Replication of Phage G4

A NOVEL AND SIMPLE SYSTEM FOR THE INITIATION OF DEOXYRIBONUCLEIC ACID SYNTHESIS*

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Conversion in vitro of single-stranded circular DNA of phage G4 (related to phage φX174) to the double-stranded replicative form (RF-II) depends on a novel and relatively simple group of three proteins: a priming protein of approximately 65,000 daltons, the DNA unwinding protein, and the DNA polymerase III holoenzyme. Stimulation by ATP and GTP suggests an RNA synthetic step in the priming of DNA synthesis. The synthetic strand in the RF-II contains a small gap at a unique position relative to the template strand; the 5' end of the gap is about 250 nucleotide residues (5% of the genome length) away from the single site of cleavage by a restriction endonuclease (Eco RI).

Studies of the conversion of single-stranded circular DNA to the duplex form in vitro have disclosed two distinctive systems for initiation of DNA synthesis in extracts of Escherichia coli (1). One, exemplified by filamentous phage M13, is inhibited by rifampicin because RNA polymerase action is required for priming of DNA replication (2). The other, illustrated by the polyhedral phage φX174, is resistant to rifampicin (3). The φX174 system depends upon seven or more proteins, including those inferred from genetic studies to be needed for initiation of the E. coli chromosome at its origin (4). Upon examining the replication of the DNA of phage G4 (5), a particle resembling φX174, we have now come upon a third system. As with φX174, the enzymes required for initiation of DNA synthesis on the G4 template are resistant to rifampicin. Unlike φX174, the G4 system is far simpler and depends on only three of the replication proteins.

In this report, we (a) compare the G4 replication system with the others, (b) describe a partially purified protein which primes G4 DNA synthesis, and (c) show that initiation is at a unique location on the viral template. A subsequent report† will present further information using purified enzymes for G4 replication and details of the novel RNA synthetic action by the priming protein, tentatively identified as the dnaG protein.

MATERIALS AND METHODS

Materials were from sources described previously (2, 6).

Bacterial and Phage Strains—Escherichia coli H560 (F+, pol A−).

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The abbreviations used are: SS, single-stranded, circular DNA; RF-II, circular double-stranded DNA of replicative form with a discontinuity in one strand.

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pmol of SS DNA (in nucleotide residues), 0.04 mg/ml of rifampicin, 0.5 mm each of rATP, rGTP, and rUTP, and 0.02 mm each of dATP, dGTP, dTTP, and [α-32P]dCTP. (In some experiments [α-32P]dCTP was used instead of [α-32P]dCTP.) Specific activities were 100 to 800 cpm/pmol. Enzyme fractions (Fraction I, Fraction II, or purified enzymes) were added last. After incubation for 15 min the reaction was terminated by chilling the tube in ice. For measurement of acid-insoluble much incubation, 0.4 ml of 0.2 M sodium pyrophosphate and 0.5 ml of 10% trichloroacetic acid were added. The precipitate was collected on glass fiber filters (Whatman GF/C), washed three times with 4 ml of 1 M HCl, 0.2 M sodium pyrophosphate, dried, and counted in a low background Nuclear Chicago gas flow counter for 32P or in 5 ml of a toluene-based scintillation fluid in a Nuclear Chicago liquid scintillation counter for 3H. For analysis of the reaction product by alkaline sucrose gradient centrifugation, 2 μl of 0.5 M EDTA and 5 μl of 3 M KOH were added to the incubation mixture. The sample was incubated for 10 min at 30° and then layered on top of an alkaline sucrose gradient.

Sucrose Gradient Analysis—Linear neutral sucrose gradients (5 to 20%) contained 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 1 M NaCl. Linear alkaline sucrose gradients (5 to 20%) contained 50 mM glycine, 0.15 M KOH, 0.4 M LiCl, and 1 mM EDTA. Gradients were centrifuged for 3 hours (neutral), for 4.5 hours (alkaline) at 55,000 rpm, and 4° using an SW 56 Ti rotor in a Spinco L2-65B centrifuge. Tubes were punctured at the bottom and fractions of either 5 or 8 drops collected. Calf thymus DNA (50 μl of 1 mg/ml in 0.01 M Tris-HCl (pH 7.5), 1 mM EDTA) was added as carrier to each fraction. DNA was precipitated by addition of 10% trichloroacetic acid, collected on glass fiber filters, and the radioactivity was measured as described above.

Other Methods—Protein was determined by the procedure of Lowry et al. (15), using a bovine serum albumin standard. Human hemoglobin, purified as described previously (12), was measured spectrophotometrically at 410 nm.

RESULTS

Rapid Conversion of G4 DNA to RF-II by Soluble Enzymes—G4 DNA served in vitro as an efficient template (Fig. 1A) and sustained a rapid rate of DNA synthesis (Fig. 1B) without addition of spermidine. With the same ammonium-sulfate fraction (Fraction II), prepared from an Escherichia coli lysate, spermidine was shown to be essential for optimal conversion of the DNA of the other φX-like phages (S13 and G14).

The conversion of G4 DNA was not inhibited by rifampicin (Fig. 1). In this regard the synthesis by this enzyme fraction resembles the rifampicin-resistant conversion of φX174 DNA to RF-II (3) and is distinct from the rifampicin-sensitive conversion of M13 DNA (3, 16).

As is the case with φX174 DNA (3), the single-stranded G4 DNA was converted to RF-II; the synthetic strand sedimented in an alkaline sucrose gradient to a position expected for a full length linear strand (Fig. 2). Some of the synthetic material (up to 20%) sedimented more rapidly for unexplained reasons.

Gap in Synthetic RF-II Is at Unique Position—Cleavage of the G4 RF by Eco RI restriction endonuclease at a single site (18) was confirmed with the enzymatically synthesized RF-II. The product obtained after complete cleavage of RF-II by Eco RI sedimented in a neutral sucrose gradient containing 1 M NaCl slightly slower than the circular RF-II, a position expected for a linear double-stranded DNA molecule (Fig. 3A). Because of the interruption in the complementary strand (Fig. 2), the same material yielded two fragments clearly separated by alkaline sucrose gradient centrifugation (Fig. 3B). A rapidly sedimenting fraction, only slightly slower than the untreated, nearly full length strand, contained about 95% of the 32P radioactivity of the synthetic strand. A very slowly sedimenting fraction contained only about 5% of the radioactivity of the full length strand. This result places the cleavage site at a unique place and close to the interruption in the complementary strand of RF-II.

To determine whether the Eco RI site is near the 3' or 5' end of the synthetic strand, the gap in the RF-II was first filled by DNA polymerase I with 3H-labeled deoxynucleoside triphosphates and then subjected to Eco RI cleavage. The bulk of the 3H radioactivity sedimented with the large fragment in an alkaline sucrose gradient marking the 5' end and the location of the Eco RI at about 5% of a genome length from the 5' end (Fig. 4).

Relatively Few Components Are Required for G4 Replication—The first suggestion of the relative simplicity of the system for conversion of G4 DNA to RF was the efficiency and speed of the reaction with the crude enzyme fraction (Fig. 1). The next was its relative insensitivity to a sulfhydryl blocking agent. After treatment with N-ethylmaleimide, the activity was fully restored upon the addition of DNA polymerase III holoenzyme (Table I). Therefore, no other N-ethylmaleimide-resistant components were required.
FIG. 3 (left). Unique Eco RI cleavage site in enzymatically synthesized RF-II. G4-RF-II labeled with $^{32}$P or $^3$H in the complementary strand was synthesized in vitro in a 14-fold standard assay mixture (0.7 ml) using Fraction I as enzyme source and [32P]dATP + dCTP or [3H]dCTP, dATP, dTTP. The reaction was stopped by chilling the tube in ice and adding $\frac{1}{2}$ volume 0.5 M EDTA solution. The RF-II was separated from the radioactive precursor by centrifugation in a neutral sucrose gradient containing 1 M NaCl (19). Proteins were removed from the combined RF-II-peak fractions by extraction with phenol and the DNA concentrated from the aqueous phase by precipitation with ethanol at $-20^\circ$. RF-II was purified further by a second neutral sucrose gradient centrifugation in 1 M salt. The combined peak fractions were dialyzed overnight against 0.01 M Tris-HCl (pH 7.5), 0.1 mM EDTA, and concentrated by covering the dialysis bag with dry Sephadex G-75. The yield of RF-II recovered after purification as acid-precipitable radioactivity was 33%, the specific activities were 0.75 Ci/mmol for the 32P-labeled and 0.5 Ci/mmol (2.3 *Ci/pg of RF-II) for the $^3$H-labeled RF-II. For restriction by endonuclease Eco RI 7.6 pg of RF-II labeled with $^{32}$P in the complementary strand were incubated in a volume of 0.03 ml containing 0.1 M Tris-HCl (pH 7.5), 0.01 M MgCl$_2$, and 0.1 mM EDTA, and 0.1 pg of endonuclease Eco RI at 37$. After 45 min an additional 0.1 pg of Eco RI was added and the incubation continued. After 45 min the reaction was terminated by adding 0.5 M EDTA to a final concentration of 10 mM. The sample was mixed with an equal volume of a control sample containing 7.6 pg of $[^3$H]RF-II which had been incubated identically but without Eco RI. The mixture was split into two parts. One part was analyzed in a neutral sucrose gradient (A), the other in an alkaline sucrose gradient (B).

FIG. 4 (right). Location of Eco RI cleavage site near the 5’ end of the complementary strand. The gap of purified 32P-labeled G4 RF-II (see Fig. 3) was filled with DNA polymerase I and dideoxynucleoside triphosphates. The repaired molecules were prepared for treatment by Eco RI as described in (19). After Eco RI cleavage, the products were analyzed by alkaline sucrose gradient centrifugation. The inset illustrates the position of the gap relative to the Eco RI site.

### TABLE I

Activity of N-ethylmaleimide-treated enzyme fraction restored by DNA polymerase III holoenzyme

<table>
<thead>
<tr>
<th>Treatment of enzyme</th>
<th>Holoenzyme added</th>
<th>DNA synthesis (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nematode</td>
<td>+</td>
<td>10.8</td>
</tr>
<tr>
<td>Nematode</td>
<td>-</td>
<td>13.6</td>
</tr>
</tbody>
</table>

sensitive component, such as the dnaC protein and protein n, both required for φX174 conversion (4), is needed for the G4 reaction.

Gel filtration through Sephadex G-150 resolved the G4 replication system into three components (Fig. 5). None of the fractions was active by itself (Fig. 5A). The excluded fraction (Fraction 29) was active when supplemented with an included fraction (Fraction 39) and pure unwinding protein (Fig. 5B); it was active as DNA polymerase III holoenzyme in a primed φX174 assay (20); and it was replaceable by purified holoenzyme. There were two included components. One (Fraction 36) was active when supplemented with the excluded fraction (Fraction 29) and an included one (Fraction 39) (Fig. 5C); and it was replaceable by pure unwinding protein. The other included component (Fraction 39) could be assayed in the presence of the holoenzyme and unwinding protein (Fig. 5D). This second included component (Fraction 39) was presumed to be responsible for the priming of G4 DNA needed for replication by the holoenzyme.

The priming component after further purification was absolutely required for G4 replication as were holoenzyme and unwinding protein (Table II). Upon sedimentation in a glycerol gradient, the bulk of the priming activity coincided with a hemoglobin marker (Fig. 6), although some activity was also located at higher S values. The peak fractions from this gradient complemented an extract of a temperature-sensitive dnaG mutant in the conversion of φX174 DNA to RF’ (data not shown).

### Ribonucleoside Triphosphates Are Required for G4 Replication

In addition to the four deoxyribonucleoside triphosphates and Mg$^{2+}$ (data not shown), both rATP and rGTP were required for G4 replication. In the presence of G4 DNA, the addition of rATP or rGTP to the reaction mixture caused a significant increase in the rate of incorporation of [3H]dCTP. This effect was observed at concentrations of both nucleoside triphosphates ranging from 1 to 10 mM. The most pronounced effect was observed when the concentration of rGTP was increased to 10 mM, where the incorporation of [3H]dCTP was increased 10-fold compared to the control reaction without ribonucleoside triphosphates.

By guest on October 15, 2017
DNA-POLYMERASE I
RNA POLYMERASE
HEMOGLOBIN
ADDITIONS TO ASSAY
(A) NONE
(B) FR. 39 + UNW. PROT.
(C) FR. 29 + FR. 39
(D) FR. 29 + UNW. PROT.

Fig. 5. Separation by gel filtration of three components required for G4 replication. Fraction II (200 µl) was filtered through a column of Sephadex G-150 superfine (1 x 26 cm) equilibrated with 0.05 M Tris-HCl (pH 7.5), 5 mM MgCl₂, 0.1 M NaCl, 10% (v/v) glycerol, 1 mM EDTA, and 1 mM dithiothreitol. Fractions of 0.25 ml were collected, of which aliquots (2 µl) were assayed under standard conditions with additions as follows: A, none; B, Fraction 39 (3 µl, containing priming activity) and purified DNA unwinding protein (UNW. PROT.) (1 µg); C, Fraction 29 (3 µl, containing DNA polymerase III holoenzyme as judged by assay with primed φX174 DNA (13)) and Fraction 39 (3 µl, containing priming activity); D, Fraction 29 (3 µl, containing DNA polymerase I holoenzyme) and purified DNA unwinding protein (1 µg). Positions of reference markers RNA polymerase (= void volume), DNA polymerase I, and hemoglobin were determined in a separate run on the same gel bed under identical conditions.

Optimal concentration about 0.2 mM, were required for optimal conversion of G4 DNA to RF-II either by crude or purified enzyme fractions (Table III and Fig. 7, A and B). UTP had little effect (Fig. 7C) and CTP appeared to be inhibitory (Fig. 7D). ATP, GTP, and UTP were each slightly inhibitory at concentrations over 1 mM.

DISCUSSION

The E. coli enzymes used for conversion of phage G4 DNA to a duplex circle are distinct as a group from those required by M13 DNA or φX174 DNA in the first stage of their replication. Unlike M13 which depends on RNA polymerase for initiation of DNA synthesis, the G4 system (like that for φX174) is rifampicin-resistant. However, replication of G4 DNA was observed even with crude enzyme preparations to be generally more rapid and efficient than that of φX174 (Fig. 1). The reason appears to be the relative simplicity of the G4 system.
which requires only three of the seven proteins which are necessary for the replication of φX174 DNA.

The basic distinction between the systems for conversion of M13, G4, and φX174 DNA circles to their respective duplex forms appears to reside in the enzymatic equipment needed for synthesis of the primer essential for initiation of the DNA chain. In all cases, the DNA chain itself is synthesized by DNA polymerase III holoenzyme in the presence of DNA unwinding protein, or spermidine or both.

The rifampicin-sensitive RNA polymerase which primes M13 DNA is inactive on φX174 DNA and on G4. The protein which primes G4 DNA synthesis fails with M13 and is inadequate by itself for φX174 DNA. The complex φX174 system also utilizes G4 DNA because it includes the G4 primer protein, but it does not act on M13 DNA. Thus, there must be in each case a recognition by an enzyme of a specific sequence or secondary structure which serves as the "promoter" for the brief transcription that initiates DNA synthesis. Direct evidence for a specific template location for the start of replication has been presented for M13 (16, 19) and was observed once again in these studies for G4 DNA.

Analysis of the G4 replication system showed that the priming component was insensitive to a sulfhydryl-blocking agent (Table I), could be separated from other components by gel filtration (Fig. 5), and was a protein of about 65,000 daltons (Fig. 6). The capacity of this priming protein to complement φX174 replication dependent on the dnaG protein, furnishes a provisional indication of its identity.

In view of the dependence on ATP and GTP, the priming protein appears to catalyze the synthesis of a short segment of RNA (Table III, Fig. 7). The lack of a requirement for UTP and CTP may be attributable to their availability as contaminants, or their dispensability in this very brief transcriptional operation in vitro. However, current studies show that incubation of the priming protein with all four ribonucleoside triphosphates results in the synthesis of an oligoribonucleotide of about 20 residues in which all ribonucleotides are represented.1 Subsequently, addition of deoxyribonucleoside triphosphates and DNA polymerase III holoenzyme results in a covalent extension of the oligoribonucleotide by DNA synthesis to produce the nearly full length complementary strand.

In the presence of amounts of unwinding protein, sufficient to mask all but a specific region of M13 DNA, the successive actions of RNA polymerase and DNA polymerase III holoenzyme, produce an RF-II with a short gap in a unique location; the 3' end of the complementary strand was positioned about 5% of a genome length from the Hind endonuclease cleavage site (19). Similarly with G4 DNA, with the exception that the priming protein replaced RNA polymerase, a short gap in the complementary strand was uniquely placed with the 5' end of the strand about 5% of a genome length (about 250 residues) from the Eco RI endonuclease cleavage site. That the gap in the RF-II marks the origin of in vitro replication is attested by the residual RNA at the 5' end of the synthetic strand of both M13 (3) and G4.1 Whether the origin identified in vitro corresponds to the one in vivo remains to be determined.

Single-stranded templates used in vitro are not likely to be found under physiological conditions because decapsidation of the phage particle is tightly coupled to replication (17, 21). Assuming that there is a physiological role for the unwinding
protein, it seems probable that in our soluble enzyme system its function is amplified to make up for the lack of capsid proteins which normally mask the DNA during the early events of infection.

By analyzing the replication systems for various phages, insights are furnished into the nature and operations of the systems used by the host cell for replication of its own DNA. The rifampicin sensitivity of the conversion of M13 DNA to RF suggests that the host system appropriated is one used for certain plasmids, such as colicinogenic factor E1 or the F factor. The complex system used for conversion of φX174 DNA resembles, as suggested by genetic data, the one employed by E. coli for the origin of replication of its own chromosome. Thus, one may imagine a close similarity between the "promoter" at the replication origin of the phage DNA and that of the host DNA. In this vein, the G4 system resembles that needed for the origins of nascent (Okazaki) fragments. The latter requires the dnaG protein as proposed by Lark (22) and does not appear to depend on the dnaC gene product; however, the need for dnaB gene function to sustain synthesis of nascent fragments (23) does distinguish it from G4 DNA conversion, for which the dnaB product seems to be dispensable.

We anticipate that among the diversity of available phages, additional probes will be found which will help in unraveling the complexities of the multienzyme systems of DNA replication.

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