Genetic and Physiological Studies of Bacteriophage T5

III. Patterns of Deoxyribonucleic Acid Synthesis Induced by Mutants of T5 and the Identification of Genes Influencing the Appearance of Phage-Induced Dihydrofolate Reductase and Deoxyribonuclease¹

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Patterns of deoxyribonucleic acid (DNA) metabolism in nonpermissive cells infected with amber mutants representing 29 genes of T5 are reported. A group of 7 contiguous genes are essential for the synthesis of phage DNA, whereas 20 other genes, when defective, permit varying degrees of phage DNA synthesis. Two further genes are essential for complete transfer of phage DNA to host cells, and therefore indirectly do not permit the synthesis of phage DNA. The structural genes for an early T5 deoxyribonuclease and for T5 DNA polymerase, as well as a gene that affects the synthesis of dihydrofolate reductase, have been identified in the genetic map of T5.

Phage-bacterial systems are unusually favorable systems in which to study intracellular mechanisms that regulate growth and development because phage mutants, which can be so readily isolated, facilitate the identification of the functional product of each phage gene and thereby permit correlations to be made between a particular gene product and a particular step in the development of the phage. This approach has been highly successful with phages T4, λ , and T7, each of which has its own peculiarities of regulation. We are using this approach to elucidate the regulatory mechanisms of the growth and development of coliphage T5, and we expect that this phage will display some regulatory mechanisms not manifested by other phages because of its certain unique features. For example, the deoxyribonucleic acid (DNA) of T5 is transferred to host cells in a stepwise manner. Initially an 8% segment is transferred (17, 23), and only after this 8% segment directs the synthesis of certain pre-early proteins (18), can the remaining 92% of the DNA be transferred. Also unique is the presence of three inter-

rupted phosphodiester bonds (i.e., "nicks") at specific locations along the DNA of T5 (1, 2, 14). In addition, T5, unlike T-even phages, does not require replication of its DNA for the normal cessation of early protein synthesis, or normal onset of late protein synthesis (13, 31). Pre-early, early, and late proteins are sequentially induced by T5 as they are by other phages (22). Finally, the genetic map of T5 does not appear to be a single linkage group even though each mature phage particle contains but a single molecule of DNA. Rather, the map consists, formally, of four apparently independent linkage groups (12).

In this paper, we report certain physiological and biochemical properties of the amber mutants which we described previously in relation to the generation of an extended genetic map of T5 (12). We examined first the pattern of DNA metabolism induced by mutants defective in each of the genes of T5 in which we have mutations. These patterns indicate that the products of at least six different genes are essential for the process of DNA replication itself, whereas the products of several other genes affect the rate and extent of DNA replication. Secondly, we report the location in our map, of the structural gene for early T5 deoxyribonuclease (29, 33), of a gene that controls the synthesis of T5-specific dihydrofolate reductase (25, 27), and of the structural gene for T5 DNA polymerase. This

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last-mentioned gene has been previously mapped (5), and the enzyme for which it codes has been characterized (28, 32).

MATERIALS AND METHODS

Bacteria, phage, and media. *Escherichia coli* F (16) is a fast-adsorbing strain for bacteriophage T5, but is a nonpermissive (su^-) host for amber mutants. *E. coli* F su^+ (henceforth termed su^+) is a permissive host for amber mutants (12). The amber mutants of T5 used in this study were those reported previously (12). HA70R4, a temperature-sensitive mutant that carries a mutation in the structural gene of the "early" deoxyribonuclease induced by T5, was kindly supplied to us from C. C. Richardson's laboratory by Gerald D. Frenkel (7). Supplemented M9 medium and M9 adsorption buffer have been defined, as has the preparation of nutrient broth and agar (13).

Mapping of tsHA70R4. Genetic mapping of HA70R4 was accomplished by infecting su^+ cells simultaneously with the temperature-sensitive mutant, HA70R4, and with a selected amber mutant. The su^+ cells were grown to 10^8 per ml, concentrated to 10^{10} per ml by sedimentation and suspension in nutrient broth containing 10^{-3} M $CaCl_2$, and infected with the temperature-sensitive and amber mutants at an input ratio of 6 plaque-forming-units (PFU) of each mutant per cell. After 10 min of adsorption at 0 C, followed by 2 min at 30 C, the infected cells were diluted to 10^6 per ml and incubated for 90 min at 30 C. Chloroform was added to ensure complete lysis of all cells, and the progeny phage were preadsorbed and plated on su^+ cells at 30 C and on su^- cells at 45 C. Per cent recombination was calculated as 200 times the progeny titer on su^- at 45 C divided by the progeny titer on su^+ at 30 C.

Preparation of extracts from infected cells. Bacteria were grown in supplemented M9 medium to a concentration of 2×10^8 per ml. After centrifugation and resuspension to 5×10^8 per ml of M9 adsorption buffer, the cells were incubated for 45 min at 37 C with gentle aeration to exhaust residual nutrients. The cells were infected with phage at an input ratio of about 5 PFU per cell, and, after incubation for 10 min at 37 C for adsorption, samples were removed for the determination of uninfected bacteria, unadsorbed phage, and infected bacteria. At this stage, the infected bacteria were at about 5×10^8 /ml, and uninfected bacteria and unadsorbed phage were both less than 1% of the input values. Phage growth was initiated by diluting the culture 10-fold into supplemented M9 medium that had been equilibrated at 37 C, and commencing aeration. Samples of 20-ml each were withdrawn at various times and added to centrifuge tubes containing 10 g of ice. The samples were centrifuged at $12,000 \times g$ for 10 min at 4 C, and the resulting pellet was resuspended in 2.0 ml of 0.05 M glycylglycine buffer (pH 7.0) containing 10^{-3} M 2-mercaptoethanol. The cells were disrupted by ultrasonic treatment, and the cellular debris, together with any unbroken cells, were separated from the solubilized cellular proteins by sedimentation at $12,000 \times g$ for 10 min to yield a translucent supernatant fluid. This supernatant fluid, referred to here-

after as the crude extract, could be stored for several months without appreciable loss of the enzymic activities with which we are concerned in this report.

Determination of enzyme activities. The activity of dihydrofolate reductase was determined by the method of Warner and Lewis (35). Each determination was carried out on a solution containing 20 μ moles of potassium phosphate buffer at pH 6.9, 8 μ moles of 2-mercaptoethanol, 0.075 μ moles of dihydrofolate, 0.06 μ moles of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), and up to 3 units of dihydrofolate reductase in a final volume of 1.0 ml. One to three such reaction solutions were equilibrated at 30 C in the cuvette chamber of a Gilford recording spectrophotometer before addition of the crude extract which contained the enzyme. After addition of the enzyme, the optical density (OD) at 340 nm was recorded at 12-sec intervals over a period of 10 to 20 min. A blank solution, which contained all constituents except dihydrofolate, was simultaneously monitored, and the rate of the reaction was corrected for this small blank reaction. One unit of dihydrofolate reductase is defined as the amount of enzyme that catalyzes a decrease of 0.01 in OD at 340 nm in 1 min at 30 C under the above conditions.

The activity of "early" T5 deoxyribonuclease was determined by the spectrophotometric method of Paul and Lehman (29). Each determination was performed on a solution containing 20 μ moles of sodium glycinate buffer at pH 9.3, 4 μ moles of $MgCl_2$, 4 μ moles of sodium ethylenediaminetetraacetate, approximately 13 μ g of heat-denatured salmon sperm DNA, and up to 6 units of deoxyribonuclease in a final volume of 0.5 ml. After incubation for 30 min at 37 C, the reaction was stopped by adding 0.5 ml of 0.5 N $HClO_4$, and then the mixture was brought to 0 C. The material precipitated by the acid was removed by centrifugation, and the OD of the clear supernatant fluid was measured at 260 nm. The OD values of blanks, which contained all constituents but to which acid was added immediately, were subtracted from those of the corresponding incubated samples. One unit of deoxyribonuclease is defined as the amount of enzyme that catalyzes the release of 10 nmoles of material that absorbs at 260 nm into the acid-soluble fraction in 30 min under the above conditions.

The activity of thymidylate synthetase was determined by method 8 of Friedkin (9) which is based on the increase in OD at 340 nm when 5, 10-methylene-tetrahydrofolic acid is oxidized to dihydrofolic acid.

DNA analyses. Bacteria were grown, concentrated, and infected as described above for the preparation of extracts from infected cells. Before dilution into supplemented M9 medium to initiate phage growth, a 0.45-ml sample of the phage-bacterial complexes at 5×10^8 per ml was removed and added to a cold mixture of 4.05 ml of supplemented M9 medium plus 0.5 ml of 50% trichloroacetic acid. This sample yielded the "zero-time" value. The remainder of the infected cells was diluted exactly 10-fold into supplemented M9 medium at 37 C, and aeration was begun. At various times after this dilution, samples of 4.5 ml were withdrawn from the culture and rapidly added to a tube containing 0.5 ml of 50% trichloro-

acetic acid in an ice-water bath. The total amount of acid-precipitable DNA in each sample was determined by the Dische diphenylamine reaction as modified by Burton (3). The samples in 5% trichloroacetic acid were incubated overnight at about 5 C to hydrolyze most of the ribonucleic acid (10) before proceeding with the Dische reaction.

Incorporation of ^{14}C -thymidine into DNA. Bacteria were grown, concentrated, and infected as described above for the preparation of extracts from infected cells. For the determination of the cumulative incorporation of ^{14}C -thymidine into DNA, phage growth was initiated by diluting the phage-bacterial complexes 10-fold into supplemented M9 medium containing 250 μg of deoxyadenosine per ml, 5 μg of uracil per ml, and 2 μg of ^{14}C -thymidine per ml (specific activity = 0.025 $\mu\text{Ci}/\mu\text{g}$). Aeration was begun immediately after dilution, and 1.0-ml samples were taken at various times and added to 1.0 ml of ice-cold 10% trichloroacetic acid. After at least 30 min at 0 C, the suspension was filtered through a membrane filter (Millipore Corp.), and the acid-insoluble material retained by the filter was washed with 5% trichloroacetic acid. The filters with the adhering acid-insoluble material were dried, and the radioactivity on each filter was determined by placing each filter in a vial containing toluene-based scintillation fluid, and counting in a scintillation counter.

For the determination of the incorporation of ^{14}C -thymidine into DNA for short time periods, bacteria were grown, concentrated, and infected as described above for the preparation of extracts from infected cells. Phage growth was initiated by a 10-fold dilution of the phage-bacterial complexes into nonradioactive supplemented M9 medium at 37 C. At various times after this dilution, 1.0 ml of the suspension of infected cells was rapidly mixed with 0.1 ml of a solution of supplemented M9 medium that contained 100 μg of deoxyadenosine, 2 μg of uracil, and 0.2 μCi of ^{14}C -thymidine (specific activity = 49.7 $\mu\text{Ci}/\text{mole}$), and incubated for exactly 2 min at 37 C. The incorporation of ^{14}C -thymidine into DNA was stopped at the end of the 2-min incubation period by adding 1.0 ml of ice-cold 10% trichloroacetic acid to the suspension. The radioactivity in the acid-insoluble material was determined in the same manner as for the cumulative incorporation of ^{14}C -thymidine into DNA.

Reagents. Dihydrofolic acid was prepared from folic acid by the method of Futterman (11), and tetrahydrofolic acid was prepared by the PtO_2 -catalyzed reduction of folic acid by molecular hydrogen by the method of Kisluk (15). Dische reagent was prepared by the method of Burton (3) with diphenylamine that had been purified by steam distillation. NADPH was purchased from Sigma Chemical Co., and ^{14}C -thymidine was from Schwarz BioResearch, Inc.

RESULTS

Patterns of DNA metabolism. The pattern of DNA metabolism in cells infected with wild-type T5 phage has been reported by several previous workers (4, 21, 30; L. A. Manson, *Bacteriol.*

Proc. 57:37, 1957) and is shown in Fig. 1 by the curve designated DN (normal DNA synthesis). The initial decrease in acid-insoluble DNA represents the degradation of host DNA, whereas the increase in acid-insoluble DNA starting at about 10 min is due to the synthesis of phage DNA. Also included in Fig. 1 are the patterns of DNA metabolism induced in nonpermissive cells by mutants defective in genes A1 and A2, which are located in the first-step-transfer (FST) segment of the DNA of T5 (12). The pattern induced in the case of a mutant defective in gene A1 is designated DDO (no host DNA degradation nor phage DNA synthesis), whereas that induced by a mutant defective in gene A2 is designated DO (no phage DNA synthesis). Similar patterns induced by mutants defective in FST genes have been previously reported by Lanni (19). These patterns substantiate our previous conclusion that the A segment (Fig. 2) of our genetic map (12) corresponds to the FST segment of the physical DNA molecule. The DO phenotype of genes A1 and A2 do not indicate that the products of these genes are essential for the process of DNA replication itself but, rather, that their products are required for the completion of transfer of phage DNA to the host cell (20) and hence for the entry into the

FIG. 1. Patterns of DNA metabolism in *su⁻* cells after infection with T5 wild-type (DN), T5·A1·amH16d (DDO), and T5·A2·amH231 (DO). The experimental and analytical procedures are described in Materials and Methods.

FIG. 2. The patterns of DNA metabolism in *su⁻* cells after infection with representative amber mutants defective in each of our known T5 genes. The genes are arranged according to the genetic map of T5 taken from Hendrickson and McCorquodale (12). Each gene is numbered above the heavy line according to genetic segment (A, B, C, and D) and order within the segment (1, 2, 3, etc.). Above each gene number is the designation for the DNA pattern that mutants defective in that gene display in nonpermissive cells. The designations DN, DA, DR, DO, DDR, DLD, and DDO are defined in the text. Recombination distances between consecutive genes in each genetic segment are given below the heavy line.

cell of T5 genes coding for those early enzymes that are required for the replication of T5 DNA.

Patterns of DNA metabolism displayed by other mutants infecting nonpermissive cells are shown in Fig. 3. Mutants defective in genes in the B segment display a pattern of DNA metabolism designated DR, in which DNA synthesis begins at or near the normal time, but proceeds at a slower rate than in T5⁺-infected cells. The rate at which DNA accumulates differs among the DR mutants as shown in Table 1. In some cases (all mutants in the B segment, for example), DNA synthesis proceeds at a low rate, the accumulation of DNA barely reaching the starting level after 80 min at 37 C. In other cases (gene D19, for example), synthesis proceeds at a more rapid, but still subnormal rate.

A DNA-arrest (DA) pattern is observed in the case of mutants defective in several genes in the D-segment (Table 2). In these cases, DNA synthesis begins at the normal time and rate, but is prematurely slowed or ended at various times for different DA mutants.

Another pattern of DNA metabolism shown in Fig. 3 is designated DDR, meaning that the onset of DNA synthesis is delayed and that the rate at which synthesis occurs is reduced. Genes D1 and D2 display such a pattern. It has been reported that in cells infected with certain T4 mutants a DNA-delayed pattern is observed in which the onset of DNA synthesis is delayed, but, once synthesis has begun, it proceeds at a normal rate (6). We have not observed this pattern with any mutants of T5.

Finally, we have encountered a pattern of DNA metabolism that we designate DLD (Fig. 4), which is displayed by mutants defective in

FIG. 3. DNA metabolism in *su⁻* cells after infection with T5 amber mutants that display DN, DA, DDR, and DR patterns. The patterns are defined in the text and genes displaying each pattern are indicated in Fig. 2.

genes D4, D6, and D11. Infection of nonpermissive cells by such mutants leads to the accumulation of a limited but variable amount of DNA which is subsequently degraded, either completely as in the case of D11, or partially as in the case of D6.

Figure 2 summarizes the data on patterns of DNA metabolism by showing the map position of each gene and its characteristic pattern of DNA synthesis. It is pertinent to note that mutants defective in genes D4 through D11, with the exceptions of D6 and D10, do not induce a net synthesis of phage DNA. We interpret

TABLE 1. *Deoxyribonucleic acid (DNA) synthesis by DR mutants^a*

Gene	Mutant	Relative amount of DNA accumulated at times indicated		
		0 min	50 min	80 min
B1	25a	1.00	0.63	0.98
B2	18c	1.00	0.50	0.78
B2	24b	1.00	0.70	1.09
B3	53	1.00	0.80	1.30
B4	6c	1.00	0.67	1.04
C2	55	1.00	0.45	0.55
D1	2	1.00	0.77	1.78
D2	7	1.00	1.36	0.69
D3	51	1.00	1.84	4.11
D13	58	1.00	1.17	1.60
D14	6b	1.00	1.11	1.65
D19	39b	1.00	1.17	2.25
Wild type		1.00	3.30	5.10

^a Nonpermissive (*su*⁻) cells were infected with the indicated mutants (or wild-type T5) at an input ratio of five phage per cell, and the relative amount of DNA per culture was determined by the diphenylamine reaction as described in Materials and Methods. For explanation of DR mutants, see text.

TABLE 2. *Deoxyribonucleic acid (DNA) synthesis by DA mutants^a*

Gene	Mutant	Relative amt of DNA accumulated at times indicated			
		0 min	30 min	50 min	80 min
D10	34c	1.00	1.00	1.50	1.85
D12	23c	1.00	1.30	2.00	2.40
D15	50	1.00	0.90	1.26	1.30
D20	30b	1.00	0.70	0.80	1.10
D21	47	1.00	0.75	0.82	1.03
Wild type		1.00	1.40	3.30	5.10

^a These data were obtained by the procedure indicated in the legend of Table 1. DA = DNA-arrest.

these results to mean that the products of genes D4, D5, D7, D8, D9, and D11 are absolutely essential for T5 DNA replication and that the product of gene D6 prevents the degradation of whatever phage DNA is synthesized. The identification of gene D9 as the structural gene for T5-specific DNA polymerase (13) fits this interpretation. The grouping in adjacent loci of genes whose products are involved in the production of a common product has been noted in a number of genetic maps (6, 34).

Identification of gene products. The identification of the proteins and their functions that are

specified by T5 genes is an essential step in elucidating the regulatory mechanisms that operate during the growth and development of the phage. Although five early enzymes are known to be induced after infection of sensitive cells by T5⁺, only mutants defective in the gene for the T5-induced DNA polymerase have been previously characterized and mapped (5, 13). In an effort to locate genes involved in the synthesis of other early enzymes, nonpermissive cells were infected with representative amber mutants, and determinations of early enzyme activities were performed on crude extracts of the infected cells.

When nonpermissive cells are infected with B3-*amH56*, only a very small increase in dihydrofolate reductase is observed, whereas in permissive cells infected with this same mutant the rate and extent of synthesis of dihydrofolate reductase are essentially the same as that of T5⁺-infected cells (Fig. 5). There is a characteristic delay of 5 min in induction, followed by a rapid increase in early enzyme activity which is slowed (or shut off almost completely in the case of other early enzymes) after about 15 to 20 min (22). Lysis normally begins at about 45 min, and the decrease in dihydrofolate reductase activity after 45 min is interpreted to reflect the lower levels of this (and other) enzyme(s) in the cells that have not lysed by this time. In the same figure is plotted thymidylate synthetase activity as a function of time in nonpermissive cells infected with B3-*amH56*. The kinetics are normal with respect to the time of onset, extent of increase, and time of cessation of synthesis of this enzyme, so it is believed that the lesion in B3-*amH56* is in a gene-controlling (either directly or indirectly) dihydrofolate reductase, and is not in a gene-controlling early enzyme synthesis in a more general way.

FIG. 4. DNA metabolism in *su*⁻ cells after infection with T5 amber mutants that display DLD patterns. The DLD pattern is defined in the text and the genes corresponding to each pattern are: ○, D4; ●, D11; X, D6.

FIG. 5. Lack of induction of dihydrofolate reductase activity in su^- cells infected with B3-amH56 (●). The rate and extent of synthesis of dihydrofolate reductase in su^+ cells infected with B3-amH56 (○) is essentially the same as in su^- cells infected with T5⁺ (□). The induction of thymidylate synthetase activity in su^- cells infected with B3-amH56 is normal (Δ).

Similarly, a gene involved in the elaboration of early T5-induced deoxyribonuclease has been located. Figure 6 shows the kinetics of synthesis of this enzyme in permissive and nonpermissive cells infected with D15-amH50. There is no increase in deoxyribonuclease activity over a 70-min period of infection in the nonpermissive host, but there is a normal increase in dihydrofolate reductase in these cells. Again we interpret this to mean that the mutation in D15-amH50 is in a gene involved only in the synthesis of deoxyribonuclease, and not in a gene that codes for a general regulatory protein. When the mutant is grown in the permissive host, high (but still somewhat subnormal) levels of deoxyribonuclease are produced, and the average burst size is several hundred compared to less than one in the case of nonpermissive cells infected with this mutant.

The deoxyribonuclease under the control of gene D15 was assayed by the method of Paul and Lehman (29), and so D15 is the gene controlling the activity described by these authors. Recently (7, 8), a deoxyribonuclease activity induced by T5 has been purified and characterized and some evidence has been presented to relate this activity to that described by Paul and Lehman. Frenkel and Richardson isolated a temperature-sensitive mutant of T5 (HA70R4) that induced a temperature-sensitive deoxyribonuclease (8). We have mapped this mutant in the T5 genome, and, as shown in Table 3, HA70R4 maps closest to amber mutations in gene D15, suggesting that it is de-

fective in the same gene that controls the induction of early deoxyribonuclease as assayed under the conditions of Paul and Lehman. Complementation tests between HA70R4 and several mutants defective in genes in and near D15 (Table 4)

FIG. 6. Lack of induction of "early" deoxyribonuclease activity in su^- cells infected with D15-amH50 (●). The rate and extent of synthesis of "early" deoxyribonuclease in su^+ cells infected with D15-amH50 (○) is somewhat less than when T5⁺ infects su^- cells (□), but nevertheless is extensive. The induction of dihydrofolate reductase activity in su^- cells infected with D15-amH50 is normal (Δ).

TABLE 3. Recombinational distances between *ts*HA70R4 and amber mutants defective in genes in and near gene D15^a

Mutant	Recombinational distances				
	D4	D13	D14	D15	D17
<i>ts</i> HA70R4	15.8	5.5	4.1	0.6	14.3
D15-amH50	>25	7.9	3.7		16.0

^a su^+ Cells were infected with five each of *ts*-HA70R4 and an amber mutant defective in one of several genes. After appropriate dilutions and incubation for 90 min at 30°C in the usual manner, progeny phage were preadsorbed and plated on su^+ at 30°C for total progeny, and on su^- at 45°C for wild-type recombinants. Values given in the table are per cent recombination calculated as (progeny on su^- at 45°C/progeny on su^+ at 30°C) × 200.

confirm that HA70R4 is defective in the product of gene D15. These data show that gene D15 is the structural gene for the early T5-specific deoxyribonuclease and that the deoxyribonuclease activity recently described by Frenkel and Richardson is due to the same enzyme described by Stone and Burton (33) and by Paul and Lehman (29).

It is interesting to note that mutants defective in gene B3, the gene controlling the synthesis of dihydrofolate reductase, exhibit a DR pattern of DNA synthesis and produce a burst of phage in nonpermissive cells that is approximately proportional to the amount of phage DNA synthesized. Gene B3 is not, therefore, essential for the growth and development of T5. On the other hand, mutants defective in gene D15, the structural gene for early deoxyribonuclease, can synthesize almost as much DNA in nonpermissive cells as mutants of gene B3, but yet they produce a burst of less than 1. Gene D15 is therefore essential for the growth and development of T5. The very low burst compared to the amount of DNA synthesized in nonpermissive cells infected with mutants of gene D15 appears to be due to an inability of the mutants to produce mature-sized DNA from larger replicating structures (7, 8).

DNA synthesis by deoxyribonuclease⁻ mutants. Mutants defective in the structural gene for early

deoxyribonuclease (D15) exhibit a DA pattern of DNA synthesis, as determined by total acid-insoluble DNA. This pattern is confirmed by cumulative (Fig. 7) and pulse (Fig. 8) labeling of the DNA synthesized in nonpermissive cells infected with D15·*amH50*. These data on the incorporation of radioactive precursors show that the synthesis of DNA begins normally but is arrested prematurely; this is in complete agreement with the pattern of DNA synthesis at a nonpermissive temperature in cells infected with HA70R4 (8).

DISCUSSION

The results presented in this paper provide information on the biochemical functions that are specified by individual genes of T5. The patterns of DNA synthesis observed in nonpermissive cells infected with mutants defective in each of the genes in which we have mutations provide a general view of the involvement of each of these genes in the important function of phage DNA synthesis. The genes whose products are essential for the process of DNA replication itself are grouped

TABLE 4. Complementation tests between HA70R4 and amber mutants of T5^a

Mutants	Burst size
HA70R4 alone	1
HA70R4, D15· <i>amH8</i>	2
HA70R4, D15· <i>amH50</i>	1
HA70R4, D13· <i>amH58</i>	147
HA70R4, D14· <i>amH6b</i>	69
HA70R4, D16· <i>amH34a</i>	30
HA70R4, D17· <i>amH34d</i>	34
D15· <i>amH8</i> , D17· <i>amH34d</i>	36
D15· <i>amH50</i> , D14· <i>amH6b</i>	83
T5 ⁺ alone	170 ^b

^a Nonpermissive (*su*⁻) cells were infected with phage at an input ratio of five of each mutant (10 if only one mutant was used). The infected cells were incubated at 43 C (±0.2 C) for 90 min in nutrient broth with aeration, after which a sample was withdrawn, added to CHCl₃, shaken vigorously, and chilled. A sample taken prior to incubation was diluted, plated on *su*⁺, and incubated at 30 C to determine the concentration of infected bacteria, and the sample withdrawn after 90 min was preadsorbed and plated to determine the concentration of progeny phage. Burst size was calculated as total progeny divided by infected bacteria.

^b Average of two determinations.

FIG. 7. Cumulative incorporation of ¹⁴C-thymidine into DNA after infection of *su*⁻ cells with T5⁺ and D15·*amH50*, a mutant defective in the structural gene for "early" deoxyribonuclease. The experimental and analytical procedures are described in Materials and Methods.

FIG. 8. Rate of ^{14}C -thymidine incorporation into DNA after infection of su^- cells with T5^+ and $\text{D15-}am\text{H50}$, a mutant defective in the structural gene for "early" deoxyribonuclease. The individual points represent the amount of ^{14}C -thymidine incorporated into DNA during 2-min periods of incubation at 37°C , and are plotted as a function of the time after infection at which the 2-min incubation period was begun. The experimental and analytical procedures are described in *Material and Methods*.

together in the D segment of the genetic map and include genes D4, D5, D7, D8, D9, and D11. Within this group of genes, gene D6 is not necessary for a small net accumulation of phage DNA but is required to prevent the degradation of the DNA that is formed (Fig. 4). Gene D6 is therefore of critical importance in DNA synthesis but is apparently not involved in the replication process itself. Gene D10, on the other hand, appears out of place in the group because mutants defective in it induce a DA pattern of DNA synthesis. Indeed, recent mapping studies indicate that D10 may be mismapped. If it is not located between genes D9 and D11, the genes D4, D5, D6, D7, D8, D9, and D11 would form a contiguous group, all the products of which play an essential role in phage DNA synthesis.

Of the five early enzymes known to be induced by T5 (26), DNA polymerase is one which is essential for the replication of T5 DNA (13). The structural gene for T5 DNA polymerase (D9) is located among the cluster of six genes that are essential or critical for the synthesis of phage DNA (Fig. 2). On the other hand, the dihydrofolate reductase induced by T5 is neither essential for DNA replication nor critical for the supply of thymidylate for the replication process. From the discussion in the previous paragraph, it might be expected that the structural gene for dihydrofolate reductase would not be located among genes D4

through D11. Indeed, the gene (B3) that controls the appearance of dihydrofolate reductase appears to be at a considerable genetic distance from the polymerase gene. The amount of DNA synthesized in nonpermissive cells infected with a mutant defective in the production of dihydrofolate reductase is about one-eighth of that synthesized in a wild-type infection; the burst size of the former type of infection is also about one-eighth that of the latter. Thus, the relative burst size produced by mutants defective in dihydrofolate reductase is proportional to the amount of phage DNA synthesized as compared to the amount synthesized in a wild-type infection. Apparently, the dihydrofolate reductase induced by T5 permits a more rapid rate of phage DNA synthesis which, in turn, leads to a larger burst size. This role for T5 dihydrofolate reductase is therefore similar to the role for T5 dihydrofolate reductase which, when active, permits about a twofold increase in the amount of T4 DNA synthesized and in the burst size (24).

By analogy to T4 in which the structural genes for dihydrofolate reductase and thymidylate synthetase are adjacent to one another (26), we suspected that the structural gene for thymidylate synthetase might also be in the B segment of the T5 map. However no mutants, which we have isolated, that are defective in genes in the B segment fail to induce significant levels of thymidylate synthetase. We have also not found a mutant defective in the production of the T5-specific deoxynucleoside monophosphokinase. However, not all of our mutants have yet been tested with respect to this kinase activity or with respect to the induction of thymidylate synthetase.

The early deoxyribonuclease of T5 is clearly essential for the growth and development of the phage. It does not, however, participate in the DNA replication process itself since appreciable amounts of phage DNA are synthesized in cells infected with mutants defective in the structural gene for this early deoxyribonuclease. Recently, Frenkel and Richardson (7) have characterized an early deoxyribonuclease activity induced by T5 as a 5'-exonuclease which can cleave not only mononucleotides but also di-, tri-, and oligonucleotides from the 5'-termini of nicks, gaps, or ends in double-stranded DNA. The temperature-sensitive mutant HA70R4 is defective in this 5'-exonuclease activity, and the mutation that causes this defect maps in gene D15 (Tables 3 and 4). Amber mutants defective in gene D15 do not induce the early deoxyribonuclease activity described by Paul and Lehman (29). These results therefore confirm the conclusion of Frenkel and Richardson (7, 8) that the 5'-exonuclease activity described by them and the early deoxyribonuclease activity described by

Paul and Lehman are properties of a single enzyme. The results also demonstrate that gene D15 is the structural gene for the enzyme that displays these nuclease activities.

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