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Replicative Form II DNA of ϕX174; Resistance to Exonucleolytic Cleavage by *E. coli* DNA Polymerase

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Abstract. The progeny replicative form II DNA isolated from *E. coli* cells infected with ϕX174 is resistant to the exonucleolytic activity associated with *E. coli* DNA polymerase. A limited endonucleolytic cleavage with micrococcal endonuclease renders the replicative form II molecule susceptible to the exonucleolytic activity associated with the *E. coli* DNA polymerase.

These experiments suggest that the resistance of the replicative form II DNA to the exonucleolytic activity is a result of the structure of these molecules at their 5′-terminus.

Introduction. An exonucleolytic activity associated with highly purified preparations of *E. coli* DNA polymerase has been described by Klett, Cerami, and Reich[1] and by Deutscher and Kornberg.[2] This activity degrades double-stranded DNA from the 5′-end producing 5′-mononucleotides and oligonucleotides[3] terminating in a 5′-phosphate group. The experiments designed to establish the characteristics of this exonucleolytic activity used, usually, poly d(A-T) or poly dA:dT as substrate. *E. coli* DNA was found to be a very poor substrate.[2]

From experiments performed in Kornberg's laboratory, it is evident that the polymerase will bind to nicked circular DNA duplexes at nicked sites regardless of the nature of the chain break.[4]

Experiments described in the present communication indicate that the *in vivo* replicative form (RF) II DNA[5] is completely resistant to the 5′ → 3′ exonucleolytic activity associated with the polymerase. Our results suggest further that the structure at the 5′-end (primary, secondary, or tertiary) or the open chain provides this resistance.

Materials and Methods. Isolation of RFII DNA: *E. coli* H502 (*Su*$^-$, *hcr*$^-$, *thy*$^-$, *Endo* I$^-$) cells infected with ϕX174*am*3 were pulse labeled, 40 min after infection, for 20 sec with [^{3}H-methyl] thymidine (Schwarz BioResearch, Orangeburg, N.Y.). In this labeling procedure radioactivity is incorporated exclusively into the viral strand of the replicative forms. The replicative form molecules were purified by sedimentation through a 5–20% neutral sucrose gradient as described previously.[6] The 16S DNA was used as the substrate for the exonucleolytic activity associated with DNA polymerase.

Assay of the exonucleolytic activity of *E. coli* polymerase: The exonucleolytic activity was assayed in a reaction mixture containing 40 μmol of glycine buffer, pH 9.2, 5 μmol $MgSO_4$, 0.5 μmol 2-mercaptoethanol, 3–10 pmol of polynucleotide as substrate, and 10 μl (approx. 5 units) of purified polymerase (prepared by Dr. L. B. Dumas as described in Dumas, Darby, and Sinsheimer, in preparation). The final volume of the reac-

tion mixture was 0.5 ml. Twenty-microliter aliquots were withdrawn from the reaction mixture after different times of incubation at 37°C. Trichloroacetic acid (5%) and thymus DNA (10 μg) were added to each and the mixtures were filtered through glass fiber filters. The filters were washed with 5 ml of 5% trichloroacetic acid, dried, and then counted in the Beckman scintillation counter.

Poly dA:dT (^{3}H) was a generous gift of Dr. F. J. Bollum and poly dG:dC (^{3}H) was purchased from Biopolymers Laboratory, Dover, N.J. All other chemicals were of analytical grade.

Results and Discussion: The exonucleotic activity associated with *E. coli* polymerase: RFII DNA as substrate: The 16S RFII DNA (see *Materials and Methods*) is double-stranded circular DNA nicked at a specific site in the viral strand.[6] The 5′-end of the nicked strand lacks a terminal phosphate; by phosphorylation with polynucleotide kinase, followed by treatment with polynucleotide ligase, about 50% of the RFII DNA can be converted into the fast-sedimenting supercoiled RFI (as a result of the closure of the open strand). The reason for the incomplete conversion of RFII into RFI is still obscure.[6]

The characteristics of the 16S DNA and the specificity of the 5′ → 3′ exonucleolytic activity associated with *E. coli* DNA polymerase,[1,2] suggested that this DNA should be a suitable substrate. However, this DNA does not serve as substrate; label on the RFII remains 100% acid precipitable after a period of 18-hr incubation under the conditions described in the *Materials and Methods* (Fig. 1). No exonucleolytic activity has been observed when one, or a combination of two, three, or four deoxynucleoside triphosphates were added. That has been the case when the incubation was performed at higher temperatures (up to 45°C), at pH values ranging from pH 7.0 to 9.2, or at higher concentrations of enzyme (fivefold).

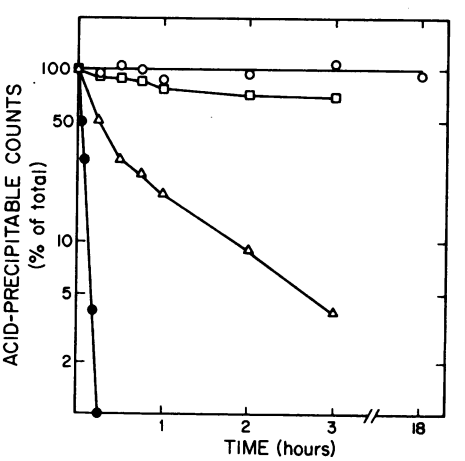

FIG. 1.—5′ → 3′ exonucleolytic activity associated with E. coli DNA polymerase: RFII DNA, poly dA:dT, and poly dG:dC as substrates. Incubation conditions were as described under *Materials and Methods;* all substrates were added at a concentration of 3 pmol of 5′-OH-termini per reaction mixture (judged by ^{32}P incorporation into the 5′-end) ○, RF II (^{3}H); □, poly dG:dC (^{3}H); △, poly dA:dT (^{3}H); ●, poly dA:dT (^{32}P counts).

Poly dA:dT as substrate: That the enzyme preparation had a 5′ → 3′ exonucleolytic activity under the conditions described was demonstrated with poly dA:dT (^{3}H) as substrate (Fig. 1). To rule out the possibility of a quantitative difference between the substrates, the poly dA:dT (^{3}H) was used in a concentration of 5′-OH ends identical to that of the RFII DNA. (The 5′-OH end concentration was estimated by incorporation of ^{32}P label at the 5′-end employing

the polynucleotide kinase.[6]) The prompt "solubilization" or such ^{32}P radioactivity (Fig. 1) indicated that the activity assayed was indeed the 5′ → 3′ exonucleolytic activity.

Poly dG:dC as substrate: The 5′ → 3′ exonucleolytic activity of *E. coli* DNA polymerase is reported to have a distinct preference for d(A-T) copolymer as substrate.[1] No exonucleolytic activity could be demonstrated on the "repaired" cohesive ends of λDNA. These cohesive ends are d(G-C) rich and 11 d(G-C) pairs are in a pure d(G-C) run.[7] The two terminal nucleotides at the 5′-end of the RFII DNA were identified in a previous study as dCMP.[6]

These observations suggest that the resistance of RFII DNA to the exonucleolytic activity might arise in the primary or secondary structure of the 5′-end. Such possibility has been supported by the very limited exonuclease activity observed with poly dG:dC (^{3}H) as substrate (Fig. 1). The apparent 10–20% degradation of dG:dC has been observed in the presence or absence of the enzyme and is, presumably, a nonenzymatic release of some radioactive contaminant in the commercial product. Sequences rich in dG's and dC's appear to be very resistant to the 5′ → 3′ exonucleolytic cleavage.

The resistance of the RFII 5′-end to exonuclease: Assuming then that the sequence at the 5′-end is resistant to the 5′ → 3′ exonucleolytic activity, a limited endonucleolytic cleavage of the circular duplex might generate susceptible sites for exonuclease. Micrococcal nuclease catalyzes the hydrolysis of DNA to produce 3′-nucleoside phosphates[8] thereby exposing new 5′-OH ends, and it acts preferentially in A-T rich regions.[9] When a 60-min preincubation was performed in the presence of 1 μg/ml of micrococcal nuclease (Worthington Co.), four new 5′-OH ends per molecule were produced (as judged by ^{32}P incorporation catalyzed by polynucleotide kinase).[6] Addition of *E. coli* DNA polymerase to the substrate at that point resulted in a rapid degradation of the DNA, whereas the DNA in the control tubes (polymerase only and endonuclease only) remained acid precipitable. When polymerase and endonuclease were added from the beginning of the incubation, the DNA started to degrade without observed lag time (Fig. 2). Assuming that the limited endonucleolytic cleavage is not an instantaneous event, this lack of lag period suggests that one endonucleolytic hit might be enough to start the exonucleolytic reaction. That is a further confirmation of the assumption that the resistance of RFII to the exonuclease lies in the structure at its 5′-end.

The resistant character of the 5′-end of RFII DNA to exonucleolytic cleavage has been further demonstrated in the following experiment; ^{3}H pulse-labeled RFII DNA was labeled at the 5′-end with ^{32}P as described before,[6] subjected to a limited endonucleolytic cleavage, and then used as substrate for the 5′ → 3′ exonuclease reaction. The much more rapid rate of decrease of acid-precipitable ^{3}H counts than of acid-precipitable ^{32}P counts and the relatively limited release of ^{32}P (Fig. 3) suggest that the 5′-end region remains intact and probably paired with the complementary strand. Evidence supporting this interpretation was obtained from the behavior of the ^{32}P-labeled digestion product in high voltage electrophoresis. It has shown no mobility at pH 3.5 (75 V/cm) for 2 hr. The product was eluted from the paper (80°C, 15 min in water) and subjected to

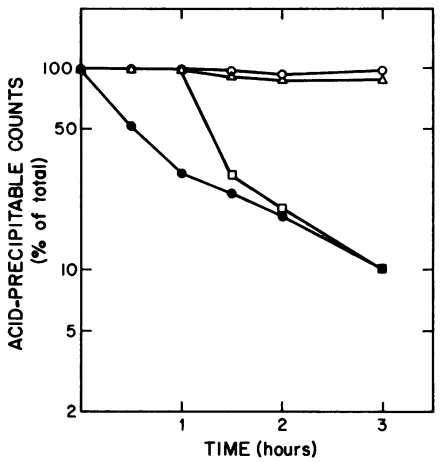

FIG. 2.—5′ → 3′ exonucleolytic cleavage of endonuclease-treated RF II. Incubation conditions were as described under *Materials and Methods*. O, polymerase only; Δ, micrococcal endonuclease only; □, micrococcal endonuclease added at the onset and polymerase after 60 min; ●, micrococcal endonuclease and polymerase added at the beginning of the incubation.

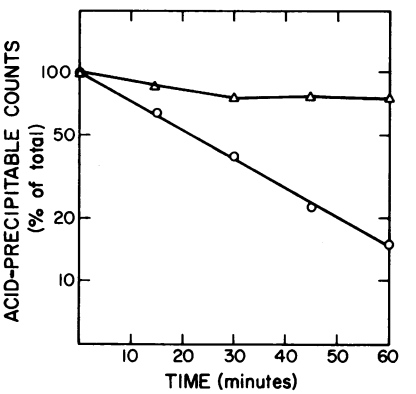

FIG. 3.—The 5′-end of RF II; resistance to 5′ → 3′ exonuclease. Incubation conditions as described under *Materials and Methods*. RFII DNA labeled with ^{3}H-thymidine *in vivo* and ^{32}P at the 5′-end *in vitro* used as substrate after a limited digestion with micrococcal endonuclease. O, ^{3}H counts; Δ, ^{32}P counts.

venom phosphodiesterase as described previously.[6] The ^{32}P-labeled product of this digestion was dCMP, as judged by its electrophoretic mobility.

The conclusion drawn from these experiments is that the 5′-terminal region of RFII DNA, isolated from *E. coli* cells infected with φX174, is highly resistant to the 5′ → 3′ exonucleolytic activity associated with *E. coli* DNA polymerase, possibly because of the presence of a cluster of G-C pairs.

The physiological significance of this phenomena is not clear. It can be speculated that this resistance might serve in the control of DNA replication in the process of production of φX174 single-stranded DNA. It might be connected to the mechanism of formation of the specific nick in the RFII DNA.[6] The limited genetic information which might be stored in the small φX174 DNA makes the possibility of a virus-coded, specific "nickase" somewhat doubtful. Our results raise the possibility of producing a specific nick by means of unspecific enzymes. A concerted catalysis by host endonuclease, 5′ → 3′ exonuclease, and DNA polymerase, would bring a translation of a nick initially in an unspecific site to a specific site where the exonuclease cannot operate further. At this point DNA synthesis will stop unless the resistant 5′-end is displaced from the complementary strand. Viral coat proteins (or other phage-coded proteins) might function in such a displacement reaction.

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