Bacteriophage T7 DNA Replication

AN ELECTRON MICROSCOPIC STUDY OF THE GROWING POINT AND THE ROLE OF THE T7 GENE 4 PROTEIN IN THE FORMATION OF DNA FRAGMENTS

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John Wolfson and David Dressler

From the Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

T7 gene 4 codes for a protein whose function is essential for the replication of the viral chromosome. The experiments reported in this paper were designed to study the mechanism of action of T7 gene 4 by assessing the change that occurs in intracellular viral DNA when the gene 4 protein is inactivated during a normal DNA replication cycle.

Following infection with phage carrying a temperature-sensitive mutation in gene 4, transfer of the cells from the permissive to the nonpermissive temperature for about 15 s generates a progressive lengthening of the regions of single-stranded template DNA in the growing points. Normal T7 chromosomes contain about 1500 bases of single-stranded template DNA on one side of the growing point; after the temperature shift, the single-stranded regions increase to a length of 3000 to 4000 bases. This effect is not observed in infections with wild type phage.

A straightforward interpretation of the data is that, upon the shift to 42°C, the T7 gene 4 protein is inactivated and DNA synthesis can occur on only one side of the growing point. Thus the T7 DNA polymerase continues to elongate the daughter strand with the 3'-OH terminus, resulting in a lengthening of the region of single-stranded template DNA on the other side of the growing point. Evidently, in the absence of functional gene 4 protein, no new fragments can be developed to convert the region of single-stranded template DNA to the duplex state.

Our in vitro results form the complement for the in vivo studies of Scherzinger and Richardson and their colleagues, which demonstrate that purified gene 4 protein is capable of priming the de novo initiation of DNA fragments.

The differential effect of the gene 4 protein on one side of the growing point, observed in the present in vivo study, supports the model of hemidiscontinuous synthesis, in which one daughter DNA strand is elongated continuously and the other discontinuously by fragments.

Electron microscopic studies have defined the sequence of topological changes that occur during the first round of replication of the T7 chromosome. DNA synthesis is initiated at a point about 17% from the left end of the viral DNA rod and proceeds bidirectionally from this origin, generating, first, an eye-shaped intermediate (−O−), then a Y-shaped molecule (−), and finally two progeny DNA rods (1, 2).

In these electron microscopic experiments the fine structure of the T7 DNA growing point was also studied (3). About 85% of the growing points could be seen to contain a region of single-stranded DNA on one side of the growing point. An example is shown in Fig. 1. The single-stranded region averaged about 1500 bases in length. Occasionally, within the single-stranded region, a short stretch of duplex DNA was visible.

These results were interpreted as showing that the synthesis of T7 DNA in the growing point is an asymmetric process; the data suggest that one daughter DNA strand is grown continuously, while the other is elongated discontinuously, by a mechanism involving DNA fragments sealed together by ligase. The daughter strand that appears continuously elongated is expected to be the one that terminates in a free 3'-OH group (Fig. 1B). The elongation of this strand can be directly carried out by the T7 DNA polymerase holoenzyme which, like other polymerases, is able to extend 3'- but not 5'-terminated strands (4). The data suggest that, accompanying the elongation of the 3'-OH-terminated daughter strand, the parental double helix is unwound to expose a region of single-stranded template DNA on the other side of the growing point. When the single-stranded region becomes sufficiently long, a DNA fragment is initiated. Thus one occasionally observes a short area of double-stranded DNA within the single-stranded region (3). The new DNA fragment is grown in the 5'-to-3' direction by the viral DNA polymerase until it reaches the daughter strand with the 5' terminus and is sealed to it by ligase.

In this paper, we use the in vivo wild type T7 DNA replication pattern as a standard for comparison with the change that results in the structure of replicating viral DNA when an essential DNA metabolism gene is inactivated. The variation from the wild type pattern is then used to infer the mechanism of action of the mutant gene.

Specifically, we have studied the function of T7 gene 4. When we began our study, it was already known from the work of Studier and Hausmann (5) that, if the product of gene 4 was not available, an after infection with a plaque carrying an amber mutation, very little radioactive thymidine was incorporated by the infected cells. Furthermore, there was no production of progeny phage. Thus T7 gene 4 had been classified as an essential DNA metabolism gene whose precise mechanism of action was unknown.

EXPERIMENTAL DESIGN

Ideally, to study gene 4, one would like to have a normal phage replication cycle underway, to remove the functional
Fig. 1. Replicating T7 chromosome. Panel A is an electron micrograph of a T7 chromosome that is 48% replicated. In the growing point, a region of single-stranded DNA is visible. The single-stranded DNA is thinner and less rigid than duplex DNA. The single-stranded region extends outward from the apex of the fork for 9% of T7 unit length; the daughter arm then becomes duplex (arrow). Panel B shows the probable strand substructure of the molecule shown in Panel A. In this line diagram, parental strands are thicker than newly-synthesized (daughter) strands. The arrowheads mark 3'OH ends of polynucleotide chains.

Fig. 2. Growth curve of gene 4 temperature-sensitive mutant. The growth curve shows that T7 phage with a temperature-sensitive mutation in gene 4 reproduce normally at the permissive temperature (○). A shift of the culture from 30°C to 42°C during the latent period prevents phage development (●). The phage content of the culture (including intracellular phage) was assayed by withdrawing a 50-μl aliquot into a 2-ml solution containing 50 mM Tris, pH 8.0, 500 mM NaCl, 10 mM EDTA, and 400 μg/ml of lysozyme (Worthington). After 2 h at 0°C, phage were titrated by diluting through buffer (500 mM NaCl, 50 mM Tris, pH 8.0) and plating on E. coli B/r.

gene 4 product, and then to determine the immediate consequence to replicating DNA. This type of experiment can be carried out by using a virus that produces a temperature-sensitive gene 4 protein. Cells are infected with this phage at the permissive temperature so that normal growth can occur. Then midway through the life cycle, when T7 chromosomes are replicating, the culture is shifted to the nonpermissive temperature. Aliquots of the culture are harvested before and after the temperature shift. The DNA is then isolated from each aliquot and examined in the electron microscope to search for a difference in the structure of the intracellular DNA as a result of the inactivation of the gene 4 protein.

As in our previous studies of T7 DNA replication, a density isotope labeling procedure has been used to isolate actively replicating viral chromosomes: isotopically light (14N'H) T7 phage are used to infect cells growing in the presence of heavy (15N'H) medium (1). As the infecting chromosomes begin to replicate, they incorporate heavy nucleotide precursors from the medium and shift in density from light (LL) toward hybrid (HL). In the first round of replication, T7 DNA molecules will have densities ranging from light to hybrid. Therefore, they can be separated from fragments of the host chromosome (HH) and from unreplicated viral chromosomes (LL) when the intracellular DNA is centrifuged to equilibrium in CsCl.

To analyze the replicating T7 DNA molecules, before and after the inactivation of the gene 4 protein, we used the electron microscope. The material banding between the light and hybrid positions of the CsCl gradient was dialyzed into a
carbonate buffer (3) and spread according to the Davie et al. modification (6) of the basic protein monolayer technique of Kleinschmidt and Zahn (7). The use of a carbonate buffer results in an enhancement in the contrast between single-stranded and duplex DNA (8). As in our previous studies, the DNA was rendered electron-scattering simply by staining with uranyl acetate; it was then possible to examine and photograph the DNA directly in the electron microscope, using dark field illumination (1). Because shadowing was unnecessary, the time required in going from the sample of DNA in the test tube to the electron microscopic analysis was usually about 10 min (Fig. 1).

RESULTS

Infections with a Temperature-sensitive Mutant in Gene 4

Isolation of the Temperature-sensitive Mutant—A T7 phage with a temperature-sensitive mutation in gene 4 was isolated by selecting a pseudorevertant of a gene 4 amber mutation. A stock of a gene 4 amber mutant (am208, a gift of Dr. William Studier) which forms plaques with equal efficiency at both 30°C and 42°C on a permissive cell line (Escherichia coli 011') was plated at 30°C on a nonpermissive cell line (E. coli B/r). Only one phage in 10⁵ proved to be capable of forming a plaque. Fifty examples of such “revertant” phage were isolated and shown to be now capable of growth at 30°C in the nonpermissive host. When these 50 revertants were tested at 42°C, three were unable to form plaques even after prolonged incubation. In these pseudorevertants, the original nonsense codon had apparently mutated, at a frequency of 10⁻⁶ which is characteristic of a single-mutational event, so as to confer temperature sensitivity on the gene 4 protein. The three mutants were characterized in complementation tests (performed by placing drops of different mutant phage stocks

FIG. 3. Lengths of the single-stranded regions in the growing points of T7 eyes and Y-shaped rods after infection with phage carrying a temperature-sensitive mutation in gene 4. Panels A and B, cells were infected with T7 and maintained at 30°C. After 15 min, one-half of the cells were harvested (Panel A); the other half of the culture was shifted to 42°C for 10 s prior to harvesting (Panel B). Panels C and D show the results of a similar experiment in which the second harvest occurred 15 s after the temperature shift. Processing of the data was as follows: each eye form or Y-shaped rod was photographed, projected, and traced (1). The length of the single-stranded region was then calculated as a fraction of the total length of the viral chromosome.
together on a lawn of nonpermissive cells at 42°C. The temperature-sensitive mutants failed to complement the original gene 4 amber mutant (am208) but fully complemented T7 phages carrying an amber mutation in other genes that affect T7 DNA synthesis—genes 1, 2, 3, 5, and 6. The results thus indicate that the temperature-sensitive lesion is located in gene 4, and that the other DNA replication genes are normal. One of the temperature-sensitive mutants (ts208-1) was chosen for the experiments described in this paper.

As shown in Fig. 2, the gene 4 temperature-sensitive mutant was capable of normal growth at the permissive temperature of 30°C. Progeny phage began to accumulate in the infected culture about 25 to 30 min after the infection, and the final burst was about 80 plaque/cell.

Experiments to Assess the Function of T7 Gene 4—For the experiments with the temperature-sensitivity, the cells were infected and maintained at 30°C. After 15 min, an aliquot of the culture was harvested to provide T7 chromosomes representative of the normal virus life cycle. At the same time, the remainder of the culture was shifted to 42°C by the addition of an equal volume of growth medium that had been prewarmed to 54°C. From 10 to 15 s after the shift to 42°C, the second aliquot was harvested. The open circles in Fig. 2 show that the temperature shift prevented the production of progeny phage.

The infected cells from each harvest were broken open with lysozyme and detergent, and the unfractonated cell lysates were digested with pronase (1). The intracellular DNA forms were then resolved on CsCl gradients. The material that came to equilibrium at a density between light and hybrid was recovered, as representative of T7 DNA molecules in the first round of replication (1, 2). This material was then examined in the electron microscope to search for a difference in the length of the single-stranded template DNA in the growing points. Fifty-three growing points contained one single-stranded region (as in Fig. 1A). The remaining five growing points contained two single-stranded regions, both on the same side of the growing point and separated from each other by a short stretch of duplex DNA.

The mean length of the single-stranded regions at 30°C was 1500 bases (see Fig. 3, A and C).

Results at 42°C—A similar but not identical pattern was found in the T7 intracellular DNA forms recovered from the cells that had been infected for 15 min at 30°C and then shifted to 42°C. The overall topology of the replicating viral chromosomes was essentially unchanged; we observed T7 eye forms and Y-shaped rods. Furthermore, the high frequency with which single-stranded regions occurred in the growing points remained unchanged: of 62 partially replicated chromosomes analyzed, only 4 did not contain a visible single-stranded region in the growing point. Fifty-three growing points contained one single-stranded region (as in Fig. 1A). The remaining five growing points contained two single-stranded regions, both on the same side of the growing point and separated from each other by a short stretch of duplex DNA.

Despite these basic similarities, the temperature-shifted molecules were in one way strikingly different: the regions of single-stranded template DNA in the growing points were 2 to 3 times longer than those in the molecules which had been maintained at 30°C. Fig. 3, B and D, shows the results of two experiments in which cells had been shifted to 42°C for 10 and 15 s, respectively.

The mean length of the single-stranded regions at 42°C was 3000 to 4000 bases (Fig. 3, B and D). Furthermore, as can be seen in the data of Fig. 3, at 42°C the increase in the percentage of growing points with long single-stranded regions was accompanied by a decrease in the percentage of growing points with short single-stranded regions (compare Fig. 3B with 3A, and 3D with 3C).

The lengthening of the single-stranded regions in the growing points is an effect that is associated with gene 4; the effect is not found after infection with wild type phage. As shown in

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**Fig. 4.** Lengths of the single-stranded regions in the growing points of T7 eye forms and Y-shaped rods after infection with wild type phage. Panel A, one-half of the culture was infected and maintained at 30°C until harvest. Panel B, the other half of the culture was maintained at 30°C and then shifted to 42°C for 10 s prior to harvest. Processing of the data is as described in the legend to Fig. 3.
Fig. 4, cells infected with wild type phage and then shifted to 42°C yielded eye forms and Y-shaped molecules that contained single-stranded regions of nearly the same length both before and after the temperature shift. For both the DNA molecules maintained at 30°C (Fig. 4A) and those shifted for about 15 s to 42°C (Fig. 4B), 85% of the replicating chromosomes contained a region of single-stranded DNA in their growing points. The mean length of these single-stranded regions was about 1500 bases, both before and after the temperature shift.

The gene 4 temperature shift data, together with the wild type control, indicate that the immediate effect of inactivating the gene 4 protein is expressed as a lengthening of the region of single-stranded template DNA that exists on one side of the DNA growing point.

INTERPRETATION

The most straightforward interpretation of the data is that, upon the shift to 42°C, the T7 gene 4 protein is inactivated and DNA synthesis can occur on only one side of the growing point. Thus the T7 DNA polymerase continues to elongate the daughter strand with the 3'OH terminus, resulting in a lengthening of the region of single-stranded template DNA on the other side of the growing point (as shown in Fig. 1). Evidently, in the absence of functional gene 4 protein, no new DNA fragments can be developed to convert the region of single-stranded template DNA to the duplex state.

These in vitro findings are fully consistent with the in vitro studies of the T7 gene 4 protein (9–16) as will be discussed below.

DISCUSSION

All current evidence indicates that in the DNA growing point either one or both daughter DNA strands are synthesized discontinuously by a mechanism involving fragments sealed together by ligase (for a review see Refs. 3, 17, 18, and 19). A fundamental problem of current interest is to determine the exact mechanism(s) by which DNA fragments can be initiated; the known DNA polymerases are not able to initiate, but only to extend, DNA strands.

Using the electron microscope, we have studied the nature of the events that occur in the T7 DNA growing point. Normally a region of single-stranded template DNA, about 1500 bases long, is visible on one side of the growing point. We have found that, after infection with phage carrying a temperature-sensitive mutation in gene 4 and a temperature shift, there is a change in the structure of the T7 growing points. Specifically, when thermolabile gene 4 protein is inactivated by a temperature shift in the midst of a normal life cycle, the growing points in the replicating eye forms and Y-shaped molecules develop enlarged regions of single-stranded template DNA. Thus the evidence of this paper indicates that, in phage T7, the process of discontinuous synthesis by fragments requires the function of the gene 4 protein.

The exact way in which the gene 4 protein participates in the conversion of single-stranded to duplex DNA in the growing point is an area of active current investigation. Considerable progress has been made through the development of cell-free systems that are able to synthesize T7 DNA in vitro. Schierzinger and Seiffert (9), and Hinkel and Richardson (12), have developed a system in which extensive synthesis of T7 DNA occurs in vitro when template T7 DNA is incubated in the presence of several purified viral proteins.

In vitro T7 synthesis depends upon three viral coded proteins: T7 DNA polymerase holoenzyme, DNA binding protein, and gene 4 protein, which are incubated together with template T7 DNA in the presence of both DNA and RNA precursors (9–16). The ribonucleoside triphosphates are essential, for if they are omitted from the reaction mixture, synthesis falls to 10% of its maximal level. Similarly, omission of the gene 4 protein drastically reduces synthesis. Schierzinger and his colleagues (11), and Romano and Richardson (15, 16), have shown that the gene 4 protein uses the ribonucleoside triphosphates to synthesize a very short RNA strand which serves as a primer that is subsequently elongated by the T7 DNA polymerase. The sequence of the short RNA piece made by the gene 4 protein is most commonly pppACCA—OH or pppACCC—OH.

These properties of the gene 4 protein as determined by in vitro studies are in complete agreement with the in vivo gene 4 studies presented in this paper. The priming of nascent DNA fragments on one side of the growing point would not be expected to occur in the absence of functional gene 4 product. Thus, after we inactivate this protein by a temperature shift in vivo, the single-stranded regions that exist on one side of the growing point cannot be made duplex. As synthesis continues on the other side of the growing point, where a 3'OH group exists, the single-stranded region in the growing point becomes longer.

The involvement of RNA primers to initiate DNA strands is very clearly indicated for T7, but is not unique to this experimental system. RNA priming is also supported by data obtained in studies of polyoma (20), SV-40 (21), bacterial plasmids (22, 23), bacteriophage T4 (24), bacteriophage G4 (25, 26), bacteriophage dX (27), and bacteriophage M13 (28), in which this observation was first reported. Interestingly, although the E. coli priming protein dna G can use DNA nucleotide precursors as well as RNA nucleotide precursors in vitro (25, 26), this does not appear to be the case for the T7 gene 4 protein (11, 16).

In addition to its RNA primer function, the T7 gene 4 protein also contributes in vitro to the active unwinding of the parental DNA double helix in the growing point (11, 13, 14, 29), a function that requires ATP but not the remaining RNA nucleoside triphosphates. In our in vivo experiments, unwinding appears to occur in the T7 growing points under conditions in which the T7 gene 4 protein is not functioning in the initiation of DNA fragments. This observation can be explained either if the gene 4 temperature-sensitive mutation were in a region of the protein interfering with initiation but not unwinding, or if the gene 4 protein were helpful but not essential for parental strand unwinding in vitro.

The data of this paper bear directly on a long-standing controversy in DNA replication. The electron microscopic analyses of DNA growing points in bacteriophages T4, λ, and T4 all support a model in which DNA fragments are synthesized on one side of the growing point, whereas chain elongation is continuous on the other side of the growing point (Refs. 3, 30, and 31 as shown in Fig. 1). In contrast, some pulse labeling studies in E. coli and virus-infected cells have been reported in which the total pulse label was recovered in DNA fragments, leading to the suggestion that fragment formation occurs on both sides of the growing point (32–35). However, the interpretation of these pulse labeling experiments is currently in question because of the finding of Tye et al. (36) that dUTP is readily incorporated into DNA during normal chain elongation. The subsequent nicking and excision of the misincorporated dUTP transiently introduces discontinuities into the daughter DNA strands, artificially increasing the number and changing the size distribution of Okazaki fragments.

In sum, the data of this paper strengthen the earlier conclusion (3) that, in the T7 growing point, one daughter DNA strand is elongated continuously and the other discontin-
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This is expressed in the differential effect that the inactivation of the gene 4 protein exerts on one side of the growing point. Were both daughter DNA strands synthesized discontinuously with fragments as obligatory intermediates, then inactivation of gene 4 should have halted the progress of the growing point altogether, and we would not have observed a progressive lengthening of the regions of single-stranded template DNA on one daughter double helix.

REFERENCES


The data presented in this paper were obtained in 1972 (cited in Refs. 3 and 37). We have been encouraged to publish the full set of data because these experiments form an in vivo complement to the in vitro studies on the purified gene 4 protein (9-16).
Bacteriophage T7 DNA replication. An electron microscopic study of the growing point and the role of the T7 gene 4 protein in the formation of DNA fragments.

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