# *MECHANISM OF DNA CHAIN GROWTH, II. ACCUMULATION OF NEWLY SYNTHESIZED SHORT CHAINS IN E. COLI INFECTED WITH LIGASE-DEFECTIVE T4 PHAGES**

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The most recently replicated portion of the bacterial and T4 phage chromosome, selectively labeled by an extremely short radioactive pulse, can be isolated, after denaturation, as short DNA chains with an average sedimentation coefficient of 7–11*S*.[1–3] This supports an hypothesis of discontinuous DNA chain growth, by which short stretches of DNA are synthesized at the replicating point and subsequently connected to the older portion of the growing strands by formation of phosphodiester linkages.[2–6] The joining of these short chains is assumed to be carried out by polynucleotide ligase, an enzyme discovered recently in normal and T4 phage-infected *E. coli*.[7–12] If DNA replicates *in vivo* by such a mechanism, the newly synthesized short DNA chains would accumulate in the cell under conditions where the function of ligase is temporarily impaired. In the present work, this prediction was tested and verified by experiments with T4 phage mutants, which produce thermosensitive polynucleotide ligase.[13]

*Materials and Methods.—Bacteria and bacteriophages:* *Escherichia coli* B/5, bacteriophage T4 *ts* A80, and T4 *ts* B20 were generous gifts of Dr. R. S. Edgar. T4 *ts* A80 and T4 *ts* B20 are gene 30 mutants, which induce temperature-sensitive polynucleotide ligase.[13] T4D (wild-type) was supplied by Dr. J. Tomizawa.

*Infection of bacteriophages:* *E. coli* B/5 was grown in a glucose salt medium (Medium A described previously[3]) at 37° to 5 × $10^8$ cells/ml, and incubated at 20° for 20 min. DL-tryptophan (40 μg/ml) and bacteriophages (m.o.i. = 10) were then added to the culture and the incubation was continued at 20° with shaking.

*Temperature shift:* When the volume of a culture was 10 ml or less, temperature shift from 20° to 30–44° was attained simply by pouring the culture into a flask being shaken in a water bath of the new temperature. For larger volumes, the culture was warmed to a new temperature by being shaken in a 50° water bath before being placed in the water bath of the new temperature. By these methods temperature shift-up was completed in 1 min. For temperature shift-down, the culture was chilled in ice water to the desired temperature. It took about 10 sec to change the temperature of a 30-ml culture from 43 to 30°.

*Other methods:* Pulse-labeling with $H^3$-thymidine, DNA extraction, and alkaline sucrose gradient sedimentation were carried out as described previously.[3]

*Results.—Pulse-labeling at 43–44°:* Our previous study (ref. 3, Fig. 6) showed that $H^3$-thymidine that had been incorporated into T4 wild-type infected cells during the period of active phage DNA synthesis (70 min after infection at 20°) first appeared in short DNA chains having an average sedimentation rate of 8–9*S* (in alkali) before transition to large chains with sedimentation coefficients of 30–60*S*. In order to see if temporal inhibition of polynucleotide ligase causes accumulation of the nascent short DNA chains, *E. coli* B/5 infected with T4 *ts* A80 or *ts* B20 (gene 30) was pulse-labeled with $H^3$-thymidine 65–70 minutes

after infection at 20°; at this time the temperature was elevated to 43–44°. Control experiments were performed with cells infected with T4D (wild-type).

As seen from Figure 1, T4 *ts* A80- or T4 *ts* B20-infected cells incorporated $H^3$-thymidine into DNA at 43° (or 44°) at a rate comparable to the rate found with T4D-infected cells during a short period (about 3 min) following the temperature shift. The rate of DNA synthesis as judged by such pulse experiments decreased quickly thereafter in T4 *ts* A80- or T4 *ts* B20-infected cells but not in T4D-infected cells.

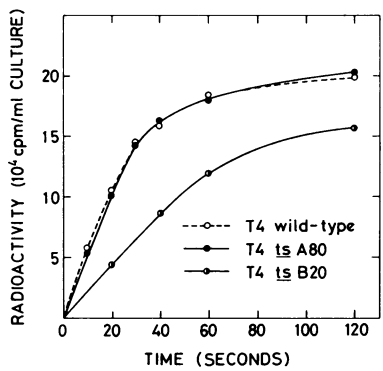


FIG. 1.—Incorporation of $H^3$-thymidine into DNA by gene 30 *ts* mutant- and wild-type T4-infected cells at 2 min after temperature shift from 20 to 43°. *E. coli* B/5 was infected with the indicated strains of T4 and incubated at 20° for 70 min. The temperature was then raised to 43°, and 2 min later $H^3$-thymidine (15 mc/μmole) was added to $10^{-7}$ *M*. Samples were taken at the indicated times after the addition of $H^3$-thymidine, and cold trichloroacetic acid-insoluble radioactivity was measured.

In the experiments presented in Figure 2, infected cells were exposed to $H^3$-thymidine for various periods beginning at two minutes after the temperature shift. DNA was extracted with 0.1 *N* NaOH–10 mM ethylenediaminetetraacetate (EDTA) and analyzed by alkaline sucrose gradient sedimentation. With T4D-infected cells, a small portion of the radioactivity was found, after a 20-second pulse, in the short chains with an average sedimentation coefficient of about 8*S*, most of the radioactivity incorporated being already found in the larger DNA chains. After 40- or 60-second labeling, virtually all the radioactivity was found in the large DNA chains with a sedimentation coefficient of more than 30*S*. This confirms the expectation from the previous experiment (ref. 3, Fig. 6), in which T4 wild-type-infected cells were pulse-labeled at 20°.

In contrast to these results with wild-type T4, when cells infected with either T4 *ts* A80 or T4 *ts* B20 were pulse-labeled with $H^3$-thymidine after temperature shift from 20° to 43–44°, virtually all the radioactivity incorporated into DNA was found after a 10- or 20-second pulse in the short DNA chains with an average sedimentation rate of about 9*S*. With an increase of pulse time (to 30, 40, or 60 sec) the label in the 5–20*S* region continued to increase and little radioactivity appeared in the material sedimenting at rates more than 35*S*. Thus, it appears that under these conditions the short DNA chains are produced, but their joining is inhibited almost completely. Occurrence of some joining reaction is suggested by a slow increase of the sedimentation coefficient of the labeled DNA. This may be due to the residual activity of phage ligase or to the action of host ligase, which is not thermosensitive but shows a much lower level of activity than the phage-induced enzyme in T4-infected cells.[10, 11]

The accumulation of the newly synthesized short DNA chains in the mutant-

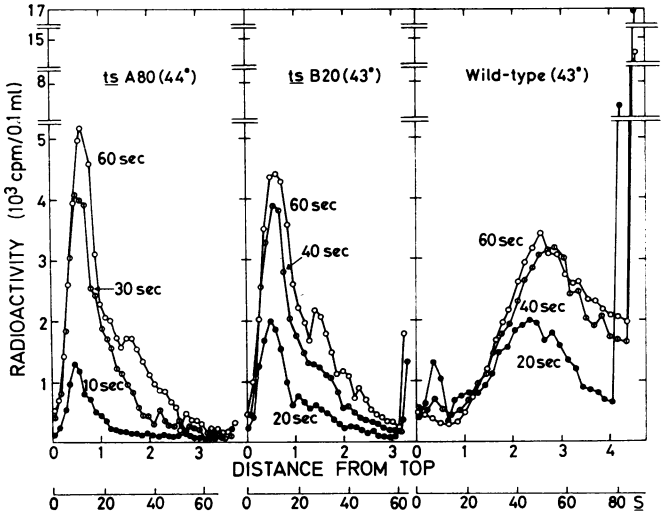


FIG. 2.—Alkaline sucrose gradient sedimentation of DNA from gene 30 *ts* mutant- and wild-type T4-infected cells pulse-labeled at high temperature. Cells were infected with the indicated strain of T4 and incubated at 20° for 70 min before the temperature was elevated to 43 or 44°. The cells were pulse-labeled with $10^{-7}$ *M* $H^3$-thymidine (15 mc/$\mu$mole) for the indicated times, beginning at 2 min after the temperature shift. DNA was extracted by NaOH-EDTA treatment[3] and sedimented through 5–20% linear sucrose gradients containing 0.1 *N* NaOH, 0.9 *M* NaCl, and 1 mM EDTA in a Spinco SW25.3 rotor for 13–16 hr at 22,500 rpm and 4°. The distance from the top is relative to that of infective δA DNA (19*S*)[3] used as an internal reference.

infected cells could also be demonstrated by pulse-labeling at one minute after the temperature shift-up (Fig. 3).

*Pulse-labeling at lower temperatures:* Accumulation of the radioactive short DNA chains as observed at 43–44° was not found when T4 *ts* A80- or T4 *ts*-B20-infected cells were pulse-labeled at 20 or 30° (Fig. 4). At these temperatures, the results obtained with the mutant and wild-type phages were indistinguishable. If the T4 *ts* A80-infected cells were pulse-labeled with $H^3$-thymidine after elevation of temperature to 40°, the result was intermediate between that obtained at 20 or 30° and that at 44°. While the radioactivity continued to increase in the 5–15*S* region for at least 60 seconds, an appreciable amount of the label also appeared in the large molecular fraction.

*Pulse-labeling at 43° followed by incubation at 20 or 30°:* In order to test the reversibility of the accumulation, T4 *ts* B20-infected cells were pulse-labeled (for 60–90 sec) at 43° as in the previous experiments (shown in Fig. 2) and then incubated at 20 or 30°. As shown in Figure 5, the sedimentation rate of the labeled DNA chains increased gradually during the incubation at low temperature, and the peak in the 10*S* region disappeared. Although a slow increase of the sedimentation rate of the accumulated short DNA chains was also found at 43°, as noted before, the rate was much faster at 30° than at 43°. Thus it is evident that the effect of high temperature was reversed by subsequent cooling, and the accumulated short chains were joined. The reversal may be due to

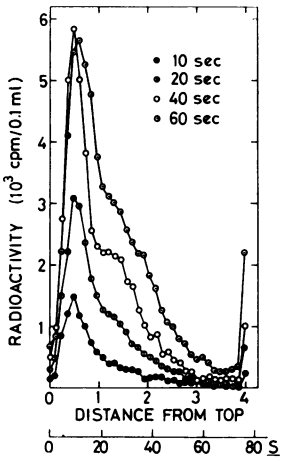


FIG. 3.—Alkaline sucrose gradient sedimentation of radioactive DNA from T4 *ts* B20-infected cells pulse-labeled at 1 min after temperature shift-up. The experiment was carried out as in Fig. 2, except that the pulse-labeling was begun at 1 min after the temperature shift from 20 to 43°.

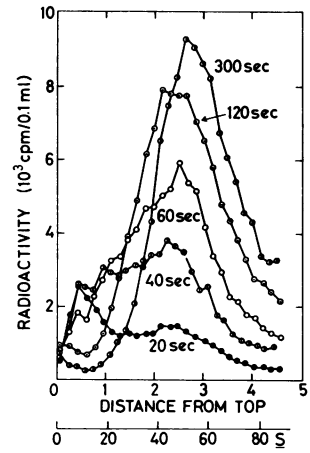


FIG. 4.—Alkaline sucrose gradient sedimentation of radioactive DNA from T4 *ts* B20-infected cells pulse-labeled at low temperature (30°). The experiment was carried out as in Fig. 2, except that the temperature shift was from 20 to 30°.

relief from the inhibition of ligase or to the synthesis of active ligase after the shift-down of temperature. With T4 *ts* A80-infected cells, such reversal of the accumulation of the nascent short chains was not observed.

*Pulse-labeling at 20° followed by incubation at 43°:* All the above results are in accordance with the idea that DNA replicates *in vivo* by a discontinuous mechanism, which involves the synthesis and joining of short stretches of both strands, and that polynucleotide ligase is involved in the joining step. However, it is still possible that the nascent short chains are degradation products formed *in vivo*, under the particular conditions of ligase inhibition, from the DNA chains which are synthesized by a *continuous* mechanism. To explore this possibility, T4 *ts* A80 or T4 *ts* B20-infected cells were pulse-labeled with $H^3$-thymidine at 20° for five minutes and then incubated at 43°. It is to be noted that five minutes at 20° may correspond to 30–60 seconds at 43°, and most of $H^3$-thymidine added to the medium is incorporated into the large DNA chains during the five-minute labeling at 20°. As shown in Figure 6, the average sedimentation coefficient may reflect breakage and joining of phage DNA normally during the three-minute period. This small but significant decrease of the sedimentation coefficient may reflect breakage and joining of phage DNA normally taking place in the cell. This change in the molecular size of the labeled long chains, however, is too small to account for the accumulation of the large amount of the pulse-labeled short chains with a sedimentation coefficient of about 10*S*. Furthermore, virtually no decrease of the sedimentation coefficient of the pre-labeled DNA is found during the first two-minute period following the temperature shift-up (Fig. 6), while the accumulation of the short DNA chains can be

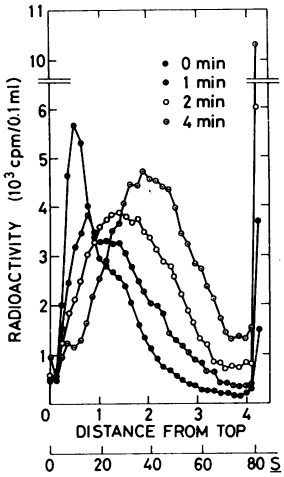


FIG. 5.—Effect of incubation at low temperature subsequent to pulse-labeling at high temperature on the sedimentation pattern of radioactive DNA from T4 *ts* B20-infected cells. Cells were pulse-labeled for 60 sec with $H^3$-thymidine at 43° as in Fig. 2 and then incubated at 30° for the indicated time. DNA extraction and alkaline sucrose gradient sedimentation were carried out as in Fig. 2.

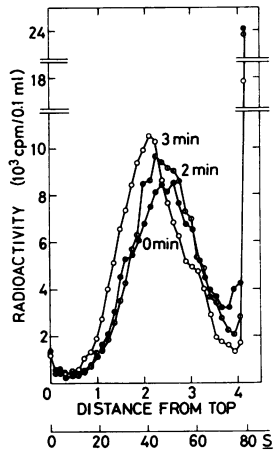


FIG. 6.—Effect of incubation at high temperature subsequent to pulse-labeling at low temperature on the sedimentation pattern of radioactive DNA from T4 *ts* A80-infected cells. Cells were pulse-labeled with $H^3$-thymidine at 20° for 5 min from 65 to 70 min after infection and then incubated at 43° for the indicated times. DNA extraction and alkaline sucrose gradient sedimentation were carried out as in Fig. 2.

demonstrated in this period (Fig. 3). It is therefore unlikely that the nascent short DNA chains are degradation products, unless there is selective breakage *in vivo* taking place in the restricted region near the growing end. This conclusion is further supported by the fact that the nascent short chains with similar properties are found normally in a smaller amount.[1-3]

*Discussion.*—Our hypothesis of discontinuous DNA replication[2, 3] assumes that short DNA chains are synthesized at the replicating point and subsequently connected to the growing strands by formation of phosphodiester linkages.

The hypothesis predicts the following: (1) The most recently replicated portion of the daughter strands can be isolated after denaturation as short DNA chains distinct from the large DNA molecules derived from the other portion of the chromosome. (2) Selective inhibition of the enzyme for the formation of phosphodiester linkages between DNA chains will result in the marked accumulation of the nascent short chains.

The first prediction was supported by the previous observations that the portion of the bacterial and T4 phage chromosome selectively labeled by an extremely short radioactive pulse can be obtained in alkali as small DNA molecules with a sedimentation coefficient of about 10*S*.[1-3] This finding, however, could be interpreted by an alternative possibility that artificial breaks may be introduced selectively in the newly replicated portion during DNA extraction.

The present work demonstrates, in accordance with the second prediction

of the hypothesis, that cells infected with T4 phage mutants of gene 30 (*ts* A80, *ts* B20), whose polynucleotide ligase is thermosensitive,[13] accumulate a large amount of the newly synthesized short DNA chains upon exposure to high temperature. Such marked accumulation of the nascent short DNA chains at high temperature is not observed with cells infected with wild-type T4. The marked accumulation is also not found with the mutant phages at low temperature. The presence of a small amount (normal steady-state amount) of nascent short chains, however, is always shown by short pulse-labeling. The short chains accumulated at high temperature in the cells infected with one of the mutants (*ts* B20) could be transformed to large molecules upon subsequent incubation of the cells at low temperature. On the other hand, the sedimentation coefficient of the radioactive large DNA chains synthesized at low temperature decreased only a little upon subsequent incubation at high temperature. Thus, the results presented here lend further support to the hypothesis of discontinuous DNA chain growth and provide evidence that the joining of the newly synthesized short chains is carried out in the cell by polynucleotide ligase. The possibility that these short DNA chains are an artifact produced during extraction is accordingly further diminished. An alternative possibility which still remains is that the DNA chains are synthesized by a continuous mechanism but selective nicks are introduced *in vivo* in the newly replicated portion, and that these nicks are subsequently sealed by the ligase reaction. This possibility would also be eliminated if one could definitively prove that the short DNA chains are synthesized only in the 5′ to 3′ direction as assumed in the original hypothesis.[2, 3] Experiments[14, 15] in which specific exonucleases degraded these chains from the 5′ and from the 3′ ends support the hypothesis.

A characteristic of the nascent DNA chains other than their small size is that an appreciable portion is isolated in the single-stranded form by mild procedures.[2, 3, 16, 17] Our recent study[17] has extended this finding, originally made with the bacterial chromosome, to the replicative T4 DNA. Moreover, a considerable portion of the short chains accumulated at high temperature in cells infected with the gene 30 mutants are found to be single-stranded after gentle extraction. Details of these observations will be presented elsewhere.[17, 18]

*Summary.*—When cells infected with temperature-sensitive T4 mutants of gene 30 (ligase) were warmed to 43–44° during the period of active phage DNA synthesis, a large amount of the newly synthesized short DNA chains, with properties similar to those found normally in smaller amounts, accumulates. This supports the hypothesis that DNA chains grow *in vivo* by a discontinuous mechanism involving synthesis and joining of the short chains and that the latter reaction is carried out by polynucleotide ligase.

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