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Regions of Single-Stranded DNA in the Growing Points of Replicating Bacteriophage T7 Chromosomes*

(DNA replication/*E. coli*/electron microscopy)

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ABSTRACT In partially replicated T7 chromosomes, the points where parental strands are separating and new DNA is being synthesized can be seen in the electron microscope to contain regions of single-stranded template DNA. The single-stranded regions are located on only one of the two daughter arms of the replicating chromosome. Inman and Schnös observed such single-stranded regions in 50% of the growing points of replicating lambda DNA, and, as reported in this paper, we find them in about 85% of the growing points of T7 DNA. Both studies support the conclusion that DNA synthesis involves the direct elongation of one daughter strand in the growing point. Evidently, this elongation is accompanied by the unwinding of the parental double helix to expose a region of single-stranded DNA which is then converted to the duplex state by a discontinuous mechanism involving the synthesis of DNA fragments.

In its first round of replication, the T7 chromosome follows a simple pattern (1, 2). Initiation begins at an internal site about 17% from the left end of the DNA rod, with replication proceeding in both directions to give rise first to an eye-shaped intermediate —O—, then to a Y-shaped form, and finally to two unit-length molecules (see Figs. 2 and 3).

The studies that revealed this overall topology also showed single-stranded regions in the growing points of 85% of the actively-replicating chromosomes (Fig. 1). This paper presents a quantitative description of the location and size of these single-stranded regions. The single-stranded DNA, when present, always occurs on a daughter arm of the partially replicated chromosome. The length of the single-stranded region varies up to about 6000 bases, with more than 80% ranging continuously from 0 to 2500 bases. We interpret these results as indicating that DNA synthesis involves the direct elongation of one daughter strand in the growing point, and the discontinuous synthesis of the other by fragments. Inman and Schnös (3) and Delius, Howe, and Kozinski (4) reached the same conclusions for replicating lambda and T4 DNA.

Isolation of replicating T7 DNA molecules

Actively replicating parental T7 chromosomes were obtained after infection of *Escherichia coli* B/r growing in heavy medium ($^{15}N^{2}H$) with isotopically light ($^{14}N^{1}H$) T7 phage (1, 2). Under these conditions of infection, replicating parental chromosomes have a density between light (LL) and hybrid (HL). Thus, they can be separated from host DNA (HH) and unreplicated viral DNA (LL) when the intracellular DNA is centrifuged to equilibrium in a CsCl gradient (1, 2).

The T7 life cycle proceeded normally in each experiment, yielding about 100 phage per cell. At 13.5, 15.5, and 17.5 min after infection at 30°, aliquots of the culture were harvested as a source of replicating T7.

Single-stranded regions in replicating T7 DNA

The molecules recovered from the HLL region of the CsCl gradient were eye forms and Y-shaped rods, representing T7 chromosomes in their first round of replication. In a typical experiment, 121 eye forms and Y-shaped molecules were photographed. Eight molecules had to be eliminated, because upon further analysis they could not be unambiguously traced. An additional 15 molecules were eliminated because, by length measurements, either they were less than unit T7 size, or they did not contain two daughter double helixes of equal length. There remained 53 eye forms and 45 Y forms whose forks were analyzed.

Eye Forms. Fig. 2 shows the location of single-stranded regions in eye forms. Only three eye forms failed to have a visible single-stranded region (*A*). The remaining 50 eye forms contained one, two, or three single-stranded regions (*B*, *C*, and *D*).

Several generalizations are immediately apparent. In each molecule, the single-stranded region is associated with a growing point. Moreover, the single-stranded region is always located on one of the daughter portions of the chromosome. The nascent strand that extends into the apex of the fork is assumed, for enzymological reasons, to have a 3′ rather than a 5′ terminus. This has been found to be the case for the single-stranded regions in forked T4 DNA (4).

Most of the eye forms have a single-stranded region in both growing points (Fig. 2*C* and *D*). Invariably, the two single-stranded regions are located on opposite daughter double helixes. This *trans* positioning of the single-stranded regions, also observed for replicating λ DNA (3), is strong evidence against the *opposing* rolling circle model (41). In view of this result it is simplest to imagine that the initiation mechanism involves nothing more than site-specific separation of intact parental DNA strands, followed by *de novo* initiation of daughter strands upon the exposed parental DNA templates. With this initiation mechanism, the daughter and parental strands are not covalently linked, a prediction fulfilled for the eye-form-like structures of replicating SV40 DNA (42).

Fig. 2*D* shows that six eye forms had two single-stranded regions associated with one growing point, and separated from each other by a short stretch of duplex DNA.

Our previous studies have shown that the eye form supports bidirectional DNA synthesis until the leftward growing point runs off the left end of the DNA rod. The replicating molecule is then converted into a Y-shaped intermediate, which contains one growing point.

* This is paper no. III in a series, the previous paper is ref. 2

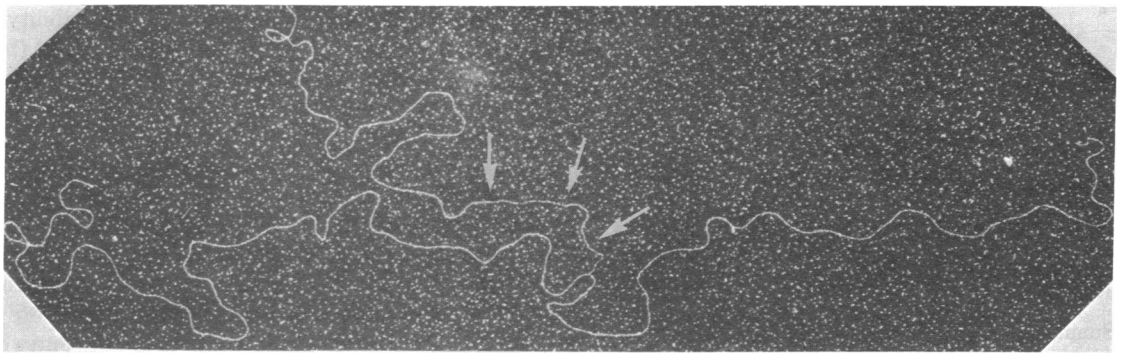

FIG. 1. The Y-shaped replicating T7 chromosome contains two regions of single-stranded template DNA (which appears thinner and less rigid than duplex DNA) associated with the growing point. They are separated from each other by a short stretch of duplex DNA *arrows* (compare this micrograph with Fig. 6*B*). In order to enhance the difference between single-stranded and double-stranded DNA, the T7 intracellular DNA forms were prepared for electron microscopy with a carbonate, rather than a Tris-EDTA, buffer. The material banding at an HLL density in CsCl was dialyzed into 0.05 M sodium carbonate (pH 9.3)-5% formamide (Matheson, Coleman, and Bell). 20 μl of this sample was mixed with 4 μl of 1 mg/ml cytochrome *c* (Calbiochem) in 1 M sodium carbonate (pH 9.3) and 25 μl of formamide. Immediately, 30 μl of this solution was spread onto a fresh hypophase of 20% formamide in 1 mM carbonate (pH 9.3). The protein–DNA film was picked up on a Parlodian film supported on a 200-mesh copper grid, stained with uranyl acetate, and examined in an electron microscope (1). The length ratio of single-stranded to duplex DNA was 0.984 for ϕX174 and 0.993 for T7 by this procedure.

Y-Shaped Molecules. An analysis of the Y-shaped intermediates for the presence of single-stranded regions in their growing points gave the results shown in Fig. 3. Only five of the 45 Y-shaped rods contained no visible single-stranded region (3*A*). The remaining 40 contained one or two single-standed regions (*B* and *C*, also see Fig. 1). These were always located in a growing point and on a daughter double helix.

Four Y forms remain. These contained one single-stranded region in the growing point, and a second at the end of the other daughter double helix (Fig. 3*D*). This type of *trans* configuration is expected if the Y were recently derived from an eye form such as that shown in Fig. 2*C*.

In all of the eye forms and Y forms analyzed, there existed 151 growing points. In total, 20 had no visible single-stranded region. 118 had one single-stranded region and 13 had two single-stranded regions, separated from each other by a short stretch of duplex DNA. These results, and the results of a similar experiment, are summarized in Fig. 4.

It is possible that those forks that contained no visible single-stranded DNA could, in fact, have had very small single-stranded regions. Regions of a few hundred bases would not have been identified with the electron microscopic techniques used.

Fig. 5 shows that the single-stranded regions ranged in length from 0 to about 15% of the length of the T7 chromosome, with more than 80% of the single-stranded regions being less than 6% unit length. Since the mature T7 chromosome contains about 40,000 base-pairs (5), 6% of unit length corresponds to about 2500 bases.

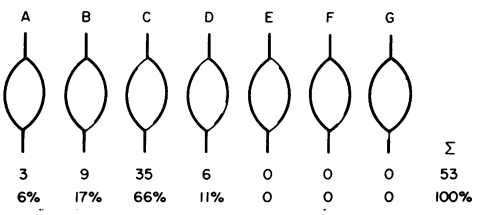

FIG. 2. The location of single-stranded regions in the growing points of T7 eye forms. *Thick lines:* double-stranded DNA; *thin lines:* single-stranded DNA.

Interpretation

We interpret our data as fitting most easily into the pattern shown in Fig. 6*B*. In the growing point, the daughter strand with the 3′OH terminus is directly elongated by T7 DNA polymerase (6), the product of gene 5. This polymerase, like all known DNA polymerases, can synthesize only by elongating the 3′OH terminus of a template-bound strand (7). As this elongation proceeds, the parental DNA in the growing point unwinds and generates a region of single-stranded template DNA on the other side of the growing point. By the time the single-stranded region has become about 2500 bases long, it has had a high probability of being converted to a double-stranded state. This conversion is imagined to involve the synthesis of a DNA fragment that grows in the 5′ to 3′ direction. We believe, but do not have sufficient data to establish, that the molecules in Figs. 1 and 4*C* are intermediates in this conversion process. The DNA fragment apparently continues to grow until it reaches, and is covalently attached to, the 5′-terminated daughter strand (Fig. 6*B*). The ways in which the fragment might be initiated will be considered below.

Discussion

Because of the antiparallel nature of the double helix, there is a chemical asymmetry in the growing point of a replicating DNA molecule (8–10). One of the two growing polynucleotide

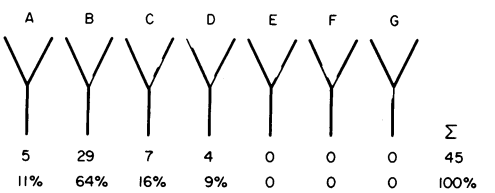

FIG. 3. The location of single-stranded regions in the growing points of Y-shaped molecules.

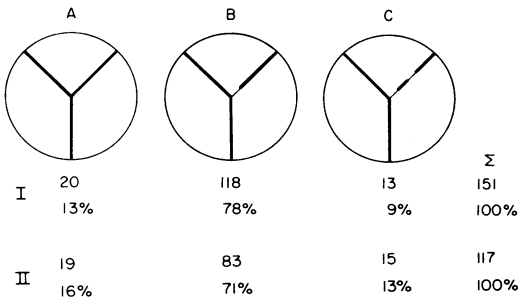

FIG. 4. Summary of the presence of single-stranded regions in the growing points of actively replicating T7 chromosomes.

strands will contain a 3′ terminus, while the other will contain a 5′ terminus. Cairns' autoradiograph of the replicating *E. coli* chromosome, and all subsequent work (11, 12), has shown that both nascent DNA strands are elongated more or less simultaneously. This poses an enzymological problem, since the known DNA polymerases are capable only of elongating strands with 3′OH termini (7).

Two general solutions to this problem have been suggested:

(*i*) There might exist DNA polymerases that are capable of directly elongating strands with 5′ termini (Fig. 6, Model *A*). However, extensive searches for enzymes of this type have proved unsuccessful (13, 14).

(*ii*) Alternatively, a net extension of the 5′ terminated strand could be accomplished by the existing class of DNA polymerases, provided that the synthesis is discontinuous rather than continuous (Fig. 6, Model *B*) (15–18). In this case, as the nascent strand with the 3′-OH terminus is directly elongated, the parental helix unwinds to expose a region of single-stranded template DNA on the other side of the growing point. At various times, a new piece of DNA starts to grow in the 5′ to 3′ direction on this single-stranded template. The growing 3′ end of the fragment eventually reaches the daughter strand with the 5′ terminus. The two polynucleotide strands are then sealed together by DNA ligase. Thus, the daughter strand with the 5′ terminus has been elongated, albeit by a discontinuous back-and-fill mechanism. Essentially, in this model, one has replaced the problem of direct elongation of a 5′-terminated strand with the problem of initiating a DNA fragment.

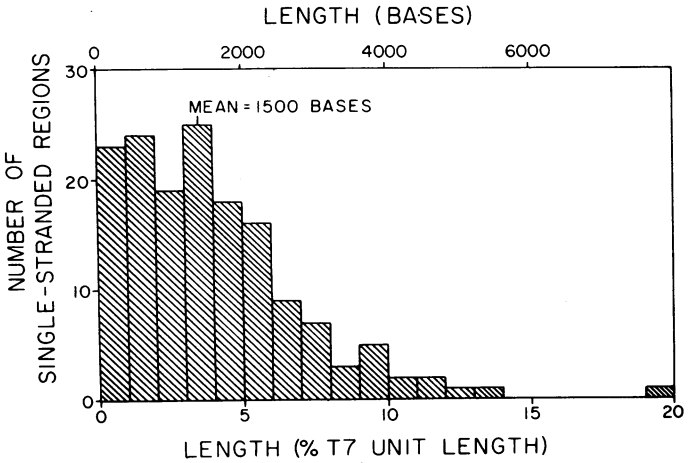

FIG. 5. Lengths of single-stranded regions in the growing points of actively replicating T7 chromosomes.

How might the fragment arise? One possibility is the knife and fork model (see Fig. 6*B*) proposed by Guild (19), Richardson (7), and Kornberg (20). In this mechanism, the polymerase extending the daughter strand with the 3′OH terminusmight migrate ("fork") onto the single-stranded region, carrying with it the end of the daughter strand it was elongating. Eventually, the nascent daughter strand would be cut behind the polymerase by an endonuclease (the "knife"). This cut regenerates a 3′-OH terminus in the apex of the fork, and leaves a growing fragment in the single-stranded region. Thus, the knife and fork mechanism allows the 3′-OH terminus of one daughter strand to be the source of primer for the synthesis of both daughter strands.

A more direct way to generate the fragment would involve *de novo* strand initiation (see Fig. 6*B*). Although none of the existing DNA polymerases is able to initiate strands *de novo* (7), macromolecules might exist inside the cell that perform a primer function for the existing DNA polymerases. Perhaps specific oligonucleotide fragments serve as primers (21). Or, the RNA polymerase could synthesize short RNA strands *in situ* that serve as primers; this is the mechanism by which M13 single-stranded circles are converted to duplex rings (22, 23). The use of an RNA primer has received further support from the findings of Sugino *et al.* (43) that nascent *E. coli* DNA frag-

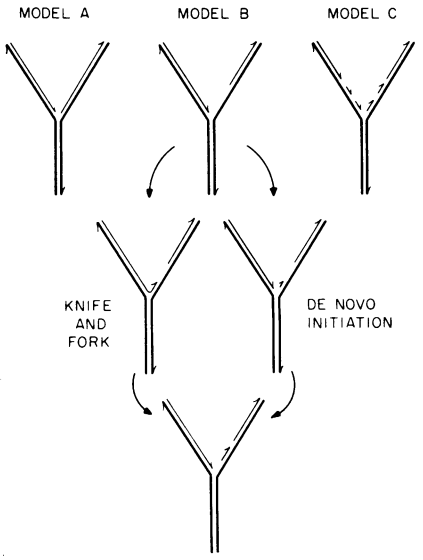

FIG. 6. Models for the fine structure of the growing point. Model *A*: Both daughter DNA strands in the growing point are elongated continuously (13, 14). No extended regions of single-stranded DNA are involved in the synthesis. Model *B*: One daughter strand, carrying the 3′OH terminus, is continuously elongated. The other daughter strand is synthesized discontinuously, by a mechanism involving fragments that are eventually sealed together by ligase (15–18). Two mechanisms are shown for the formation of the fragments: the knife and fork mechanism (7, 19, 20) (*left*) and the *de novo* initiation mechanism (*right*). There is no experimental evidence indicating whether the initiation event that creates the fragment occurs randomly or at specific sites (perhaps in intergenic regions) along the DNA. Model *C*: Both template strands are used to support DNA synthesis by fragments that are ultimately sealed together by ligase (18). Parental DNA strands are drawn thicker than daughter strands. The 3′OH ends of the DNA strands are represented by *arrowheads*.

Other models for the fine structure of the DNA growing point are possible, and several interesting ideas have been put forth. See, for instance, Haskell and Davern (39) and Morgan (40).

ments contain a short stretch of RNA at their 5′ termini. Alternatively, a protein might be found that works directly with the DNA polymerase as an initiation factor (21, 24).

Finally, if the cell is able to initiate fragments *de novo*, then the elongation of not just one, but both, daughter strands might involve fragments (Fig. 6, Model *C*) (18, 25). This mode of replication has been considered attractive because it would allow several relatively slow polymerases to synthesize simultaneously on each template strand, thus permitting the growing point to move forward relatively rapidly. However, many simultaneously growing fragments would appear to be unnecessary in view of the findings (26, 44) that the *in vivo* rate of chain growth in *E. coli* and the *in vitro* rate of polymerization by polymerase III (*dna* E) are about as fast as the forward movement of the growing point itself.

Two experimental approaches have been used to distinguish between Models 6A,B,C, one involving pulse-labeling and the other electron microscopy. The electron microscopic approach has been consistent in its support of Model *B:* the growing points in both replicating T7 and lambda chromosomes have been seen to possess regions of single-stranded template DNA. These single-stranded regions are always located on a daughter double helix, and are confined to one side of the growing point. For phage lambda, the single-stranded regions have been observed with a frequency of 50%, and for T7, with a frequency of 85%. Occasionally for T7 (10%), and more frequently for lambda (29%), the single-stranded regions are seen to contain a duplex segment within them. Such single–double–single regions in the growing point are readily interpretible in terms of Model *B*, where synthesis on one side of the fork is expected to involve the formation of a DNA fragment. Studies of replicating mitochondrial DNA also support Model B (45).

As shown in Model *B*, the fragment could arise either from *de novo* initiation or from the knife and fork mechanism. If the knife and fork mechanism is correct, then our data require that the endonuclease must act immediately after the polymerase has switched template strands, and the newly formed 3′-OH end in the apex of the fork must be immediately elongated. Thus, we see growing points with the structure shown in Fig. 4*C*, but not growing points of the type shown in Figs. 2*F* and 3*E*.

The electron microscopic observation of a physical asymmetry in the growing point (that is, a region of single-stranded template DNA on one side of the fork) argues against Models *A* and *C* as representations of the growing point. For instance, in Model *A*, where both of the daughter DNA strands are elongated continuously, the forks are not expected to contain single-stranded regions. Even if, under Model *A*, the daughter strands were to be elongated continuously, but at unequal rates, the resulting single-stranded regions in the growing points would have the property that they increase in size in proportion to the degree of replication. This is clearly not the case for the single-stranded regions in replicating T7 DNA. Finally, the appearance of growing points with two single-stranded regions separated by a short stretch of duplex DNA (as in Fig. 4*C*) is not expected on the basis of Model *A*.

Could the regions of single-stranded template DNA seen in the electron microscopic studies reflect a loss of fragments from a Model *C*-type growing point during the isolation of the DNA? We consider this very unlikely, for the loss of fragments would have to be highly asymmetric in order to generate growing points that have regions of single-stranded template DNA on just one side. To explain the electron microscope results on the basis of Model *C*, it would be necessary to place a restriction of asymmetry on the growth of the fragments: e.g., that the fragments on one side of the growing point are synthesized much more rapidly than those on the other side. This would lead to a tandem array of completed fragments on one side of the fork, giving the appearance of continuous strand elongation.

Nonetheless, based on the most straightforward interpretation of the electron microscopic data, Model *B* appears to represent the fine structure of the DNA growing point.

Analysis of growing points by means of pulse-labeling studies has also supported replication models involving DNA fragments (18, 27–31).† However, some pulse-labeling studies support the discontinuous synthesis of one daughter DNA strand (Model *B*), while other studies support the discontinuous synthesis of both daughter DNA strands (Model *C*). The initial pulse-labeling studies of Okazaki, Okazaki, Sakabe, Sugimoto, Kainuma, Sugino, and Iwatsuki (18), and several subsequent studies (27–29), indicated that both daughter strands are synthesized in fragments: most, or all, of the newly-synthesized DNA was present in replicating intermediates in fragments of about 1000–2000 bases in length. With time, the fragments were joined to larger, preexisting DNA strands. These data generated the model shown in Fig. 6*C* and are its primary support. However, the claim that 100% of the pulse-label is initially incorporated into DNA fragments is, except in one instance to be discussed below, based on pulses of very brief duration: in these, so few counts are incorporated into fragments that a continuum of more rapidly sedimenting strands would be very difficult to detect above background. There is only one instance, in which the *E. coli* strain lacking DNA polymerase I was pulse-labeled, where solid evidence has been obtained that 100% of the pulse-label is initially incorporated into DNA fragments. In the Pol A mutant (34), Kuempel and Viomett (35), and later Okazaki, Arisawa, and Sugino (31), found that the joining together of the fragments is considerably slowed, allowing them to see that, under these conditions, both daughter strands of the *E. coli* chromosome first appear as fragments. In addition, the results obtained with the Pol I$^-$ strain indicate that DNA polymerase I plays a role in the finishing of the fragments before their sealing by ligase.

Although the results obtained with the Pol A mutant clearly support Model *C*, there are several pulse-labeling studies that argue directly in favor of Model *B*. One such study is the analysis of replication in *E. coli* strain 15T$^-$ by Iyer and Lark (30). These investigators found that after a brief period of pulse-labeling, only one half of the nascent pulse-label was present in DNA fragments. The other half of the label was recovered at the 3′-OH ends of much-longer DNA strands. These are the results expected on the basis of Model *B*. Moreover, recent ex-

† See also the experiments of Werner (32) who has shown that pulse-labeling data vary significantly, depending on the exact experimental conditions (for instance, whether thymine or thymidine is used to pulse-label, how the pulse is stopped, etc.). It is also important to remember that, although fragments are generally observed by sedimentation of replicating DNA through alkaline sucrose, up to 30% of the pulse-label can appear as fragments even in neutral sucrose sedimentation. The implication of this result (18, 33) is that replicating chromosomes with intact growing points may be very difficult or even impossible to isolate.

periments of Okazaki and his coworkers with *Bacillus subtilis*, and phages P2 and PM2 (personal communication) have supported Model *B*, where only one daughter DNA strand is synthesized discontinuously.

To summarize, virtually all current experimental evidence supports the synthesis of either one or both daughter DNA strands by a mechanism involving fragments. The electron microscopic approach has been consistent in its support of Model *B*, while pulse-labeling studies have generated support for both Models *B* and *C*. Further experiments or, more likely, an independent approach such as the *in vitro* study of the proteins involved in the formation of the fragments will be required to settle the issue.

Very brief pulse-labeling experiments have not yet been performed to provide evidence as to whether T7 DNA synthesis involves fragments. After a rather long period of labeling, Barzilai and Thomas have detected another intracellular DNA form, possibly relevant to the structure of the growing point. They found that after labeling T7-infected cells for 30 sec at 37°, about 40% of the label was recovered in T7 DNA strands that, after denaturation, could rapidly self-anneal (36). Such self-annealing DNA is predicted by the knife and fork model, so long as the endonuclease does not cut too soon after the polymerase has switched template strands. Our evidence, however, argues that the T7 self-annealing DNA does not come from the growing point: 85% of the observed growing points contain a region of single-stranded DNA extending into the apex of the fork (Fig. 4); in these growing points, the two daughter DNA strands can be seen not to be physically connected. In addition, the type of growing point expected to have self-annealing DNA (Fig. 6*B*, knife and fork intermediate) was not seen in our study (see Figs. 2*F* and 3*E*). Perhaps the self-annealing DNA found in T7 by Barzilai and Thomas arises from enzymatic reactions not involved in replication. For instance, Weiss has found an activity in the extracts of T7- and T4-infected cells that crosslinks DNA (37). Further studies will be required to clarify the relationship between the self-annealing T7 DNA and the fine structure of the DNA growing point.

A fundamental problem remains. It is to define the mechanism of initiation for the DNA fragments that convert regions of single-stranded DNA to duplex DNA. By whatever mechanism, the process in T7 appears to involve the product of gene 4, which is one of the viral genes essential for DNA metabolism (38). We have found that when cells are infected with T7 containing a temperature-sensitive mutant in gene 4, and transferred from the permissive to the nonpermissive temperature, the single-stranded regions in the growing point become longer. Since this effect is not seen in infections with wild-type phage, it would appear that the gene-4 product plays a direct role in the events on the side of the T7 growing point that involve synthesis by fragments. This phenomenon will be discussed in a succeeding paper.

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