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The Structural Gene for a T7 Endonuclease Essential for Phage DNA Synthesis*

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Abstract. Infection of *Escherichia coli* with bacteriophage T7 results in the appearance of an endonuclease activity capable of hydrolyzing both double- and single-stranded DNA. Treatment with chloramphenicol prevents the induction of the endonuclease. *Amber* mutants of phage T7 defective in gene 3 are unable to produce the enzyme after infection of the nonpermissive host, and mutants that produce a heat-labile endonuclease were found, indicating that this gene is the structural gene for the enzyme. Gene 3 mutants synthesize only a limited amount of DNA. In addition, they are defective in carrying out the degradation of host DNA, suggesting that the gene 3 endonuclease is involved in this function.

Introduction. Bacteriophage T7 has recently received extensive genetic and physiological analysis.[1–4] After infection, T7 specifies approximately 25–30 proteins, and so far 19 genes have been identified and mapped by isolation and characterization of *amber* mutants. We have been studying an endonuclease activity induced in *E. coli* after infection with T7.[5] This activity, which has been purified 1000-fold, hydrolyzes both double- and single-stranded DNA. Degradation of native DNA to a limit produces fragments which, when sedimented in alkaline sucrose, have a molecular weight of approximately 2×10^4. The purified endonuclease degrades single-stranded DNA more extensively, producing fragments having a molecular weight of 10^4 and less. The present report provides evidence that this endonuclease is the product of gene 3 and that it participates in the breakdown of host DNA after T7 infection. Hausmann and Gomez[6] have previously presented preliminary evidence for a T7-induced DNase.

Materials and Methods. Bacterial strains: Strains permissive for *amber* mutants were *E. coli* 011′ and *E. coli* K12 endo$^-$ (thiamine-requiring), a strain deficient in endonuclease I. Nonpermissive strains were *E. coli* B and *E. coli* ER22, a B strain also deficient in endonuclease I.

Bacteriophage: Preparation of wild-type and *amber* mutants of T7 has been described.[2] Phage λ was obtained[7] from a heat inducible lysogen *E. coli* W3102 (λ*C*i857). Phage $\phi X174$ was prepared as described by Sinsheimer.[8] Labeling of phage DNA with ^{3}H-thymidine or ^{32}P (New England Nuclear Corp.) was performed in 3*XD* medium and in 3*XD* medium with the phosphate concentration of TCG medium,[9] respectively. Most phage stocks were partially purified by differential centrifugation while the labeled phages were further purified by banding in CsCl.[7]

DNA: Phage DNA was extracted with phenol saturated with 0.01 *M* Tris-HCl (pH 7.6)–0.001 *M* EDTA. ϕX-RFI DNA was prepared as described by Sinsheimer[8] and isolated after centrifugation in CsCl and ethidium bromide as described by Kiger *et al.*[10]

Sedimentation analysis: Sedimentation analyses were performed in linear 5–20% sucrose gradients using either the SW39 or SW50.1 rotor. Alkaline sucrose was prepared in 0.3 *M* NaOH–0.7 *M* NaCl–0.01 *M* Tris-HCl–0.001 *M* EDTA. Neutral sucrose was prepared in 1.0 *M* NaCl–0.01 *M* Tris buffer (pH 7.6)–0.001 *M* EDTA.

Protein: Protein was determined according to the method of Lowry *et al.*[11]

Enzyme assays: DNA ligase activity was determined as described previously.[12]

Endonuclease activity: One assay measures the endonuclease activity towards native DNA by following the conversion of ϕX-RFI to a form which can be trapped on nitrocellulose membrane filters (type B-6, Schleicher and Schuell) after heating at 100°C. The standard reaction mixture (0.2 ml) contained 0.25 mμmoles ^{3}H-ϕX-RFI (3.0×10^4 cpm/mμmole), 50 mM Tris buffer (pH 8.0), 7 mM $MgCl_2$, 5 mM β-mercaptoethanol, 10 μg bovine serum albumin, and 2–8 units of enzyme. After incubation for 10 min at 37°C, 6 μmoles of EDTA and 1.0 ml SSC[13] were added to the reaction mixture. After mixing, the solution was heated for 3 min in a boiling water bath and then chilled. The solution was mixed with 5.0 ml of 6 × SSC[13] and filtered slowly through a nitrocellulose membrane. After filtration, the membrane was washed with an additional 5 ml of 6 × SSC, dried, and the radioactivity was determined. A unit of enzyme activity is defined as the amount of enzyme which can convert 1 mμmole of ϕX-RFI in 10 min at 37°C to a form which, after being heated, can be trapped on nitrocellulose membrane filters.

The second assay measures the endonuclease activity towards single-stranded DNA by measuring the conversion of ϕX DNA to a form which is susceptible to hydrolysis by *E. coli* exonuclease I.[14] The standard reaction mixture was as described above except that RFI was replaced with 0.05 mμmoles of ^{3}H-ϕX DNA (3.0×10^4 cpm/mμmole). After a 10-min incubation period at 37°C, EDTA was added to a final concentration of 7 mM and the reaction mixture was heated for 10 min at 60°C. Under these conditions (in the presence of EDTA), the endonuclease is inactivated.[5] After cooling, $MgCl_2$ was added to give a final concentration of 7.5 mM. Exonuclease I (5.2 units)[15] was added and the reaction mixture was incubated at 37°C for 20 min. After incubation, 0.1 ml salmon sperm DNA (0.25 mg/ml) and 0.2 ml cold 1 *N* trichloracetic acid were added, and the tubes were centrifuged for 5 min at 10,000 × *g*. The radioactivity in the supernatant liquid was measured as previously described.[16] A unit of enzyme activity is defined as the amount of enzyme which, together, with exonuclease I, catalyzes the conversion of 1 mμmole of $\phi X174$ DNA to an acid-soluble form in 10 min at 37°C. Activity is proportional to enzyme concentration when 0.2–1.5 enzyme units are used in the reaction.

Results and Discussion. Detection of endonuclease activity in cells infected with T7: As shown in Figure 1, extracts of uninfected *E. coli* K12 endo$^-$ do not cause significant degradation of either double-stranded λ or single-stranded ϕX DNA. However, after infection with T7, both DNA's are hydrolyzed to a considerable extent. In the presence of chloramphenicol, no endonuclease activity can be detected with either DNA substrate (Fig. 1).

Identification of the structural gene for the endonuclease: (1) *Amber mutants:* *E. coli* ER22 (nonpermissive) was infected with a variety of T7 *amber* mutants, and endonuclease and DNA ligase activities were determined in extracts of these cells. Of all the *amber* mutants examined, only those mutants in genes 1 or 3 did not induce an increase in endonuclease activity above the level observed in uninfected cells (Table 1), and only the mutant in gene 1 failed to induce DNA ligase activity.

In studies by Studier and Maizel[3] it was shown that gene 1 controls the syn-

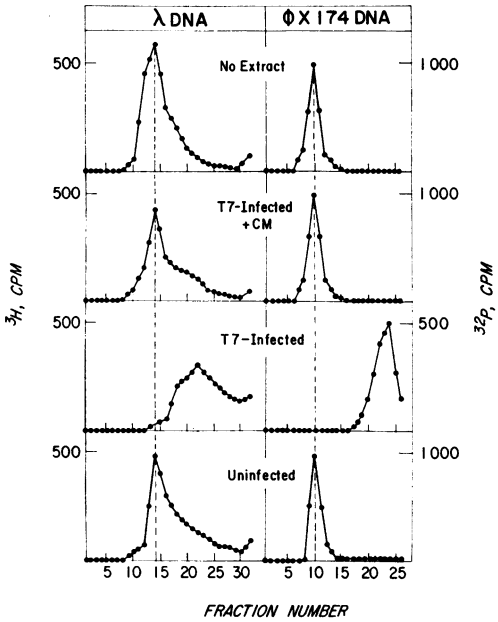

FIG. 1—Demonstration by alkaline sedimentation analysis of T7 endonuclease activity on λ and $\phi X174$ DNA. *E. coli* K12 endo$^-$ was grown in T-broth at 37°C to a density of 7×10^8 cells/ml. T7 phage was added at a multiplicity of 5, and 20 min after infection the cells were harvested and extracts were prepared. Extracts were also prepared from cells collected before infection. Chloramphenicol as indicated was added at a concentration of 50 μg/ml just prior to the addition of phage. The standard reaction mixture contained 1.5 mμmoles of ^{3}H- λDNA (1×10^4 cpm/mμmole) or 0.5 mμmole ^{32}P-$\phi X174$ DNA (3.0×10^3 cpm/mμmole) and 10 μg of protein. After incubation for 15 min at 37°C, 3 μmoles of EDTA were added. λ and ϕX DNA were centrifuged in alkaline sucrose gradients in the SW39 rotor for 16.5 hr at 22,000 and 30,000 rpm, respectively. The dotted line indicates the position to which marker λ or $\phi X174$ DNA sedimented.

thesis of most T7 proteins. Recently the gene 1 protein has been identified as an RNA polymerase factor which is required for the transcription of the major portion of the T7 genome.[17] Mutants in gene 3, on the other hand, synthesize essentially all T7 proteins, presumably lacking only the protein specified by gene 3. These observations, in combination with the present results, indicate that neither the endonuclease nor DNA ligase is one of the earliest proteins of T7, and suggest that gene 3 is the structural gene for the endonuclease.

The appearance of endonuclease and ligase activities at various times after infection of *E. coli* K12 endo$^-$ and *E. coli* ER22 with T7 *am*29 is shown in Figure 2.

TABLE 1. *Endonuclease activities in extracts of E. coli ER22 infected with T7 amber mutants.*

Mutant	Gene	Endonuclease Activity — Substrate: $\phi X174$ DNA (units/mg protein)	Endonuclease Activity — Substrate: $\phi X174$ RFI DNA (units/mg protein)
Uninfected cells		<10	220
T7	(Wild type)	210	660
*am*23	1	<10	180
*am*64, 20, 28, 147, 182, 10	2, 4, 5, 6, 18, 19	250–430	700–1200
*am*29	3	<10	220
*am*128	3	<10	250
*am*285	3	<10	250

E. coli ER22 was grown in M-9 medium at 30°C to a cell density of 2.5×10^8 cells/ml. T7 *amber* mutants were added at a multiplicity of 5, and the cultures were incubated for an additional 20 min at 30°C. At the end of this period the cells were harvested by centrifugation, suspended in 0.02 *M* Tris-HCl (pH 7.6)–0.01 *M* β-mercaptoethanol, and subjected to sonication with a Branson S75 sonifier. The sonically treated cells were centrifuged 20 min at $10{,}000 \times g$ and the supernatant liquid was recovered. The standard assays were used to measure endonuclease activity.

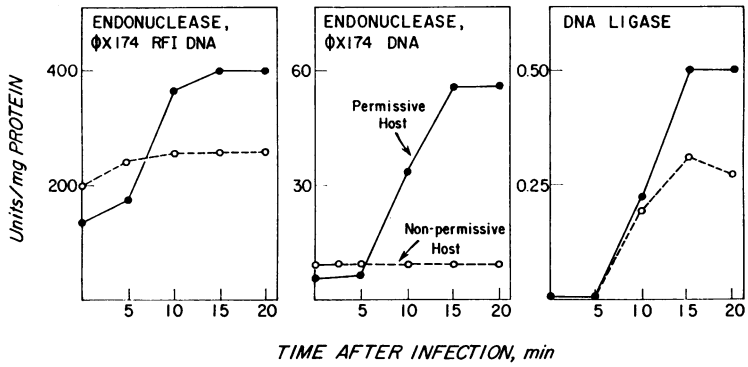

FIG. 2.—Appearance of endonuclease activity after infection of *E. coli* with T7*am*29. *E. coli* K12 endo$^-$ (permissive host) and *E. coli* ER22 (nonpermissive host) were grown in T-broth at 30°C to a cell concentration of 2.5×10^8 cells/ml. At that time the cells were infected with T7*am*29 at a multiplicity of 5. Just prior to the addition of phage, and at various times after infection, portions were collected and extracts were prepared. The standard assays were used to measure endonuclease activity.

After infection of the permissive host, the endonuclease activity with $\phi X174$ DNA as substrate increases 20-fold while the activity toward ϕX-RFI is three times that observed in the uninfected cells. In contrast, *am*29 does not induce any detectable endonuclease activity towards ϕX DNA after infection of the nonpermissive host. However, with ϕX-RFI DNA a slight increase in endonuclease activity is observed in the nonpermissive host. This activity toward ϕX-RFI DNA could be due to a second T7 induced endonuclease.

Further studies on the endonuclease activity present after infection with mutants defective in gene 3 were performed by examining the hydrolysis of native T7 and $\phi X174$ DNA by sedimentation in sucrose gradients. There is no change in the sedimentation pattern of $\phi X174$ DNA after incubation with extracts from uninfected or infected *E. coli* ER22 (Fig. 3). In contrast, extracts from infected *E. coli* K12 endo$^-$ catalyze the hydrolysis of $\phi X174$ DNA to fragments of low molecular weight.

When T7 DNA is treated with extracts from uninfected or infected *E. coli* ER22, there is no change in the sedimentation pattern in neutral sucrose gradients, as compared to intact T7 DNA (Fig. 4). In contrast, extracts from the permissive host infected with T7 *am*29 cause considerable degradation of T7 DNA. Sedimentation of the DNA in alkaline sucrose gradients has revealed that single-strand breaks are introduced into the DNA by extracts of both the permissive and nonpermissive hosts infected with T7 *am*29, further indicating that a second endonuclease is induced by T7.

(2) *A heat-labile endonuclease:* The activity of the wild-type endonuclease increases with temperature until 70°. Perhaps because of this stability we have been unable to isolate temperature-sensitive mutants from revertants of *amber* mutants in gene 3, except in the case of *am*128, which also contains a second, closely-linked mutation. The endonuclease activity induced by this temperature-sensitive strain is normal at 42°. However, at 60° the activity is decreased

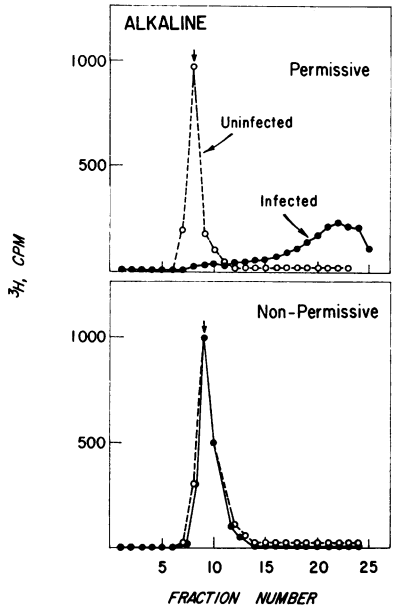

FIG. 3.—Alkaline sedimentation analysis of $\phi X174$ DNA treated with extracts of cells infected with T7*am*29. *E. coli* K12 endo$^-$ and *E. coli* ER22 were infected with T7*am*29 as described in Fig. 2. Extracts were prepared from cells taken 20 min after infection. The reaction mixture was as described in Fig. 1, except that 0.25 mμmole of ^{3}H-$\phi X174$ DNA (2.8×10^4 cpm/mμmole) was used as substrate. Incubation time was 15 min at 37°C. After the addition of 3 μmoles of EDTA, the sample was centrifuged in alkaline sucrose for 16.5 hr at 30,000 rpm in the SW39 rotor.

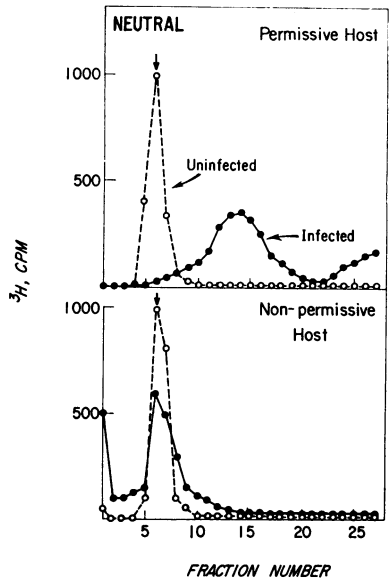

FIG. 4.—Neutral sedimentation analysis of T7 DNA treated with extracts of cells infected with T7*am*29. *E. coli* K12 endo$^-$ and *E. coli* ER22 were infected with T7*am*29 as described in Fig. 2. Extracts were prepared from cells taken 20 min after infection. The reaction mixture was as described in Fig. 1, except that 2 mμmoles of ^{3}H-T7 DNA (3.0×10^3 cpm/mμmole) was used as substrate. After incubating for 15 min at 37°C, 3 μmoles of EDTA were added to the reaction mixture, and the samples were centrifuged in neutral sucrose for 17 hr at 22,000 rpm in the SW50.1 rotor. The arrow indicates the position to which intact T7 DNA sediments.

relative to that induced by wild-type T7. When the second mutation is removed from the temperature-sensitive strain, the ability to grow at 42° is restored, but the endonuclease activity is still reduced at 60°. The experimental details are given in Table 2. The interpretation of these results is that a reversion at the *am*128 site in gene 3 has produced a missense mutation that alters the stability of the endonuclease molecule.

Degradation of bacterial DNA after infection: Infection of the nonpermissive host with *amber* mutants in gene 3 leads to the synthesis of only a limited amount of phage DNA and DNA synthesis stops prematurely.[2] These effects might be expected if the endonuclease is necessary for the breakdown of host DNA, since T7 DNA is formed primarily from the nucleotides of the host DNA.[18–20] In order to test this hypothesis, we have examined mutants in genes 1–6, which affect the synthesis of T7 DNA, for their ability to render host DNA acid-soluble (Fig. 5). In a wild-type infection, phage DNA is synthesized so rapidly that little, if any, host DNA can be found in acid-soluble form (Fig. 5). However,

TABLE 2. *A temperature-sensitive endonuclease induced by a mutant of T7.*

Mutant	Endonuclease Activity (units/mg) Assayed at 37°C	Assayed at 60°C	$Q_{37°}^{60°}$
T7 wild type	45	110	2.5
T7*rev*(*am*128)A	28	7.5	0.30
T7*rev*(*am*128)B	21	7.5	0.35

E. coli K12 endo⁻ was grown in T-broth at 25°C to a cell density of 2.5 × 10^8 cells/ml. The cells were infected with phage at a multiplicity of 5, and 20 min after infection the cells were harvested and extracts were prepared as described in legend to Table 1. Endonuclease activity was measured with $\phi X174$ DNA as substrate as described in *Materials and Methods*.

Attempts to find temperature-sensitive mutants among revertants of *amber* mutants in gene 3 were successful only with *am*128, which maps at the same position as *am*29 and *am*434. However, it soon became apparent that the original *am*128 strain carries a second mutation, *var*, which causes it to make small, variable-sized plaques on the permissive host. This original strain will be referred to as T7*am*128, *var*. The temperature-sensitive revertant also carries *var* and will be referred to as T7*rev*(*am*128), *var*, where *rev*(*am*128) refers to a reversion of *am*128. The *var* mutation was removed from T7*am*128, *var* by crossing it with wild type. The resulting strain, T7*am*128, has normal plaques on the permissive host and still does not recombine with *am*29 or *am*434. The temperature-sensitive revertant, T7*rev*(*am*128), *var*, when crossed with its parent strain, T7*am*128, *var*, produces no recombinants which plate on the restrictive host at 42°C. However, when crossed with T7*am*128, T7*am*29, or T7*am*434, approximately 0.5% of the burst will grow on the restrictive host at 42°C; but these recombinant plaques are somewhat smaller than wild type. These pseudo-wild recombinants must have the genetic composition T7*rev*(*am*128). Stocks were made of two such recombinants, designated T7*rev*(*am*128)A and T7*rev*(*am*128B). These apparently carry a missense mutation at the *am*128 site.

infection with mutants in gene 5,[21] which do not make phage DNA, causes 50 per cent of the host DNA to become acid soluble. To study the involvement of other genes in the degradation of host DNA, double mutants were constructed, each containing an *amber* mutation in gene 5 plus a second mutation in genes 1–4 or 6. The construction of these double mutants eliminates any incorporation of degraded host nucleotides into phage DNA. The results obtained with these double mutants were also obtained with single mutants in these genes. As shown in Figure 5, host DNA becomes acid soluble only after infection with mutants in genes 2, 4, or 5. There is essentially no decrease in acid-precipi-

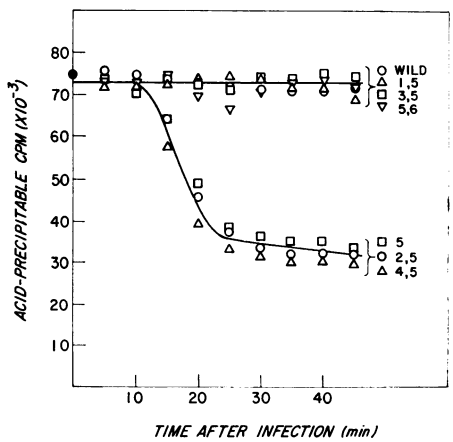

FIG. 5.—Degradation of host DNA by different mutants of T7. *E. coli* B was grown in M9 medium at 30°C to 10^8/ml, and 0.5 mCi/ml of ^{3}H-thymine (6.7 Ci/mmole) was added. Uptake of ^{3}H into material which was precipitated by 0.5 *N* trichloracetic acid was linear for approximately 5 min and had stopped by 10 min after addition of label. After incubating another hour during which the cells slightly more than doubled, T7 phage was added at a multiplicity of 8–10. At 5-min intervals 1-ml samples were precipitated by 1 ml of 1 *N* trichloracetic acid, the precipitates were collected on glass filters, dried, and counted. The point at "0 minutes" refers to uninfected cells. The genetic composition of the T7 strains is given in the figure; for example, "1,5" refers to a strain carrying mutations in both genes 1 and 5. The *amber* mutations used were[2]: *am*23 in gene 1, *am*64 in gene 2, *am*29 in gene 3, *am*20 in gene 4, *am*28 in gene 5, *am*147 in gene 6.

table material after infection with wild type or mutants in genes 1, 3, or 6. The result obtained with the gene 1 mutants reflects the inability of such mutants to synthesize T7 proteins as discussed above. The results obtained with mutants in genes 3 and 6 do suggest, however, that the proteins specified by these genes are involved in the degradation of host DNA.

Since the purified gene 3 endonuclease produces little acid-soluble material by itself,[5] the gene 6 protein could be an exonuclease which acts at the breaks produced by the gene 3 endonuclease. The mechanism by which T7 selectively degrades *E. coli* DNA *in vivo* remains unclear, particularly in view of the observation that the purified enzyme can hydrolyze T7 DNA *in vitro*.

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[1] Hausmann, R., and B. Gomez, *J. Virology*, **1**, 779 (1967).

[2] Studier, F. W., *Virology*, **39**, 562 (1969).

[3] Studier, F. W., and J. V. Maizel, *Virology*, **39**, 575 (1969).

[4] Studier, F. W., and R. Hausmann, *Virology*, **39**, 587 (1969).

[5] Center, M. S., and C. C. Richardson, manuscript in preparation.

[6] Hausmann, R., and B. Gomez, *J. Virology*, **2**, 265 (1968).

[7] Richardson, C. C., *J. Mol. Biol.*, **15**, 49 (1966).

[8] Sinsheimer, R. L., in *Procedures in Nucleic Acid Research*, eds. G. L. Cantoni and D. R. Davies (New York: Harper and Row, 1966), p. 569.

[9] Thomas, C. A., and J. Abelson, in *Procedures in Nucleic Acid Research*, eds. G. L. Cantoni and D. R. Davies (New York: Harper and Row, 1966), p. 553.

[10] Kiger, J. A., E. T. Young, II, and R. L. Sinsheimer, *J. Mol. Biol.*, **33**, 395 (1968).

[11] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

[12] Weiss, B., A. Jacquemin-Sablon, T. R. Live, G. C. Fareed, and C. C. Richardson, *J. Biol. Chem.*, **243**, 4543 (1968).

[13] The following abbreviations are used: SSC, solution containing 0.15 *M* NaCl–0.015 *M* sodium citrate; 6 × SSC, six times the concentration of SSC; CM: chloramphenicol.

[14] Lehman, I. R., *J. Biol. Chem.*, **235**, 1479 (1960).

[15] Exonuclease I is not limiting in the reaction since, under conditions where $\phi X174$ DNA is hydrolyzed completely by a purified endonuclease,[5] 100% of the radioactivity is rendered acid soluble by 5.2 units of exonuclease I.

[16] Richardson, C. C., *J. Biol. Chem.*, **241**, 2084 (1966).

[17] Summers, W. C., and R. B. Siegel, *Nature*, **223**, 1111 (1969).

[18] Labaw, L. W., *J. Bacteriol.*, **62**, 169 (1951).

[19] Labaw, L. W., *J. Bacteriol.*, **66**, 429 (1953).

[20] Putnam, F. W., D. Miller, L. Palm, and E. A. Evans, Jr., *J. Biol. Chem.*, **199**, 177 (1952).

[21] Evidence has been obtained which indicates that gene 5 specifies a T7-induced DNA polymerase (Masamune, Y., personal communication).