*STUDIES OF DNA REPLICATION IN VIVO, III. ACCUMULATION OF A SINGLE-STRANDED ISOLATION PRODUCT OF DNA REPLICATION BY CONDITIONAL MUTANT STRAINS OF T4**

By M. Oishi

DEPARTMENT OF GENETICS, THE PUBLIC HEALTH RESEARCH INSTITUTE OF THE CITY OF NEW YORK, INC.

Communicated by George K. Hirst, May 14, 1968

The preceding papers of this series[1, 2] reported that the initial product of bacterial DNA replication is isolated as low-molecular-weight single-stranded DNA. This first intermediate is converted, in the cell, into the second intermediate whose structure when isolated appears to be that of a double-stranded DNA moiety containing a small but significant single-stranded portion as well as gaps in the newly synthesized strand. Okazaki and his associates showed that an appreciable amount of newly synthesized DNA is isolated as single-stranded DNA.[3] An important question at this time concerning the conversion between these two different stages of DNA replication is whether an unknown enzyme(s) is, directly or indirectly, involved. As a first step in seeking to answer this question, strains of bacteriophage harboring conditional lethal mutations in genes implicated in DNA replication were examined. This paper reports that bacteriophage T4 strains which contain conditional mutations of gene 41, under nonpermissive conditions, accumulate newly synthesized DNA that is isolated as single-stranded DNA.

Materials and Methods.—Bacteria and bacteriophage strains: *E. coli* B3 (*thy*), kindly provided by Dr. E. M. Witkin, was used as a host strain for temperature-sensitive mutants of bacteriophage T4 and as a nonpermissive host strain for amber mutants of bacteriophage T4. A temperature-sensitive gene 41 mutant of T4 (A14), and amber mutants of gene 41 (N81, N57) were generously supplied by Dr. R. S. Edgar. A permissive (for T4 amber mutants) *E. coli* strain CR63, wild-type bacteriophage T4D, and most of the amber mutants tested in the preliminary work (A453, gene 32; N81, gene 41; N101, gene 43; N82, gene 44; HL628, gene 59; E1140, gene 62) were kindly supplied by Dr. J. F. Speyer. Dr. J. Hosoda kindly sent two more amber mutants (H39X, gene 30, and E10, gene 45). Frequencies of plaque formation by these mutants under nonpermissive conditions were less than 0.05%.

Media: H broth[4] was used for preparation of phage stock. Modified TCG medium[5] (0.2% glucose), which contains 20 μg/ml of uracil, 10 μg/ml of thymine, and 2 μg/ml of thymidine, was used for the growth of host bacterial strain when incorporation studies were performed.

Infection of bacteriophage and labeling of DNA: One ml of overnight culture of *E. coli* strain (*E. coli* B3 (*thy*$^-$) or CR63) in nutrient broth was transferred to 50 ml of modified TCG medium with supplement and incubated at 37°C (25°C or 30°C when a temperature-sensitive mutant was used) until middle-log phase (viable cells, approximately 2.5×10^8/ml). Bacteriophages (m.o.i. = 10) were then added with L-tryptophan (20 μg/ml). For studies at temperatures other than that used for growing bacteria, the cultures were preincubated at the appropriate temperature for 2–4 min prior to infection. At 4 min after infection, H^3-thymidine (10–20 μc/ml, spec. act. 16.7 c/mM, New England Nuclear Co.) was added and incubation continued until 10 min after infection. The cultures were then poured into 5–10 times their volume of ice-cold buffer D (0.01 *M* Tris, pH 7.8, 0.01 *M* EDTA, 0.1 *M* NaCl, 0.02 *M* NaN_3, and 0.02 *M* NaF). After centrifugation, cells were resuspended in the small volume (1–2 ml) of cold buffer D.

Isolation of DNA from phage-infected cells: Isolation of DNA is the same as reported previously,[1] except that lysozyme treatment was carried out at 0°C for 20 min and then at 43°C for 2 min.

Hydroxyapatite chromatography of DNA:[6,7] Chromatography procedures were basically the same as reported previously.[1] It was found that 0.10–0.40 *M* gradient of phosphate buffer (pH 7.0) is optimal for separation of the different kinds of DNA after bacteriophage T4 infection. Columns of 1 × 3 cm were used.

DNA-DNA hybridization: DNA-DNA hybridization was carried out at 56°C for 20 hr in 5 ml of 2× SSC with 20% dimethyl sulfoxide according to the method of Legault-Démare *et al.*[8] After incubation membrane filters were washed with 3.3×10^{-3} *M* tris (hydroxymethyl)aminomethane (Tris) buffer pH 9.4.[9]

Preparation of labeled standard DNA: C^{14}-*E. coli* DNA was prepared according to modified phenol procedure.[10] P^{32}-labeled DNA from T4 and T7 phage was prepared by the method of Grossman *et al.*[11]

Results.—If there is an enzyme(s) that is related in some way to the conversion of the first intermediate (isolated as single-stranded DNA) into the second intermediate, it was reasonable to hope that infection of a host by a phage strain carrying a mutation of the responsible gene would result in an accumulation of single-stranded DNA. Therefore, after infecting *E. coli* cultures under non-permissive conditions with various T4 strains bearing conditional mutations of genes involved in DNA replication[12] the newly synthesized DNA was analyzed with respect to the secondary structure and origin (host DNA or phage DNA).

Hydroxyapatite chromatography is known to separate single-stranded DNA from double-stranded DNA[6] and was applied to isolate the first intermediate of DNA replication as single-stranded DNA.[1] If the proper conditions of phosphate buffer gradient are employed, native *E. coli* DNA can also be well separated from native bacteriophage T4 DNA. As shown in Figure 1, C^{14}-labeled

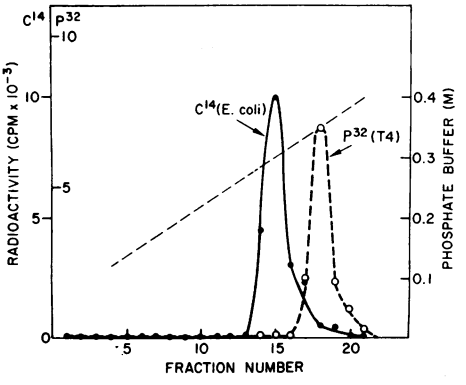

FIG. 1.—Separation of *E. coli* DNA from T4 DNA by hydroxyapatite chromatography. C^{14}-labeled *E. coli* DNA (9.0 μg) and P^{32}-labeled wild-type T4 DNA (1.1 μg) were mixed and fractionated by hydroxyapatite column. Gradient was 0.10–0.40 *M* of phosphate buffer, pH 7.0. Recovery of DNA is usually more than 95% for *E. coli* DNA and 70–80% for T4 DNA under these conditions. ●——●, C^{14}-radioactivity; O- - -O, P^{32}-radioactivity; ——, phosphate buffer concentration.

E. coli DNA was separated from P^{32}-labeled bacteriophage T4 DNA when the phosphate buffer gradient is 0.10–0.40 *M*. *E. coli* DNA was eluted at approximately 0.29 *M* and T4 DNA started to elute at about 0.34 *M* in our preparation of hydroxyapatite. Since it was also found that *E. coli* DNA was separated from T2 DNA but not from T7 DNA, it is possible that glucosylation of T2 and T4 DNA play a role in the separation. A more detailed analysis of this phenomenon will be reported elsewhere.[13] Under the same conditions, single-stranded DNA from T4 was eluted earlier at approximately 0.15 *M* phosphate buffer concen-

tration. It was shown previously that *E. coli* single-stranded DNA can also be eluted at about the same buffer concentration. Therefore it became possible to analyze at least three different species of DNA synthesized after T4 infection in a single-column chromatogram. All the mutants tested were amber or temperature-sensitive T4 mutants whose DNA synthesis after infection under nonpermissive condition was known to be impaired[12] but with the specific function of the mutated gene in DNA replication as yet unknown. These included gene 32 (*am* A453),[14] gene 44 (*am* N82), gene 45 (*am* E10), gene 59 (*am* HL628), and gene 41 (*ts* A14, *am* N81, and *am* N57). Amber (*am*) mutants were infected in nonpermissive *E. coli* B3 (*thy*) at 37°C, and temperature-sensitive (*ts*) mutants were infected in the same *E. coli* strain but at 43°C (nonpermissive temperature). H^3-thymidine was added at four minutes after infection, and at ten minutes DNA was isolated and analyzed by hydroxyapatite chromatography as described above. It should be borne in mind that the experiments to be described here represent analyses of the very limited amount of DNA synthesized after infection by the mutant strains of T4.

Among all the strains studied, only DNA synthesized after infection of *E. coli* by gene 41 mutant strains showed significantly increased radioactivity at the position where single-stranded DNA should be eluted. As shown in Figure 2*A*, DNA synthesized after infection by the gene 41 *ts* mutant (A14) at 43°C can be separated into three distinct peaks of radioactivity, one big peak (fraction I) and two small peaks (fractions IIa and IIb). Since the position where fraction I DNA is eluted is about the same as that of single-stranded DNA, it seemed quite possible that this mutant accumulates single-stranded DNA at high temperature where normal phage DNA synthesis is inhibited almost completely (Fig. 3). Further characterization of this DNA is given later. At 37°C, as is seen in Figure 2*B*, the radioactivity in fraction I is considerably reduced compared to the increase of radioactivity in fraction IIb where double-stranded phage DNA should have appeared. This tendency is still more evident in the profile of DNA synthesized at 30°C (Fig. 2*C*), where only small amounts of radioactivity are present in fraction I compared to that in fraction IIb. As a control experiment (shown in Fig. 2*D*), DNA synthesized after infection by wild-type T4 at 43°C showed primarily a single radioactive peak which is eluted at the position of double-stranded phage DNA. (Note the high levels of DNA (IIb) synthesized.) Essentially the same profile as in Figure 2*A* was obtained with newly synthesized DNA that was labeled after infection by amber gene 41 mutant strains (N81 and N57) of the nonpermissive *E. coli* B (B3 *thy*) at 37°C, except that the amount of the radioactivity in fraction IIb is almost equivalent to that of fraction I. This is probably due to some leakiness and reversions of the mutations in these strains.

The strains with mutations of genes other than gene 41 can be separated into two classes. One group shows only residual host DNA synthesis (fraction IIa only). Infection by strains of the second group results in both residual host DNA synthesis and double-stranded phage DNA synthesis. Among strains harboring mutations of genes with known function, DNA synthesized[15] under nonpermissive conditions by phage bearing a mutation of the ligase gene (gene

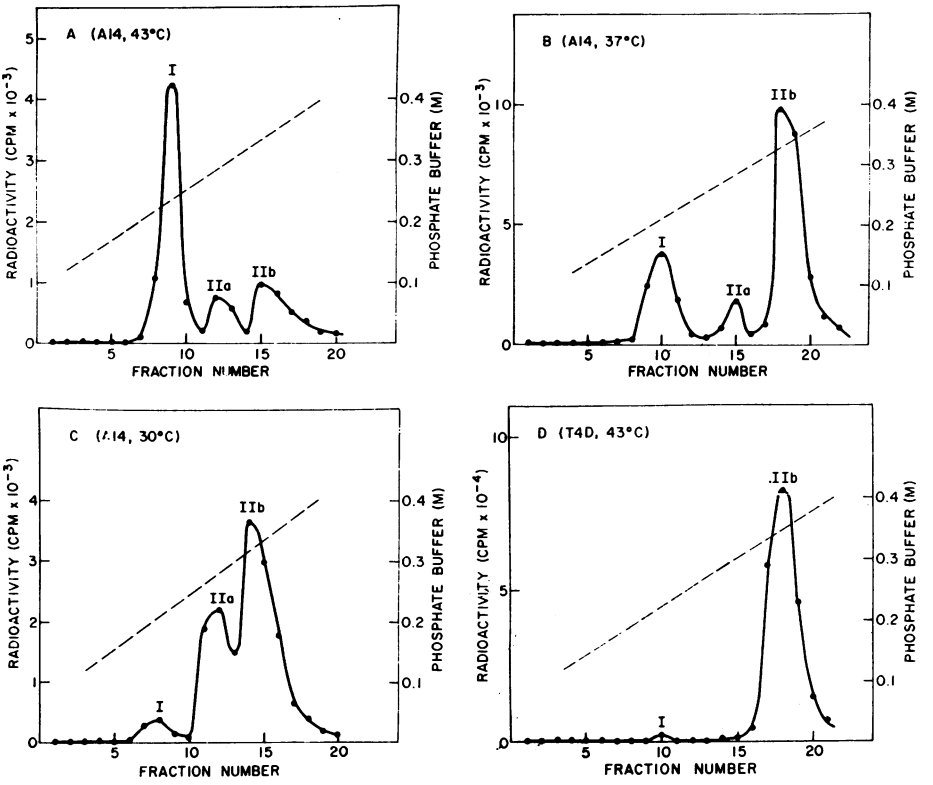

FIG. 2.—Hydroxyapatite chromatography of DNA synthesized after phage infection. DNA isolated from 5 ml of cultured cells infected by the *ts* mutant A14 (*A*, *B*, and *C*) and by wild-type T4 (*D*) was fractionated. Infection was carried out at 43°C (*A* and *D*), 37°C (*B*), and 30°C (*C*).

●——●, H^3-radioactivity, ——, phosphate buffer concentration.

30, *am* H39X)[16] has a small amount of radioactive label in fraction I, as well as a major accumulation in fraction IIb. Further details of the analysis of DNA synthesized after infection of various phage mutant strains will be published later.[13]

The fraction I DNA that was isolated from the DNA synthesized after infection of gene 41 mutant (*ts* A14) at high temperature was characterized. First, the phage origin of this DNA was confirmed by DNA-DNA hybridization. The three DNA fractions (I, IIa, and IIb) of Figure 2*A* as well as control DNA were tested for their ability to hybridize with *E. coli* DNA and T4 DNA. As is shown in Table 1, fraction I DNA hybridizes exclusively with T4 DNA, indicating the T4 origin of this fraction. Fraction IIa DNA hybridizes mostly with *E. coli* DNA, as expected, while fraction IIb DNA hybridizes solely with T4 DNA.

Furthermore, fraction I DNA is resistant to pancreatic RNase, and at least 80 per cent of it is sensitive to exonuclease I (Fig. 4) that attacks only single-stranded DNA.[17] The size of fraction I DNA was tested by alkaline sucrose gradient centrifugation. As is shown in Figure 5, the DNA of this fraction seems

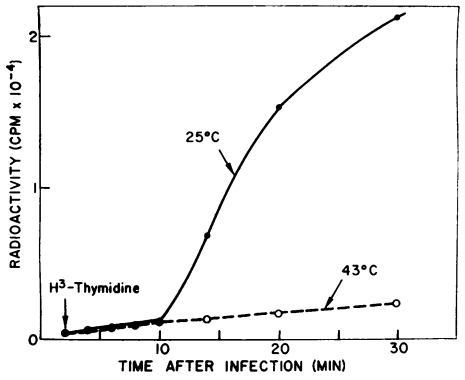

FIG. 3.—Incorporation of H^3-thymidine into DNA after infection by the gene 41 *ts* mutant at low and high temperature. *E. coli* B3 (*thy*) was infected (m.o.i. = 10) by the *ts* mutant (A14) with L-tryptophan (20 μg/ml) supplement at low (25°C) and high (43°C) temperature. At 2 min after infection, H^3-thymidine (1 μc/ml) was added with uracil (5 μg/ml), L-methionine (5 μg/ml), and carrier thymidine (2 μg/ml). Samples (0.5 ml) were withdrawn at various time intervals and trichloroacetic acid-insoluble radioactivity was measured.
●——●, at 25°C; O——O, at 43°C.

to be relatively homogeneous and sediments much slower (approximately 18*S*, molecular weight 2.2 million according to Studier's equation),[18] than reference T7 DNA. The relation of the size of these molecules to that obtained with the ligase mutant strain will be discussed in a subsequent publication.

Discussion.—It is evident that infection of *E. coli* under nonpermissive conditions by bacteriophage T4 bearing conditional mutations of gene 41 results in limited synthesis of new phage DNA, most of which when isolated proves to be single-stranded and of low molecular weight. The results presented here suggest that, at least in bacteriophage T4, a new enzyme dependent upon gene 41 function is involved in DNA synthesis, perhaps in the conversion of a first intermediate into a form that can be isolated as double-stranded DNA. It is es-

TABLE 1. *Hybridization of fractions I, IIa, and IIb with T4 and E. coli DNA.*

No.	DNA immobilized	Input labeled DNA	Hybridization efficiency (%)	Difference
1	—	T4 (P^{32})	1.9	
2	T4	T4 (P^{32})	14.8	12.9
3	*E. coli*	T4 (P^{32})	2.9	1.0
4	—	*E. coli* (C^{14})	1.0	
5	T4	*E. coli* (C^{14})	0.7	−0.3
6	*E. coli*	*E. coli* (C^{14})	9.1	8.1
7	—	Fraction I (H^3)	1.5	
8	T4	Fraction I (H^3)	25.4	23.9
9	*E. coli*	Fraction I (H^3)	1.3	−0.2
10	—	Fraction IIa (H^3)	1.2	
11	T4	Fraction IIa (H^3)	3.9	2.7
12	*E. coli*	Fraction IIa (H^3)	8.4	7.2
13	—	Fraction IIb (H^3)	1.2	
14	T4	Fraction IIb (H^3)	21.1	19.9
15	*E. coli*	Fraction IIb (H^3)	0.9	−0.3

Each labeled fraction (Fig. 2*A*) and labeled control DNA (T4 and *E. coli*) was heat-denatured (100°C, 10 min) before use. DNA (20 μg/filter) was immobilized on filters[21] and hybridization was carried out basically according to the procedures of Legault-Démare *et al.*[8] The amount of DNA input per 5 ml was P^{32} T4 DNA, 1.5 μg; C^{14}-*E. coli* DNA, 2.3 μg; fraction I, 0.2 ml (5800 cpm); fraction IIa, 0.2 ml (1400 cpm); and fraction IIb, 0.2 ml (3000 cpm). Hybridization efficiency is defined as relative amount of DNA hybridized out of total input labeled DNA. For details of hybridization see *Materials and Methods.*

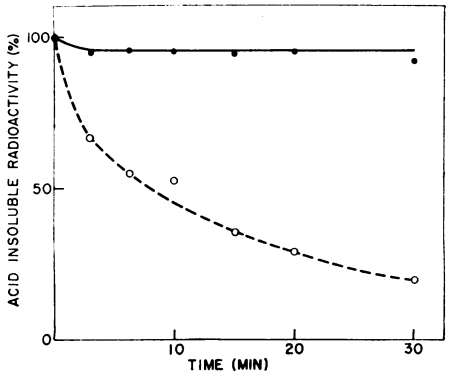

FIG. 4.—Exonuclease I treatment of fraction I DNA. H^3-labeled fraction I DNA (Fig. 2A) and P^{32}-labeled native T4 DNA (1.5 μg/0.3 ml) were mixed and treated with exonuclease I (6 units/0.3 ml, DEAE fraction, kindly provided by Dr. I. R. Lehman) as described by Lehman.[22] Trichloroacetic acid (10%)-insoluble radioactivities were assayed. Radioactivities are shown as relative values compared to the value at zero time incubation.

●——●, P^{32}-radioactivity; O——O, H^3-radioactivity.

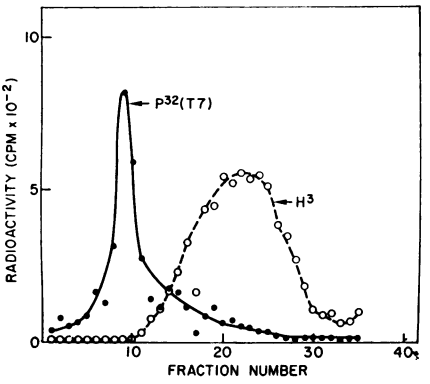

FIG. 5.—Alkaline sucrose gradient of fraction I DNA. H^3 fraction I DNA (Fig. 2A) and P^{32} T7 DNA (as a reference) were layered on an alkaline sucrose gradient (28 ml, 5–20% sucrose in 0.1 *M* NaOH, 0.9 *M* NaCl, 0.001 *M* EDTA)[18] and centrifuged at 22,000 rpm for 16 hr at 5°C in a Spinco SW25.1 rotor. Drops were collected from the bottom of the tubes.

●——●, P^{32}-radioactivity; O——O, H^3-radioactivity.

pecially tempting to consider the gene 41 product as having such a direct role in a conversion, in view of the results previously found with bacteria[1–3] that indicate that the first intermediate of DNA replication is isolated as a low-molecular-weight, single-stranded DNA. However, there is as yet no evidence for the occurrence of a first and second stage in the DNA replication of wild-type T4.

On the other hand, the relationship of the gene 41 product to the formation of stable double-stranded DNA may be much less direct. For example, its primary function could be related to the unwinding of the template DNA with only an indirect coupling effect causing the accumulation of a single-stranded isolation product upon loss of gene 41 function. An example of another possibility is that the gene 41 product catalyzes formation of a protein, the function of which is to stabilize the coupling of the newly synthesized DNA to its template. Loss of gene 41 function would result in an unstable newly synthesized DNA segment—a state not present in the wild-type replicative process. Alternatively, the lack of gene 41 function may result in attack by nucleases which leads to the appearance of single-stranded DNA. In any event, since the combination of DNA polymerase and ligase has been shown to produce biologically active single-stranded ϕX174 DNA *in vitro*,[19] it seems likely that the function of the gene 41 product is specifically related to the replication of double-stranded DNA. The possibility must also be recognized that gene 41 is not necessarily a structural gene for an enzyme. The role of gene 41 is currently under investigation in this laboratory.

The relatively small molecular size of the single-stranded DNA (fraction I), revealed by sucrose gradient centrifugation, is also interesting. This may represent the size of the unit of DNA replicated. In this sense, the comparison of the size of this DNA with the product of ligase mutant, which is also known to sediment slowly in alkaline sucrose gradient centrifugation,[3, 20] is now in progress with the use of a double mutant strain bearing mutations in both gene 41 and gene 30 (ligase gene).

Summary.—Infection of *Escherichia coli* cells under nonpermissive conditions by bacteriophage T4 having a conditional mutation of gene 41 results in the accumulation of newly synthesized DNA which when isolated is found to be mainly single-stranded. The results suggest the involvement of an as yet unidentified enzyme in the replication of double-stranded DNA. The possible function of the gene 41 product in DNA replication is discussed.

I am grateful to Miss Gloria Slywka for her skillful technical assistance. I also wish to thank Dr. I. R. Lehman for sending exonuclease I, and Dr. P. Margolin for his stimulating discussion and help in preparing this manuscript.

* This work was supported by research grant GM 14424-01 from the National Institutes of Health, U.S. Public Health Service.

[1] Oishi, M., these PROCEEDINGS, **60**, 329 (1968).

[2] *Ibid.*, **60**, 691 (1968).

[3] Okazaki, R., T. Okazaki, K. Sakabe, K. Sugimoto, and A. Sugino, these PROCEEDINGS, **59**, 598 (1968).

[4] Steinberg, C. M., and R. S. Edgar, *Genetics*, **47**, 187 (1962).

[5] Kozinski, A. W., and W. Szybalski, *Virology*, **9**, 260 (1959), with the modification described by Thomas, C. A., Jr., and J. Abelson in *Procedures in Nucleic Acid Research*, ed. G. L. Cantoni and D. R. Davies (New York: Harper and Row, 1966), p. 553.

[6] Bernardi, G., *Nature*, **206**, 779 (1965).

[7] Miyazawa, Y., and C. A. Thomas, Jr., *J. Mol. Biol.*, **11**, 223 (1965).

[8] Legault-Démare, J., D. Desseaux, T. Heyman, S. Séror, and G. P. Ress, *Biochem. Biophys. Res. Commun.*, **28**, 550 (1967).

[9] Warnaar, S. O., and J. A. Cohen, *Biochem. Biophys. Res. Commun.*, **24**, 554 (1966).

[10] Saitô, H., and K. Miura, *Biochim. Biophys. Acta*, **72**, 619 (1963).

[11] Grossman, L., S. S. Levine, and W. S. Allison, *J. Mol. Biol.*, **3**, 47 (1961).

[12] Epstein, R. H., C. M. Steinberg, E. Kellenberger, E. Boy de la Tour, R. Chevalley, R. S. Edgar, M. Susman, G. H. Denhardt, and A. Lielausis, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 28 (1963), p. 375.

[13] Oishi, M., manuscript in preparation.

[14] Tomizawa, J., N. Anraku, and Y. Iwama, *J. Mol. Biol.*, **21**, 247 (1966).

[15] Hosoda, J., *Biochem. Biophys. Res. Commun.*, **27**, 294 (1967).

[16] Fareed, G. C., and C. C. Richardson, these PROCEEDINGS, **58**, 665 (1967).

[17] Lehman, I. R., and A. L. Nussbaum, *J. Biol. Chem.*, **239**, 2628 (1964).

[18] Studier, F. W., *J. Mol. Biol.*, **11**, 373 (1965).

[19] Goulian, M., A. Kornberg, and R. L. Sinsheimer, these PROCEEDINGS, **58**, 2321 (1967).

[20] Hosoda, J., personal communication.

[21] Gilespie, D., and S. Spiegelman, *J. Mol. Biol.*, **12**, 829 (1965).

[22] Lehman, I. R., in *Procedures in Nucleic Acid Research*, ed. G. L. Cantoni and D. R. Davies (New York: Harper and Row, 1966), p. 203.