaCl containing 0.001 M EDTA. The dialysate was subjected to an equilibrium centrifugation in cesium chloride, and the fractions having buoyant density around 1.765, corresponding to that of ϕ X174 DNA, were pooled and the combined fractions were dialyzed against 0.1 M Tris-acetate buffer, pH 8.0. The dialysate was passed down a sephadex G 200 column to remove γ -³²P-ATP. The first small ³²P-peak eluted was collected and concentrated. To the DNA solution thus obtained was added

TABLE I. DISTRIBUTION OF ³²P IN DEOXYRIBONUCLEOSIDE-5'-MONOPHOSPHATES

Preparation of ³²P-labeled linear viral strand, digestion with pancreatic DNase and venom phosphodiesterase are described in the text. Digestion products were separated by ascending paper chromatography together with four deoxyribonucleotide markers in saturated ammonium sulfate-1 M sodium acetate-isopropanol (80:18:2, by vol.) and in isopropanol-HCl (68:33.3, by vol.). Each spot was cut out and counted in a gas flow counter. The values listed are the average of several experiments obtained from the two different paper chromatographic systems.

	CTS/min	%
dTMP	700	22
dCMP	1378	41
dAMP	574	17
dGMP	675	20

50 μ g *E. coli* DNA, and the mixture was digested with bovine pancreatic DNase (20 μ g) and snake venom phosphodiesterase (10 μ g). The digestion products were separated by the ascending paper chromatography on Toyo Roshi filter paper No. 50 together with four deoxyribonucleoside-5'-phosphate markers in saturated ammonium sulfate-1 M sodium acetate-isopropanol (80:18:2, by vol.) or in isopropanol-6-N HCl (68:33.3, by vol.).

Four spots corresponding to dAMP, dTMP, dGMP and dCMP were cut out and counted for the radioactivity in a gas flow counter. The results were shown in Table I. An amount of 41% of the total counts was found in dCMP and remaining radioactivity distributed evenly among dAMP, dTMP and dGMP. Even though with use of RF II and linear ϕ X174 DNA which were extensively purified, we could not eliminate the background of remaining three nucleotides. This was considered to be due to the contaminating host DNA fragments. Nevertheless, we may conclude that linear ϕ X174 DNA in resting RF II has a deoxycytidylic acid at the 5'-terminus of the molecule.

Two explanations are possible; (1) there is a

8) R. Wu and A. D. Kaiser, *Proc. Natl. Acad. Sci., Wash.*, **57**, 170 (1967).

specific initiation site (or sites) which is GC cluster (or GC clusters) on the complementary strand of RF II for DNA synthesis, and (2) a specific endonuclease attacks the viral strand of RF molecule and gives a nick (or nicks), producing DNA chain with C at the 5'-end (or 5'-ends). Our unpublished data⁹⁾ show that ϕ X174 DNA is synthesized through short chains. This will favor the possibility (1).

However, more detailed experiments are desirable to elucidate the meaning of the presence of deoxycytidylic acid in 5'-terminus of linear ϕ X174 DNA.

Acknowledgement. The authors wish to express their sincere thanks to Dr. M. Takanami and Dr. M. Sugiyama, Institute of Chemical Research, Kyoto University, for their kind advice during the course of preparation of γ -³²P-ATP. This work was partly supported by a scientific grant from the Ministry of Education, Japan.

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