Biological Effects of Substituting Cytosine for 5-Hydroxymethylcytosine in the Deoxyribonucleic Acid of Bacteriophage T4

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Received for publication 30 June 1969

Previous work from this laboratory has shown that the cytosine-containing T4 deoxyribonucleic acid (DNA) made by deoxycytidine triphosphatase (dCTPase) *amber* mutants is extensively degraded, and that nucleases controlled by genes 46 and 47 participate in this process. In this paper, we examine other consequences of a defective dCTPase. Included are studies of DNA synthesis and phage production, and of the control of both early and late protein synthesis after infection of *Escherichia coli* B with various T4 mutants defective in genes 56 (dCTPase), 42 (dCMP hydroxymethylase), 1 (deoxynucleotide kinase), 43 (DNA polymerase), 30 (polynucleotide ligase), 46 and 47 (DNA breakdown) or *e* (lysozyme). By varying the temperature of infection with a temperature-sensitive dCTPase mutant, we have been able to control intracellular dCTPase activity, and thus vary the cytosine content of the phage DNA. We have produced and characterized viable T4 phage in which cytosine replaces 20% of the 5-hydroxymethylcytosine (HMC) in the DNA. We present evidence which suggests that intact, cytosine-containing T4 DNA is much less efficient than is normal T4 DNA in directing the synthesis of tail-fiber antigen. Lysozyme production is much less affected by progressively decreasing dCTPase activity; however, complete substitution of cytosine is correlated with a depression of lysozyme synthesis greater than expected from the defective synthesis of DNA. Low but significant lysozyme synthesis is observed late after infection of *E. coli* B with T4 *amber* mutants defective in a number of genes controlling DNA synthesis. The "20% cytosine" T4 phage, once produced, can initiate an apparently normal infection at permissive temperatures; the synthesis of early enzymes, DNA, and phage does not appear to be impaired. Two roles for HMC in T4 DNA have been indicated previously: (i) involvement in host-controlled restriction of the phage, in which glucosylation of the hydroxymethyl group plays a crucial role (16, 29, 53, 58), and (ii) protection of vegetative DNA against phage-controlled nucleases, a protection not dependent on glucosylation (41, 66, 67). A third role is suggested by our present results: transcription of at least some late genes can occur only from HMC-containing DNA and not from cytosine-containing DNA.

The deoxyribonucleic acid (DNA) of bacteriophages T2, T4, and T6 contains 5-hydroxymethylcytosine (HMC) rather than cytosine [C, (71)]. Most of the hydroxymethyl groups are glucosylated after DNA synthesis (39, 42), which protects the HMC-containing DNA from restriction and degradation during injection into most strains of *Escherichia coli* (16, 29, 53, 58), a restriction which apparently does not affect C-

containing coliphages such as T5. The substitution of glucosylated HMC for C requires the phage-directed synthesis of a number of new enzymes not found in the uninfected bacterium, including deoxycytidine triphosphatase [dCTPase; E.C.3.6.1.12 (38, 39)], deoxycytidylate hydroxymethylase [dCMP HMCase (18)], 5-hydroxymethyl-deoxycytidine, 5'-monophosphate (dHMP) kinase (39, 55), and glucosyl transferases (34, 39, 40, 72).

The small amount of phage DNA produced by dCTPase *amber* (*am*) mutants of T4 (shown to

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contain C; H. Warner, *personal communication*) is degraded extensively and no phage are produced; this degradation is due at least in part to phage-directed enzymes involved in degrading the C-containing host DNA (41, 66, 67). Normal (HMC-containing) T4 DNA is not subject to this intracellular degradation, whether or not it is glucosylated (67). We now describe the synthesis and characterization of viable T4 phage in which C partially replaces HMC, using a temperature-sensitive dCTPase mutant, *tsA90* $\times$ 5, at semi-restrictive temperatures. The rate of synthesis of at least one late protein, the tail-fiber antigen, is found to be markedly reduced when the vegetative DNA contains C, even when most of the DNA made appears to be normal in both amount and size. However, the kinetics of early enzyme synthesis do not seem to be affected by these levels of C in either the parental or the vegetative DNA.

MATERIALS AND METHODS

Phage and bacterial strains. Stocks of *E. coli* B (non-permissive for T4 *am* mutants) and *E. coli* CR63 (permissive for *am* mutants) were obtained from R. Epstein via J. M. Buchanan. Strain JC411 and its ultraviolet (UV)-sensitive, recombination-deficient derivatives JC1553 and JC1554 were obtained from A. J. Clark (7). Strain AB1157 and its UV-sensitive, recombination-deficient derivatives AB2462 and AB2470 were obtained from P. Howard-Flanders (7, 33). Strain W3102 and its UV-sensitive, recombination-deficient derivative W3102 *rec⁻* (M. Meselson, *personal communication*) were obtained from M. Meselson. *E. coli* B strain MT1101, permissive for non-glucosylated T-even phage (47), was obtained from B. Molholt, as was *E. coli* K-12 strain W3110, originally isolated in the laboratory of E. M. and J. Lederberg. The latter strain is nonpermissive for T* phage (T-even phage in which the DNA is unglucosylated) and partially restricts normal T2 (29). *Shigella dysenteriae* strain Sh16, permissive for T* phage (29), was obtained from S. Hattman.

T4 *am*M69 was obtained from W. B. Wood. Wild-type T4 (T4D⁺) and all other T4 mutants were obtained from R. S. Edgar. The following *am* mutants are often identified only by the function controlled by the given gene, or by gene number (represented by numbers in brackets; e.g., [1] = gene 1): *amB24* $\times$ 5 [1], deoxynucleotide kinase (12, 66); *amH39* $\times$ 5 [30], polynucleotide ligase (17); *amN55* $\times$ 5 [42], dCMP HMase (11, 68, 69) *amB22* [43], DNA polymerase (10, 63); *amB14* $\times$ 5 [46] and *amA456* $\times$ 5 [47], presumptive deoxyribonuclease (DNase) (41, 66); *amE51* $\times$ 5 [56], dCTPase (67); *amH26* [e], lysozyme (57). The mutation in *tsA90* $\times$ 5 is in gene 56; this mutant has been shown to make an altered dCTPase (50). The designation "X5" indicates that these mutants have been made isogenic to the wild type (except for the *am* or *ts* mutation) by five successive backcrosses to the wild type (67). A temperature after a mutant name indicates the temperature at which the given stock was grown; stocks of *tsA90* $\times$ 5 were prepared

from *E. coli* B infected at 30 C unless otherwise indicated. The preparation of other phage stocks, the procedure for the construction of multiple mutants, and assay procedures for viable phage and bacteria have been described (66, 67).

Media and infection procedures. Bacteria were grown and most infections were carried out in the glycerol-Casamino Acid (GCA) medium of Fraser and Jerrel (23). "GCA phosphate buffer" is the phosphate portion of GCA medium and is 0.07 M Na₂HPO₄ and 0.033 M KH₂PO₄. Stent and Fuerst's H medium (56), which contains only 6 mg of phosphate per liter, was used for studies involving ³²P incorporation. *Shigella dysenteriae* Sh16 was grown in M medium, a mixture of one part 0.8% nutrient broth plus 0.5% NaCl and one part GCA, in which it grew much better than in GCA alone. Log-phase cultures of *E. coli* B were grown at 37 C as described previously (66). Phage were added at a multiplicity of about 5 (unless otherwise indicated), both at zero time and at 2 to 3 min to ensure lysis inhibition (2).

Chemicals. Unlabeled nucleotides and nucleosides were purchased from Calbiochem, with the exception of dHMP which we prepared enzymatically (69). Thymidine-methyl-³H (3.0 c/mole) and thymidine-2-¹⁴C (either 51.2 mc/mole or 6 mc/mole) were obtained from Schwarz BioResearch, Inc. ³²P was obtained from Nuclear Science and Engineering Corp. Lysozyme (Muramidase), pancreatic ribonuclease (RNase) and pancreatic DNase were obtained from Worthington Biochemical Co. Tris(hydroxymethyl)-aminomethane (Tris) was obtained from Sigma Chemical Co. Dowex-1-X8-formate ion exchange resin (200 to 400 mesh) was prepared from Baker's analytical grade Dowex-1-Cl (J. T. Baker Chemical Co.). Ethylenediaminetetraacetate (EDTA) was the disodium salt (Eastman Kodak Co.) adjusted to pH 8 with NaOH. ¹⁴C-HCHO was from New England Nuclear Corp., and D,L-tetrahydrofolate, prepared by catalytic hydrogenation of folic acid, was from Nutritional Biochemicals Corp.

DNA assays. The spectrophotometric assay for total DNA was that described earlier (67), except that CuSO₄ was added to the indole-HCl reagent to a concentration of 10⁻⁵ M; Short, Warner and Koerner (54) have shown that this increases both the reproducibility and the total color obtained. The incorporation of radioactive thymidine into DNA was measured as described previously (41).

Determination of DNA base composition. The first step was the preparation of ³²P-labeled phage DNA. *E. coli* B at 5 $\times$ 10⁸ cells/ml in H medium was infected at 39 C with eight phage per cell. Six min after infection, 1.5 μ C of ³²P (as orthophosphate) was added per ml. After 60 min, the ³²P was diluted with ³¹P by addition of one-tenth volume of 5 $\times$ concentrated GCA-phosphate buffer. Five min later, the culture was lysed with chloroform, and pancreatic DNase to a concentration of 5 μ g/ml and MgCl₂ to 0.004 M were added. The culture was then incubated without agitation at 37 C for 1 hr, by which time it had lost its viscosity. Bacterial debris was sedimented out at 5,000 $\times$ g for 15 min and the phage were collected by centrifuging for 2 hr at 16,000 $\times$ g. The phage

were suspended in 0.1 M NaCl, a 1 to 5 dilution GCA-phosphate buffer, 0.004 M $MgCl_2$ and treated with 5 μg each of DNase and RNase per ml for 1 hr at 37 C. The cycle of low- and high-speed centrifugation was repeated, and the phage pellet was resuspended in 0.1 M NaCl, 0.01 M Tris chloride, pH 7.5, at a concentration of about 10^{12} phage/ml, giving an absorption per cm at 260 nm (A_{260}) of 10.

The phage DNA was extracted with phenol by the method of Mandell and Hershey (45), and then concentrated and dialyzed in the cold for 36 hr against four changes of 0.01 M Tris chloride (pH 7.5). The A_{260} values of the final DNA solutions were 19 to 25.

The second step was DNA hydrolysis. The DNA was hydrolyzed essentially by the procedure of Lehman and Pratt (42); *E. coli* exonuclease I was the generous gift of I. R. Lehman.

The third step was nucleotide separation. The hydrolyzed ^{32}P -labeled DNA (0.3 to 0.6 ml) was applied to a 20 by 0.8 cm column of Dowex-1- $\times$ 8-formate, 200 to 400 mesh, along with 4 μ moles each of dCMP, deoxythymidylate (dTMP), deoxyuridylate (dUMP), deoxyadenylate (dAMP), and deoxyguanylate (dGMP), and, for *tsA90* $\times$ 5, 0.75 μ moles of dHMP, to serve as markers. The column was washed with 100 ml of water. The various nucleotides were then eluted with a step gradient of pH 4.3 ammonium formate buffer (Fig. 2). This procedure gives complete separation of all of the nucleotides except dTMP and dUMP, which overlap. The eluate containing both dTMP and dUMP (about 200 ml) was adjusted to pH 10 with ammonium hydroxide, and the entire volume was applied to a second 20 by 0.8 cm Dowex-1-formate column. Elution with 0.175 M NH_4HCO_3 [pH 9.5, (8)] then gave good separation of dTMP and dUMP (Fig. 3).

The nucleotides in each peak were identified by comparing their UV absorbance spectra with standard spectra. The radioactivity in 3 ml of each sample (dried on a planchet) was determined in a Nuclear-Chicago Corp. gas-flow counter. Appropriate corrections were made for ^{32}P decay during the course of the experiment.

Killer titer. The killer titer is the concentration of phage particles able to kill bacteria, whether able to form a plaque or not. A culture of bacteria (at a concentration of 5×10^8 cells/ml) in GCA was prewarmed for 3 min at 37 C and infected with about 3 phage per cell. After 10 min, the concentration of surviving bacteria was determined (67), and the killer titer was calculated from the latter value using the Poisson distribution (2).

Sucrose density-gradient centrifugation. The procedures for radioisotopic-labeling of phage DNA and for neutral and alkaline sucrose gradient centrifugation were those used previously (41) except that no shelf of 60% sucrose was used at the bottom of the gradients. The gradients were centrifuged at 15 ± 5 C, both here and in our previous work (41).

Enzyme assays. Cell extracts were normally prepared directly from the chilled cultures by sonic oscillation (66). The assay for dCMP hydroxymethylase (HMase) was that of Wiberg and Buchanan (68), using 0.05 M Tris acetate (pH 7.5) as the reaction

buffer, and incubating enzyme with reaction mixture for 45 min at 37 C. The assay for dTMP synthetase was similar to that described previously (69), but was modified to make it comparable to the newer HMase assay (68). The reaction mixture contained in a 0.5-ml volume: dUMP (disodium salt), 0.7 μ mole; EDTA, 1 μ mole; 2-mercaptoethanol, 50 μ moles; tetrahydrofolate, 0.5 μ mole; ^{14}C -formaldehyde, 0.5 μ mole (1.24 μ C/ μ mole); Tris acetate, 25 μ moles at pH 7.5, and 0.10 ml of cell extract. The final reagent was warmed at 37 C for 5 min before dispensing to allow complete reaction of formaldehyde with tetrahydrofolate (68). After incubation with extract for 60 min at 37 C, the reaction was terminated by adding 1 ml of 0.003 M hydroxylamine hydrochloride and then by placing the tube in boiling water for 2 min. The reaction mixture was applied at room temperature to a 50 by 3.3 mm column of Dowex-1- $\times$ 8-formate. The reaction tube was rinsed with 2 ml of water, which was applied to the column. The column was then washed with 2 ml of 0.2 M NH_4 formate at pH 4.35. The dTMP was eluted with 3 ml of 0.3 M NH_4 formate at pH 4.35. Collecting of eluted residues onto planchets, spreading of residue, and counting of planchets were done as for the dTMP kinase assay (67).

Extracts to be assayed for lysozyme (*E. C.* 3.2.1.17) activity were prepared by sonic oscillation (see above), except for the experiment of Fig. 7 (see the legend to Fig. 7). The lysozyme assay was essentially the method of Sekiguchi and Cohen (52), except that a B/4 mutant of *E. coli* (i.e., a strain which does not adsorb T4) isolated in this laboratory was used as the substrate to avoid possible "lysis from without" (2) by phage in the extracts.

Serum blocking power assay. The assay for serum blocking power (SBP) was patterned after that of De Mars (9). Antiserum against bacteriophage T4 was prepared and titered as described by Adams (2). Because we have observed that sonic oscillation (66) markedly inactivates SBP, extracts were prepared by mixing 2 ml of infected culture with 0.25 ml of chloroform (to lyse the cells) plus 0.25 ml of 40% ammonium sulfate [to prevent adsorption of phage to bacterial debris, (24)]. To avoid interference by viable phage in the assay procedures, the lysates were exposed to UV irradiation which was sufficient to reduce phage viability by a factor of over 10^6 ; this did not affect SBP. T4D⁺ served as the test phage (with survivors plated on *E. coli* B) and *amN55* $\times$ 5 was used as the standard SBP.

RESULTS

Production and characterization of cytosine-containing T4 phage. The temperature-sensitive dCTPase mutant *tsA90* $\times$ 5 produces normal amounts of both DNA and phage at 37 C in *E. coli* B. At 39 C, *tsA90* $\times$ 5 still makes nearly normal amounts of DNA, but phage are produced at only about one-third the normal rate (Fig. 1a). This discrepancy between DNA and phage production is not due to the production of nonviable phage particles. The phage titer agrees well with

FIG. 1. (a) DNA and phage production by *tsA90* $\times$ 5 and *T4D*⁺ at 39 C in *E. coli* B at a concentration of 5.2×10^8 cells/ml. DNA was assayed spectrophotometrically. Symbols: $\circ$, $\bullet$, DNA; $\triangle$, $\blacktriangle$, phage; $\diamond$, DNA of uninfected bacteria. (b) Production of DNA by *tsA90* $\times$ 5 and *T4D*⁺ at higher temperatures in *E. coli* B at a concentration of 4.9×10^8 cells/ml. Symbols: *T4D*⁺, 40.0 C ($\triangle$), 41.0 C ($\blacktriangledown$), and 42.6 C ($\bullet$); *am E 51* $\times$ 5: 42.6 C ($\diamond$).

the titer of killer particles; for example, ratios of 0.87 and 0.95 plaque-forming units per killer particle were obtained for *tsA90* $\times$ 5 and *T4D*⁺, respectively, in the phage preparations used for base analysis (see below). The A_{260} of these purified phage preparations was also consistent with the titer; suspensions of 10^{11} plaque-forming units/ml gave values of 1.15 and 0.96 for *tsA90* $\times$ 5 and *T4D*⁺, respectively (62). A single-burst analysis, performed by the method of Wiberg (66), established that all of the cells infected with *tsA90* $\times$ 5 at 39 C produced progeny, with each cell making about one-third the normal number of phage (data not shown).

The rate of *tsA90* $\times$ 5 phage production was even lower at higher temperatures, with maximal yields of about 47, 27, and 1.5 phage per cell at 40, 41, and 42.6 C, respectively. Again, DNA synthesis was less markedly affected than was phage production, although substantial subsequent degradation of the vegetative DNA was observed at 42.6 C after 20 min (Fig. 1b).

It appeared probable that this variation of phage and DNA synthesis with temperature was a function of the C content of the progeny DNA, which in turn depended on the extent to which the unstable dCTPase made by *tsA90* $\times$ 5 could function in vivo at the given temperature. We, therefore, purified the DNA from ³²P-labeled phage produced by *tsA90* $\times$ 5 at 39 C and determined its base composition to show directly that a malfunctioning dCTPase permits substitution of C for HMC in T4 DNA and to obtain an

indication of the extent of C substitution compatible with the formation of viable phage.

We found that C replaces 20.6% of the HMC in the DNA from the *tsA90* $\times$ 5 (39 C) phage, whereas no C could be detected in *T4D*⁺ DNA (Fig. 2 and Table 1); in both cases, all of the HMC residues were glucosylated. There is support by three facts that the observed C actually came from *tsA90* $\times$ 5 phage DNA and not from contaminating bacterial DNA. (i) The overall base composition agrees closely with published values for T4 and not with those for *E. coli* B (Table 1).

FIG. 2. Elution pattern from Dowex 1 $\times$ 8 (formate) of a deoxyribonuclease digest of ³²P-labeled DNA from *T4D*⁺ and *tsA90* $\times$ 5 phage grown at 39 C on *E. coli* B. (a) *T4D*⁺; the hydrolysis procedure rendered 92% of the ³²P acid-soluble; 94% of the acid-soluble material was recovered as identifiable mononucleotides. (b) *tsA90* $\times$ 5; the hydrolysis procedure rendered 95% of the ³²P acid-soluble; 99% of the acid-soluble material was recovered as identifiable mononucleotides.

TABLE 1. Base composition of the DNA from *tsA90* $\times$ 5 (39C) and *T4D*⁺ phage

Base	Mole per cent			
	Experimental data		Literature values	
	<i>T4D</i> ⁺	<i>tsA90</i> (39 C)	<i>T4</i> ^a	<i>E. coli</i> ^b
C	<0.2	3.4		25
HMC	17.2	13.2	16.3	0
G	16.1	16.1	18.1	25
T	33.4	34.4	33.3	26
A	33.4	32.9	32.3	24

^a Values to the left were obtained from Wyatt and Cohen (71), and those to the right from Hershey, Dixon, and Chase (31).

^b Values obtained from Hershey, Dixon, and Chase (31).

(ii) The phage preparation was treated with DNase, which should have destroyed any DNA not incorporated into phage particles. (iii) Any contaminating bacterial DNA should not be radioactive, since the ^{32}P was added 6 min after infection, when host DNA synthesis has been completely inhibited (31).

The extent of C substitution observed in our experiment confirmed the results of an earlier, less sensitive, experiment in which *unlabeled* $tsA90 \times 5$ (39 C) and $T4D^+$ were grown on *E. coli* B in GCA medium and their base composition analyzed similarly, except that the amount of each base was determined spectrophotometrically. In that experiment, it was estimated that C had been substituted for 21.2% of the HMC residues. This also shows that the extent of C substitution, as well as the relative efficiency of phage production, is similar, whether $tsA90 \times 5$ (39 C) is grown in GCA medium or in H broth.

W. Szybalski (*personal communication*) has used Cs_2SO_4 gradients (16, 59) to examine the DNA isolated from $tsA90 \times 5$ phage grown in this laboratory at 39 and 41 C on *E. coli* B in GCA. He finds that the phage DNA bands in a single peak in each case, with an average density of 1.4396 g/cm³ for $tsA90 \times 5$ (39 C) phage and 1.4379 g/cm³ for $tsA90 \times 5$ (41 C) phage. Since the density of $T4D^+$ DNA is 1.4430 g/cm³ and that expected for 100% C T4 DNA is 1.4213 g/cm³ (60), he concludes that approximately 16 and 24%, respectively, of the glucosylated HMC residues have been replaced by C in $tsA90 \times 5$ (39 and 41 C). The fact that each DNA preparation bands in a single peak suggests that the phage produced at a given temperature are relatively homogeneous with respect to the extent of C substitution. It is also apparent that more C is substituted, the higher the temperature of growth.

We have not measured the extent of C substitution in the vegetative DNA produced by $tsA90 \times 5$ at 39 C and above, most of which is not packaged into phage particles (Fig 1). We would expect that it is at least as high as that in the phage, since DNA production continues longer than phage production, and the $tsA90$ dCTPase has been shown to be unstable, at least in vitro (50).

Warner and Barnes (64) showed that the T4-directed dCTPase is responsible for the dUTPase-dUDPase activity first described by Greenberg for T2 (26), and that dCTPase mutants of T4 also lack this dUTPase-dUDPase. M. Goulian found that deoxyuridine triphosphate (dUTP) can substitute for deoxythymidine triphosphate (dTTP) as a substrate for T4 DNA polymerase in vitro, using denatured salmon sperm DNA as a primer, although the rate of DNA synthesis

with dUTP was only 26% of that with dTTP (*personal communication*). Therefore, it appeared possible that some substitution of uridine (U) for thymine (T) might occur in the DNA made by dCTPase mutants in vivo, and that this substitution could be responsible for some of the abnormal behavior of the dCTPase mutants. However, we found no dUMP in the $tsA90 \times 5$ (39 C) DNA (Fig. 3); substitution of U for 2% of the T could have been detected. H. Warner (*personal communication*) has since examined the small amount of DNA produced by a dCTPase *am* mutant and also finds no U, although C is substituted for over 90% of the HMC. Thus, there is no evidence for substitution of U for T when the T4-directed dUTPase is defective. Moreover, this argues that either the bacterial dUTPase (4, 27) or the preference for T over U of both the kinase (27) and the DNA polymerase, or both, suffice to exclude U from T4 DNA.

Ability of C-containing T4 phage to initiate infection. The $tsA90 \times 5$ (39 C) phage, in which C replaced 20% of the HMC, plated efficiently on *E. coli* B. However, the possibility remained that their C content made these phage susceptible to nucleases such as those which degrade unglucosylated T4 DNA; the time needed for repair of such (sublethal) damage might then be reflected in the kinetics of DNA or phage production under permissive conditions of infection. *E. coli* B was therefore infected at 30 C with a multiplicity of infection of about 0.2 with samples of the $tsA90 \times 5$ (39 and 41 C) phage prepared for W. Szybalski (Fig. 4). Infections also were carried out with other $tsA90 \times 5$ (39 C) phage preparations at multiplicities of infection of 2.5 and 5 (*data not shown*). In all cases, the rates of phage and DNA production were essentially normal. The kinetics of production of HMase and dTMP kinase also were measured at the latter two multiplicities (*data not shown*) and were very similar to those with $T4D^+$, although the cessation of synthesis came slightly later, resulting in a maximal level of enzyme activity that was con-

FIG. 3. Separation of the dTMP and dUMP obtained from the columns used for Fig. 2.

sistently 5 to 30% higher with the *tsA90*×5 (39 C) than with either T4D⁺ or *tsA90*×5 (30 C). Various bacterial strains defective in genetic recombination and the repair of UV damage also appear to support essentially normal growth at 30 C of the *tsA90* (39 C) phage (Table 2). *E. coli* K-12-W3110, which restricts T*4 phage and partially restricts T2, does not restrict *tsA90* (39 C) at 30 C (Table 2); however, this does not rule out the possibility that T4 phage containing substantially more cytosine (if they could be constructed) might be restricted by such strains.

Size of the phage DNA produced by *tsA90*×5 at 39 C. Several explanations seemed possible for the poor efficiency with which the C-containing phage DNA produced by *tsA90* at 39 C is incorporated into phage particles. (i) Some of the DNA might be partially degraded by the nucleases known to degrade vegetative DNA when dCTPase is completely inactive (41). (ii) The synthesis of late (i.e., maturation) proteins might be defective.

FIG. 4. Ability of *tsA90*×5 phage produced at different temperatures (and thus containing different amounts of C in their DNA) to direct the production of DNA and of viable phage during subsequent infection of *E. coli* B at 30 C. The multiplicities were kept low to avoid possible multiplicity reactivation of damage; the actual multiplicities, determined by measuring infective centers, were 0.15, 0.13, 0.20, and 0.15, respectively, for the T4D⁺ and *tsA90* (30, 39, and 41 C). Note that results are expressed per infected cell. At 3 min after infection, 250 μ g of deoxyadenosine per ml and 0.12 μ g of ¹⁴C-deoxythymidine (6 mc/mole) per ml were added. At 4 min, the bacteria (at a concentration of 4.9×10^8 /ml) were superinfected with a [42, 46, 47] triple mutant, at a multiplicity of 5, to cause lysis inhibition in the infected cells (2) and to kill the remaining uninfected bacterial cells in such a way as to prevent further DNA synthesis or degradation in such bacteria; temporal exclusion (13) prevents the expression of any genes of these superinfecting phage in those cells already infected by *tsA90* or T4D⁺. Symbols: (○, ●), T4D⁺ (39 C); (◇, ◆), *tsA90* (30 C); (△, ▲), *tsA90* (39 C); (▽, ▼), *tsA90* (41 C).

(iii) Packaging of the DNA into phage particles might be impaired.

To test the first of these possibilities, we used sucrose density gradient centrifugation to determine the size of the DNA newly synthesized after infection with *tsA90*×5 at 39 C (Fig. 5). Fifteen minutes after infection, when phage synthesis has just started, the sedimentation pattern of the *tsA90*×5 (39 C) DNA in neutral sucrose is very similar to that of T4D⁺ DNA, with about 30% of the DNA in the rapidly sedimenting "replicating form" described by Frankel (19, 21) and little material smaller than mature T4 DNA. By 30 min after infection, most of the *tsA90*×5 (39 C) DNA still sedimented with mature T4 DNA, although a small shoulder was apparent on the low molecular weight side of the *tsA90* DNA (Fig 5c). However, about 36% of the *tsA90* DNA was not recovered under these conditions, as compared with about 6% for the T4D⁺ DNA. That this unrecovered *tsA90* DNA was in the replicating form is indicated by two observations. (i) Replicating-form DNA sediments to the bottom and is generally lost under the experimental conditions graphed in Fig 5c (19). (ii) When *tsA90* DNA from an identical infection was centrifuged through a 20 to 70% neutral sucrose gradient for 100 min at 32,000 rev/min (*data not shown*), 67% was found in the region corresponding to mature T4 DNA. A second component, comprising about 20%, was found which sedimented very rapidly—as rapidly as did all of the DNA produced in a parallel infection with *amN134*×5 (gene 33), a maturation-defective mutant. Frankel showed that this latter DNA has a sedimentation coefficient of about 400S as compared with 200S for the normal T4D⁺ replicating form (20).

Most of the single strands from the *tsA90* DNA (39 C) also sedimented (in alkaline sucrose) like those produced by T4D⁺, although *tsA90* did show an appreciable shoulder of slightly smaller material (Fig 5d). Therefore, we conclude that fragmentation of vegetative DNA is inadequate to explain the poor production of phage by *tsA90* at 39 C. It is not known whether formation of the 400S form by *tsA90* is merely a consequence of the poor maturation of DNA into phage or whether it has some more direct bearing on the low phage production.

Alkaline sucrose gradient studies also were performed on ¹⁴C-deoxythymidine labeled DNA from purified phage produced by *tsA90*×5 at 39 C, and no single-strand breaks were found (*data not shown*).

Production of late proteins after infection with *tsA90*×5. Since the low phage production of *tsA90* at 39 C did not appear to be explainable

TABLE 2. Ability of C-containing *tsA90* $\times$ 5 to grow on various bacterial strains

Strain	Recombination	UV repair	Relative plating efficiency ^a			Phage production per colony-forming cell in liquid culture ^b		
			<i>tsA90</i> (39 C)	T4D ⁺	T*4 ^c	<i>tsA90</i> (39 C)	T4D ⁺	Colony formers per ml $\times 10^{-8d}$
JC411	+	+	1.13	1.12		28	33	9.1
JC1553	—	—	0.82	0.79		25	29	6.2
JC1554	—	—	1.03	0.97		28	36	7.3
W-3102	+	+	1.09	1.01		53	47	6.1
W-3102-rec ⁻	—	—	1.05	0.94		60	64	4.1
AB1157	+	+	0.92	0.85		16	25	2.5
AB2462	—	—	0.94	0.79		20	35	1.2*
AB2470	—	—	0.63	0.61		41	45	1.2*
B	+	+				104	120	6.0
CR63			0.99	1.11	0.24			
W3110			0.80	0.84	0.008			

^a Plating efficiency relative to that on *E. coli* CR 63, using M top and bottom agar at 30 C, except for strains CR63 and W3110, in which case the plating efficiency was relative to that on strain MT1101, which is permissive for T*4 (47).

^b At 30 C; 45 min after infection (repeated at 2 min) with 2.5×10^8 phage per ml.

^c The T*4 phage used were the gift of S. Hattman. The plating efficiency of T*4 on CR63 is far higher than that (0.01) observed by Hattman (28) and probably reflects differences in growth conditions.

^d Preinfection.

* Colonies were small and very variable in size; the actual number of cells was probably higher.

in terms of the size of the DNA produced, we next examined the second possible explanation, namely, that the incorporation of C into T4 DNA impairs its ability to direct the synthesis of late proteins. We found that *tsA90* $\times$ 5 does indeed produce SBP at a much lower rate at 39 C than does T4D⁺ (Fig. 6). SBP production was reduced even more at higher temperatures, closely paralleling the level of phage production (Table 3). Franklin (22) has shown that anti-T4 serum reacts predominantly with the tail fibers and that free tail fibers block antiserum about six times as effectively as do tail fibers which are attached to phage particles. Therefore, the excess of tail fibers over phage produced by either phage may not be as large as it appears from the SBP values in Fig. 6. We conclude that decreased production of tail fibers can nearly account for the low phage production by *tsA90* $\times$ 5 at 39 C and also at the higher temperatures examined.

We have not yet measured the levels of other phage structural proteins under these conditions. However, two facts argue that phage synthesis by *tsA90* at high temperatures is not limited by tail fibers, and, thus, that other late proteins are probably also deficient. (i) The ratio of A_{260} to plaque formers was nearly the same for the purified T4D⁺ and *tsA90* (39 C) phage preparations, indicating that few or no fiberless or non-plaque-forming phage particles were formed. (ii) Wood and Henninger (70) indicated that T4 phage particles with two and three tail fibers form

plaques with probabilities of 5 and 50%, respectively, and particles with more than three fibers are 100% infectious. Thus, the various *tsA90* preparations might possibly contain only three to four fibers per phage. We have measured the rates of adsorption of *tsA90* (39 and 41 C) to *E. coli* B and to *E. coli* CR63 at 37 C (data not shown) and find them equal to those of normal T4D⁺; in the same experiments, as a control, we confirmed Wood and Henninger's observation (70) that the viable, fiber-deficient plaque-formers produced by *amM69* (in gene 63) on *E. coli* B exhibit an abnormally low rate of adsorption. We conclude that both the *tsA90* (39 and 41 C) phage particles contain a full or nearly full complement of tail fibers.

The synthesis of lysozyme, an atypical late protein (see below), appears to be much less sensitive to the increasing defectiveness of dCTPase (Fig. 7). At all temperatures, the initial rate of lysozyme synthesis is normal; its synthesis begins to decrease only at about the time that the rate of accumulation of DNA begins to decrease (Fig. 1b).

There is nothing in our data to indicate that C substitution interferes directly with the packaging of T4 DNA; however, we cannot exclude the possibility that there is some effect, especially when the substitution is greater than 20%.

Kinetics of early enzyme and DNA synthesis in dCTPase am mutant infections. We next questioned whether substitution of C for HMC

FIG. 5. Density gradient profiles of the phage DNA produced by *tsA90* $\times$ 5 and by *T4D*⁺ at 39 C. (a and b) Phage DNA 15 min after infection at 39 C. Centrifugation was through a 20 to 70% gradient of neutral sucrose for 2.5 hr at 32,000 rev/min. The recovery of the *T4D*⁺ and *tsA90* DNA was 99% and 100%, respectively. The scale at the top of a, b, and c indicates approximate molecular weight times 10^{-8} , calculated using the equation of Burgi and Hershey (6) as described by Kutter and Wiberg (41), and should give a reasonable indication of the size, unless the conformation of the DNA differs markedly from that of mature T4 DNA. $\circ$, ^3H -deoxythymidine-labeled intracellular DNA; $\triangle$, ^{14}C -deoxythymidine-labeled marker DNA from mature *T4D*⁺ phage. (c) Phage DNA 30 min after infection at 39 C, when *tsA90* had made 94% as much DNA as had *T4D*⁺. Centrifugation was through a 5 to 20% neutral sucrose gradient for 2.5 hr at 32,000 rev/min. Here and in (d) below, the tritiated *T4D*⁺ and *tsA90* DNA forms were sedimented in separate tubes at the same time, each with ^{14}C -labeled marker DNA, and the positions of the last drops collected (i.e., the tops of the gradients) were superimposed. This procedure resulted in superposition of the marker peaks. The recovery of the *T4D*⁺ and *tsA90* DNA was 94 and 64%, respectively; the *tsA90* DNA not recovered here was undoubtedly in a fast-sedimenting form and was recovered in the alkali gradient (below). Symbols: $\circ$, ^3H -deoxythymidine *tsA90* $\times$ 5 DNA; $\triangle$, ^3H -deoxythymidine *T4D*⁺ DNA; $\bullet$, ^{14}C -deoxythymidine T4 DNA marker.

(d) DNA from the lysates as in (c), but denatured with alkali and then sedimented through a 5 to 20% gradient of alkaline sucrose for 2.25 hr at 30,000 rev/min. The recovery of both DNA forms was 103%. The scale at the top indicates the approximate molecular weight times 10^{-8} of the single strands, calculated using the equation for single-stranded DNA determined by Abelson and Thomas (1). The single-strand breaks evident in the ^{14}C -deoxythymidine-labeled T4 marker DNA, not present in the freshly-prepared ^3H -labeled *T4D*⁺ DNA, undoubtedly were produced by ^{14}C decay during the 4 months between the preparation of these phage and their use in this experiment (61).

in vegetative DNA would affect the shutoff of early enzyme synthesis. A strong correlation has been observed between the synthesis of T-even phage DNA and the cessation of synthesis of early phage enzymes (69) and their messenger ribonucleic acids (mRNA), (5). Some DNA is made in *E. coli* B by dCTPase *am* mutants (and by double mutants also defective in HMase or dHMP kinase) but it subsequently is degraded (Figs. 8 and 11); the DNA made by the two double mutants must contain only C rather than HMC, since no dHTP can be produced. In contrast, no DNA is made after infection with a

[43, 56] double mutant (DNA polymerase⁻, dCTPase⁻); i.e., the rate of incorporation of ^{14}C -deoxythymidine is no higher than the "background" level seen with HMase, kinase, or polymerase mutants alone (Fig. 8). Both the host and the phage-directed DNA polymerases have been shown to use both dHTP and dCTP as substrates in vitro (51); our results show that the phage polymerase can use dCTP in vivo as well.

The basis for the slight "background" incorporation of ^{14}C -deoxythymidine, which we reported earlier (41) and see regularly with "true DNA-negative" *am* mutants, is not clear.

FIG. 6. Production of tail-fiber antigen (SBP) and of viable phage by *tsA90* × 5 at 39°C in *E. coli* B (5×10^8 cells/ml).

TABLE 3. Production of tail-fiber antigen^a and of viable phage by *tsA90* × 5 and *T4D*⁺ at various temperatures in *E. coli* B^b

Temp ^c	30 min				60 min			
	<i>tsA90</i>		<i>T4D</i> ⁺		<i>tsA90</i>		<i>T4D</i> ⁺	
	phage	SBP	phage	SBP	phage	SBP	phage	SBP
39	61	83	170	180	90	175	390	450
40	47	56	100	130	47	73	300	320
41	23	32	140	120	27	44	250	310
42.6	1.3	10	139	170	1.5	20	280	280

^a Measured as serum blocking power (SBP).

^b All values are expressed in phage equivalent units per infected bacterium. The level of unadsorbed phage was 0.3 ± 0.1 phage per cell 10 min after infection.

^c Values expressed in degrees C.

It may be due, at least in part, to any of the following: (i) early addition of nucleotides to parental phage DNA, recently reported by Murray and Mathews (49); (ii) bacterial DNA synthesis in uninfected cells, which, e.g., comprised less than 0.4% of the total cells in the experiment summarized in Fig. 8; (iii) residual bacterial DNA synthesis in a small proportion of cells that may be slow to become inhibited by adsorbed phage, but which are destined to die eventually; (iv) some artifact of the method involving a systematic variation in the ability of

unincorporated label to be washed out of the filters; (v) slight incorporation of deoxythymidine or a labeled contaminant into non-DNA material. The rate of the second incorporation (ii) would be expected to increase with time, however, due to continued growth of such bacteria; in fact, the rate is seen to decrease in Fig. 8.

In Fig. 9, it is seen that the correlation between DNA synthesis and early enzyme shutoff appears to hold even when this DNA contains C; all of those mutants which make some C-DNA show the same kinetics of H_Mase or dTMP synthetase production, or both, as does *T4D*⁺, whereas the double mutant (DNA polymerase, dCTPase) shows the pattern of greatly extended synthesis of both enzymes which is typical of other DNA-

FIG. 7. Lysozyme production by *tsA90* × 5 in the infection described for Fig. 1. The lysozyme levels are not strictly comparable between the 39°C and the 40 to 42.6°C experiments, since the infection at 39°C was a separate experiment and its lysozyme assays were performed with a different batch of substrate. Symbols: Δ, *T4D*⁺; ○, *tsA90*; □, *amE51* × 5. The plotted values represent assays on extracts made with CHCl_3 -ammonium sulfate for the SBP assay. For a number of time points, extracts were also made by sonic oscillation. For *T4D*⁺ and *tsA90*, the lysozyme values agreed well ($\pm 10\%$) between the two types of extract. However, for *amE51*, sonification yielded values higher by 40, 25, 20, and 18%, respectively, at 15, 20, 45, and 60 min after infection. The reason for this is not clear, but it should be stressed that the difference is observed only at very low levels of lysozyme activity.

FIG. 8. DNA synthesis after infection of *E. coli* B (at 6×10^8 cells/ml) at 37 C with T4D⁺ or with *am* mutants unable to produce various enzymes involved in phage DNA synthesis. At 3 min, deoxyadenosine was added (to give a concentration of 250 μ g/ml) followed immediately by ¹⁴C-deoxythymidine (6 mc/mmole), to give a concentration of 0.12 μ c/ml. The following symbols are used to denote mutants defective in the given function(s): $\circ$, dCTPase; $\triangle$, (dCTPase, HMase); ∇ , (dCTPase, kinase); $\square$, (dCTPase, DNA polymerase); $\blacktriangle$, HMase; $\bullet$, T4D⁺; $\diamond$, *amE114*, a dCTPase mutant which maps at a site in gene 56 distinct from that of *amE51*.

negative mutants. However, it is not clear that the normal shutoff mechanism is operative when the DNA contains C (*su* Discussion).

Lysozyme production by dCTPase and DNA-negative *am* mutants. We next questioned whether lysozyme synthesis is affected by defective dCTPase activity; as one type of control, DNA-negative *am* mutants were also examined. The synthesis of most T4 late proteins appears to be absolutely dependent on prior phage DNA synthesis (5, 15, 32, 44). Although lysozyme is not a typical late protein (3, 35, 36, 37, 60), its synthesis also has been reported to require DNA synthesis (15, 35, 46, 52). However, we find that significant lysozyme activity is produced late in infection by *am* mutants defective in HMase, kinase, or DNA polymerase, although it corresponds to only 0.4 to 1.2% of that produced by wild type T4, 60 min after infection (Fig. 10). Similar amounts of lysozyme are produced by mutants defective in gene 44 or 45 (data not shown); *am* mutants in all

five of these genes are classified as "true DNA-negative," i.e., absolutely no DNA synthesis [(15, 65) and Fig. 8]. A lysozyme *am* mutant, and its double mutants with HMase, DNA polymerase, or dCTPase *ambers*, produced no detectable lytic activity (Fig. 10), indicating that the lysozyme being measured is the product of the *e* gene. It seemed possible that this low level of lysozyme synthesis was a response to a low level of DNA synthesis [a small amount of ¹⁴C-deoxythymidine incorporation is seen (Fig. 8)] which might reflect a weak suppression of the

FIG. 9. Synthesis of dCMP hydroxymethylase and dTMP synthetase after infection at 37 C of *E. coli* B (at 5.4×10^8 cells/ml) with T4D⁺ or with *am* mutants unable to produce the phage-directed dCTPase and other enzymes involved in phage DNA synthesis. One unit of activity equals one μ mole product formed per min in the standard assay. The following symbols are used to indicate mutants defective in the given function(s): $\circ$, dCTPase; $\triangle$, (dCTPase, HMase); ∇ , (dCTPase, kinase); $\square$, (dCTPase, DNA polymerase); $\blacktriangle$, HMase; ∇ , kinase; $\blacksquare$, DNA polymerase; $\bullet$, T4D⁺.

FIG. 10. Production of lysozyme. All symbols except $\times$ represent the same extracts as in Fig. 9. Symbols: $\oplus$ = T4D⁺ $\times$ 1/25; $\times$ = [e], [e, 42], [e, 43], and [e, 56] each infecting *E. coli* B separately; these data are from a separate but similar experiment, in which the lysozyme activities produced by T4D⁺ and by [42] were comparable to those shown.

DNA-negative *am* lesion. Such suppression would be expected to be far less efficient in a double *am* mutant. However, we find (*data not shown*) that the double DNA-negative *am* mutants [42, 43], [1, 42], and [1, 43] all produce the same low level of lysozyme activity as do the single DNA-negative mutants, arguing against weak *am* suppression as the basis for the lysozyme synthesis.

Mutants containing a dCTPase *am* lesion, alone or with a HMAse or kinase *amber* lesion [i.e., those mutants which make some unstable, C-containing DNA (Fig. 8)], make several times as much lysozyme as do the "true DNA-negative" mutants, and synthesis appears to start at the normal time (Fig. 10). However, lysozyme still appears at a rate less than 4% of that with T4D⁺. Thus, it appears that DNA synthesis can stimulate lysozyme production to a small extent even when the DNA made contains only C rather than HMC, and is in small fragments.

The rather poor lysozyme synthesis seen when the newly synthesized DNA contains C might, for example, be due directly to its C content or to its fragmentation. A possible way to test this question was suggested by the observation of Hosoda and Levinthal (32) that the DNA made by a [30, 46] mutant is fragmented, and the fragments are even smaller than those made by the [46, 56] mutant [(41) Hosoda, *personal communication*]. The DNA made by the [30, 46] mutant contains HMC, whereas that made by the [46, 56] mutant contains C. We find that the gene

30 mutants make substantially more lysozyme than do the corresponding gene 56 mutants (Fig. 11), although the gene 56 mutants make far more DNA, especially when gene 46 is blocked. These results suggest that the presence of C in T4 DNA depresses its ability to direct lysozyme synthesis to an extent not attributable merely to fragmentation or decreased levels of the DNA produced.

Three other features of Fig. 11 are worth noting. (i) DNA synthesis was measured both spectrophotometrically and by incorporation of ³H-deoxythymidine to reveal possible artifacts in the latter assay, due, for example, to mutationally induced changes in nucleotide pools, or in thymidine uptake (as distinguished from incorporation). No such artifacts are apparent. (ii) We have observed in many experiments that deoxyadenosine, added to facilitate uptake of deoxythymidine (Fig. 11a), depresses net DNA and phage production by as much as 50%; e.g., compare phage production in Fig. 4 with that in Fig. 6. However, in the experiment of Fig. 11a, only a 16% depression of T4D⁺ production was observed (compare sets I and II in legend to Fig. 11). The basis for this variably depressant effect is not clear. (iii) We confirm the work of Hosoda and Levinthal (32) in showing that a gene 30 *am* mutant makes a small amount of unstable DNA and that inclusion of a gene 46 *am* mutation blocks this instability.

FIG. 11. Production of DNA and lysozyme in *E. coli* by various *am* mutants. The following symbols are used to indicate mutants defective in the given gene(s): ♦, [46]; ▽, [56]; △, [30]; ●, [43]; ▼, [46, 56]; ▲, [30, 46]; ○, [30, 46, 56]; ◇, T4D⁺; ×, uninfected cells. Part a is derived from infection set I: for each phage, 1.5 ml of *E. coli* B (at 4.2×10^8 cells/ml) were infected at 37 C. At 5 min, 0.05 ml of deoxyadenosine solution was added to give a concentration of 250 µg/ml. At 5.5 min, 0.05 ml of ³H-deoxythymidine (3 c/mmole) was added to give a concentration of 1.7 µc/ml. Parts b and c are derived from infection set II: for each phage, 12.5 ml of the same culture of *E. coli* B used in set I were infected at 37 C. Samples of 1 ml each were taken at the times noted, for the DNA and lysozyme assays. Phage production per cell at 60 min for sets I and II, respectively, were: T4D⁺, 264, 317; gene 46, 10.3, 11.5. The values for the other mutants were between 0.11 and 0.34 and probably represent unadsorbed input phage.

DISCUSSION

We now have established that C can be substituted for HMC in T4 DNA when the phage-directed dCTPase is defective, and that viable T4 phage can be produced in which C replaces at least 20% of the HMC. However, we find that the incorporation of C into T4 DNA appears to be deleterious in at least two respects. (i) T4 DNA which contains high levels of C undergoes extensive degradation, due at least in part to enzymes involved in degrading the C-containing host DNA (41, 66). Normal, HMC-containing T4 DNA is not subject to this form of degradation, whether it is glucosylated or not (67). We report here that C can replace at least 20% of the HMC without this DNA undergoing much degradation (Fig. 5). The somewhat greater accumulation of both larger and smaller material with *tsA90*×5 at 39 C (Fig. 5) may simply reflect the fact that less of the DNA is packaged into phage particles; this could cause a continued increase in size of the pool of replicative DNA on the one hand, and a small extent of fragmentation by nucleases in general, on the other hand. More DNA is known to be found in the replicating pool when phage maturation is blocked (19, 20). Also, Frankel has observed (19) that the single strands of replicating-form DNA prepared by this technique are no longer than those from mature T4 DNA; longer single strands are obtained by lysing infected cells in the cold (21), a procedure which, however, does not release the DNA from mature phage particles (Frankel, *personal communication*). It should be noted that this T4-directed degradation of C-containing DNA is distinct from the restriction and degradation which occurs during injection of unglucosylated (but not glucosylated) T4 DNA into *E. coli* (25, 28). This latter form of restriction appears to involve only bacterial enzymes, as yet unidentified, and does not appear to affect C-containing coliphage such as T5 or our "20% C" T4.

(ii) The low rates of production of tail-fiber antigen (measured as SBP) in infections (39 to 42.6 C) with *tsA90*×5 (Fig. 6 and Table 3) suggest that the incorporation of C into vegetative T4 DNA may interfere with the transcription of one, if not more, of the genes for phage structural proteins. Lysozyme synthesis is much less affected than is SBP production when C partially substitutes for HMC in the vegetative DNA made by the *ts* mutant, and appears to correlate well with net synthesis of DNA (Fig. 1, 7); this small effect on lysozyme synthesis may simply reflect the general correlation between DNA synthesis and the synthesis of late mRNA and protein (5, 15, 43). However, when substitution of HMC by

C in the vegetative DNA is essentially complete, as with the [46, 56] *am* mutant, lysozyme synthesis is depressed drastically (Fig. 11). The fact that more lysozyme is made by a [30, 46] *am* mutant (Fig. 11), despite the latter's lower production of DNA that is even more fragmented than that made by the [46, 56] mutant, suggests that C in the vegetative DNA can interfere directly with transcription also of lysozyme; however, this interpretation must be taken with caution, in view of our meager knowledge of the mechanism relating DNA synthesis and transcription of late genes and of the effects of fragmentation of the DNA template on this transcription.

After completing this work, we learned that Hosoda and Levinthal (32) and Levinthal et al. (44) also have evidence that the extensive substitution of C for HMC in T4 DNA impairs its ability to direct the synthesis of late proteins. They studied late-protein production after infection of *E. coli* B with *am* mutants defective in gene 30 (polynucleotide ligase) or in gene 56 (dCTPase). Gene 30 *am* mutants, like those in gene 56, make only a little new phage DNA, which subsequently is degraded again to acid-soluble form unless gene 46 or 47 also is defective. They find that dCTPase *am* mutants, with or without a gene 46 lesion, make no late proteins detectable by their gel electrophoresis technique, whereas ligase *am* mutants produce them in substantial quantities. This difference between ligase and dCTPase mutants does not correlate with the relative amounts or sizes of the DNA produced, since gene 30 mutants make less DNA than do those in gene 56, and the double mutant [30, 46] accumulates much less DNA in smaller fragments than does the [56, 46] double mutant [(32, 41) and Fig. 11a]. The only other known difference between the DNA forms produced by these two mutants is the base composition; the DNA produced by dCTPase mutants contains C, whereas that made by ligase mutants must contain only HMC.

Both Szybalski (60) and Bolle et al. (5) have examined the pattern of RNA synthesis after infection with gene 56, [46, 56], and [46, 47, 56] *am* mutants. They find that these mutants are defective in the synthesis of late types of mRNA. Thus, the latter authors suggested that dCTPase *am* mutants make DNA which is incompetent for late transcription. None of these workers could eliminate the possibility that fragmentation of the C-containing phage DNA was responsible for the block in late mRNA and protein synthesis; our work with *tsA90*×5 provides the first evidence of an effect of a dCTPase lesion on late

protein synthesis where most of the C-containing T4 DNA remains intact.

There are several possible explanations for the observed effects of impaired dCTPase activity on the production of late proteins. (i) The RNA polymerase responsible for synthesizing late messenger may have a marked specificity for HMC as compared with C in the template DNA at polymerase-binding sites or throughout transcription, or both. (ii) A DNase may bind at isolated C molecules without breaking the DNA, thus inhibiting transcription. (iii) We cannot rule out the possibility that the impaired late-protein production is a secondary effect of, for example, altered nucleotide pool levels, rather than a direct result of the C in the progeny DNA.

We find that HMase and DNA polymerase *am* mutants and others which make no DNA still make a small amount of phage-directed lysozyme (Fig. 10), although they do not make the typical late proteins (5, 15, 32, 44). The kinetics of lysozyme production differ greatly from those of lysozyme mRNA in infections by DNA-negative mutants, as described by Kasai and Bautz (36), who report only a brief, very early burst of lysozyme mRNA which then decays rapidly and is not accompanied by the appearance of active enzyme. The function of this early lysozyme mRNA is not yet clear. One interesting possibility is that an inactive form of lysozyme is synthesized from the early mRNA; we are measuring its later activation rather than de novo synthesis. Mark has independently observed low levels of lysozyme synthesis after infection of an *am*-restrictive host by a DNA-negative mutant in gene 42, *amN122*, and has shown further that it is proportional to the multiplicity of infection (Mark, Kai-Keung, Ph.D. Thesis, Univ. of Oregon, 1965).

Shutoff of early enzyme synthesis. It is possible that the apparently normal inhibition of early enzyme synthesis after infection with dCTPase *am* mutants actually results from some abnormal mechanism, such as degradation of the parental DNA (which might occur in response to the insertion of C into the complementary strand during replication). Such a mechanism would be analogous to that proposed by Hattman, Revel, and Luria (30) to operate in infections of restrictive hosts by nonglucosylated T-even phage. They showed that cessation of early enzyme synthesis occurred despite failure of DNA synthesis, and correlated with degradation of the infecting DNA. Recent experiments by one of us (J.S.W., unpublished data) show that about half of the parental DNA of a gene 56 *am* mutant is acid-solubilized upon infection of *E. coli* B, whereas that of a [43, 56] double *am* mutant is

not; this implies that replication is a prerequisite for such degradation. Therefore, acid-solubilization of parental DNA may contribute to the cessation of early enzyme synthesis in infection by dCTPase *am* mutants. However, in other experiments not shown, we have found that the arrest of HMase synthesis after infection of *E. coli* B by a [46, 56] mutant was as rapid and complete as with T4D⁺; with this mutant the large amount of vegetative, C-containing DNA is degraded only down to a molecular weight of about 10⁷ (41). Therefore, if we assume that the (HMC-containing) parental DNA also is degraded no further than 10⁷ size (not yet tested), we can conclude that here the arrest of early enzyme synthesis is not due to acid-solubilization of DNA template.

Mosig and Revel (48) demonstrated that early enzymes are transcribed efficiently from two-third size fragments of T4 DNA. On the other hand, Ebisuzaki observed that phenotypic expression of genes for early enzymes is 40 to 50% as sensitive to ³²P decay in the parental DNA as is phage production (14); this can be interpreted to argue that one-third to one-half size fragments cannot be transcribed. It is not unlikely that the location and quality of breaks in the DNA may profoundly influence its template properties *in vivo*. Therefore, at present we cannot decide whether the arrest of early enzyme synthesis with the [46, 56] mutant occurs via the normal mechanism or is due to fragmentation of the parental DNA.

ACKNOWLEDGEMENTS

We wish to express our appreciation to W. Cohn for helpful suggestions on separating dTMP from dUMP; to M. Goulian for testing the ability of T4 DNA polymerase to use dUTP as a substrate in place of dTTP; to J. Hosoda for information on her work prior to publication; to W. Szybalski for determining the density distributions of our *tsA90* × 5 (39 to 41 °C) phage in cesium-sulfate gradients; to both W. Szybalski and P. Geiduschek for unpublished information on the effect of dCTPase *am* lesions on late-messenger production; to H. Warner for information on the base composition of the DNA produced by dCTPase *am* mutants; to R. Swift for useful discussions and excellent technical assistance, particularly with the lysozyme assays; and to I. R. Lehman, for *E. coli* exonuclease I. We thank R. Benzinger, K. Hercules, B. Kretsinger, C. Mickelson, and J. Munro for thoughtful criticism of the manuscript.

This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, and has been assigned Publication No. UR-49-1117. One of us (E.M.K.) was a National Science Foundation Predoctoral Fellow (1962-1966) and a Rochester Atomic Energy Commission Graduate Laboratory Fellow (1966-1968). Most of this work is from a thesis submitted by this author in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

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