Bacteriophage T4-Directed DNA Synthesis in Toluene-Treated Cells

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DNA synthesis has been studied in T4-infected *Escherichia coli* cells made permeable to nucleotides by treatment with toluene. The rate of incorporation of labeled deoxyribonucleoside triphosphates into DNA at various times after infection is proportional to the *in vivo* rate. This *in vitro* incorporation is dependent on all four deoxyribonucleoside triphosphates (5-hydroxymethyldeoxycytidine triphosphate can substitute for dCTP) and Mg^{2+} . It is stimulated by rATP, partially inhibited by pancreatic DNase, and abolished by *N*-ethylmaleimide and 1- β -D-arabinofuranosylcytosine triphosphate. T4 amber DO (DNA negative) and temperature-sensitive DO mutants under nonpermissive conditions of infection fail to induce DNA synthesis *in vitro*. The synthesizing activity is intracellular and the DNA product is exclusively T4 DNA. The *in vitro* synthesis proceeds in a discontinuous manner involving synthesis and subsequent joining of small DNA fragments (about 10S in alkaline sucrose gradients) into larger molecules predominantly one-half the length of mature T4 DNA. No restriction of C-containing or nonglucosylated HMC-containing T4 DNA product is observed in this system.

The complex process of T4 DNA replication proceeds through several stages and depends on the participation of a variety of viral gene products. Some of the enzymes of T4 DNA metabolism have been purified and their function is reasonably well known (29, 20). However, the role of the majority of these gene products, a few of which were recently isolated (1, 4), remains undefined. Another intriguing problem of T4 DNA replication, besides its enzymology, is the characterization of the discrete steps involved in the mechanism of chain growth. T4 DNA replication involves synthesis in a 5' to 3' direction of small DNA fragments and their subsequent joining by polynucleotide ligase (25). Recent findings indicate that DNA synthesis is primed by RNA in *Escherichia coli* and certain phages (31, 35, 30), but it is not known whether RNA synthesis is also a prerequisite for T4 DNA replication.

To study in detail the steps involved in T4 replication, we sought an *in vitro* system which maintained the features of the *in vivo* process. This report describes DNA synthesis in T4-infected *E. coli* cells treated with toluene. This synthesis is specific for T4, requires deoxyribonucleoside 5'-triphosphates and Mg^{2+} , and is absent after infection by T4 mutants unable to

synthesize DNA *in vivo*. Also, evidence is presented that the DNA is replicated in a discontinuous manner.

MATERIALS AND METHODS

Phages. The T4 amber mutants used are as follows: M41 (gene e), C42 (gene 1), E13 (gene 30), C21 (gene 33), N81 (gene 41), N122 (gene 42), N101 (gene 43), N82 (gene 44), E10 (gene 45), N130 (gene 46), and NG485 (gene 62). The temperature-sensitive (*ts*) T4 mutants are A80 (gene 30), P7 (gene 32), A14 (gene 41), and L56 (gene 43). All the T4 mutants are from the Edgar collection.

Bacteria. The *E. coli* B strains ER1 (Benzer strain) and ER47 (permissive for nonglucosylated T4) were provided by J. Eigner (11). The K-12 strains HR110 ($r_{2,4}^+$, r_s^+) and the permissive strain HR112 ($r_{2,4}^-$, r_s^-) were obtained from H. Revel (28). Strain W4597 (a UDPG-pyrophosphorylase mutant) was provided by A. Markovitz. *E. coli* CR63, permissive for T4 amber mutants, served as host for preparation of phage stocks.

Growth medium. The growth medium contained 7 g of Na_2HPO_4 , 3 g of KH_2PO_4 , 1 g of NH_4Cl , 0.5 g of $NaCl$, 0.24 g of $MgSO_4$, 2 g of glucose, and 5 g of Casamino Acids per liter.

Nucleotides and other chemicals. Unlabeled nucleoside triphosphates were purchased from P-L Biochemicals, Milwaukee, Wis. Ara-CTP (1- β -D-arabinofuranosylcytosine triphosphate) was a gift of

M. Goulian. [^3H]dTTP and [$\alpha\text{-}^{32}\text{P}$]dTTP were obtained from New England Nuclear Corp., Boston, Mass., and [^3H]thymidine was from Schwarz/Mann, Orangeburg, N.Y. Nalidixic acid and *N*-ethylmaleimide were obtained from Sigma Chemical Co., St. Louis, Mo. Rifampin was a gift from L. Silvestri. DEAE-Sephadex was purchased from Pharmacia Fine Chemicals Inc., Piscataway, N.J. The RNase was preheated at 80 C for 10 min to inactivate any contaminating DNase.

Preparation of HMdCMP and HMdCTP. HMdCMP(5-hydroxymethyldeoxycytidylic acid) was synthesized by the addition of formaldehyde to dCMP at 75 C in 5 M KOH by the method of Alegria (2). Complete separation of dCMP from HMdCMP was effected by ion-exchange chromatography with Dowex-1 formate. The hydroxymethylated derivative was identified by its UV absorption spectrum and by paper chromatography of its corresponding nucleoside obtained by dephosphorylation with bacterial alkaline phosphatase; the chromatographic solvent was *n*-butanol-5% ammonia (15). HMdCTP was formed by coupling the tributylammonium salt of HMdCMP with triethylammonium pyrophosphate in a reaction promoted by 1,1'-carbonyldiimidazole (19). The triphosphate was purified by DEAE-Sephadex chromatography (8).

Toluene treatment. Cells were grown at 37 C to a density of about $2 \times 10^9/\text{ml}$; L-tryptophan was added to 25 $\mu\text{g}/\text{ml}$ and phage was added to a multiplicity of infection (MOI) of 10. Cells were harvested by centrifugation 20 min after infection, unless otherwise indicated, and resuspended in 70 mM potassium phosphate (pH 7.5) at a cell density of about $1.2 \times 10^{10}/\text{ml}$. Toluene was added to 1%, and the suspension was shaken occasionally at 0 C. The optimum time of exposure to toluene for *E. coli* B strains was about 45 min and for K-12 strains was about 20 min.

Assay of DNA synthesis in toluene-treated cells. The reaction mixture contained in a total volume of 60 μl iters, 70 mM potassium phosphate (pH 7.5), 16.7 mM MgCl_2 , 1.67 mM rATP, 167 μM dATP and dGTP, 333 μM dCTP (or 33 μM HMdCTP when indicated), 67 μM [^3H]- or [$\alpha\text{-}^{32}\text{P}$]dTTP, and 50 μl iters of toluene-treated cells. After a 5-min incubation period at 30 C, the reaction was stopped by chilling to 0 C and the addition of 0.5 ml of 0.5 M NaOH, 1% sodium dodecyl sulfate, and 10% sodium pyrophosphate. After heating at 80 C for 5 min the DNA was precipitated by the addition of 1 ml of 10% trichloroacetic acid-0.1 M sodium pyrophosphate and collected on Whatman GF/C glass fiber filters. The filters were washed with 10 ml of 1.0 M HCl-0.1 M sodium pyrophosphate, followed by 5 ml of ethanol, dried, and counted in a liquid scintillation spectrometer.

Extraction of DNA from toluene-treated cells. For preparation of denatured DNA, the toluene-treated cells were lysed by incubation in 0.3 M NaOH, 30 mM EDTA, and 0.5% Sarkosyl for 30 min at 37 C. For preparation of native DNA, toluene-treated cells were resuspended in a solution containing 0.15 M NaCl, 15 mM sodium citrate, 10 mM EDTA, 20 mM KCN, and 500 μg of lysozyme per ml and incubated at

0 C for 10 min. Sodium dodecyl sulfate was then added to 1.5%, and incubation was continued at 37 C for 20 min. Sarkosyl at 1% was substituted for sodium dodecyl sulfate in the preparation of DNA for CsCl gradients.

Preparation of ^3H -labeled reference DNA. For the preparation of mature T4 DNA, *E. coli* B at a density of 2×10^8 cells/ml was infected at 37 C with T4D at an MOI of 10. Four min after infection, 2 to 5 μCi of [^3H]thymidine per ml was added and 2 h later lysis was completed by the addition of chloroform. Cell debris was removed by a 10-min centrifugation at $8,000 \times g$, and the phage were sedimented at $18,000 \times g$ for 1 h. The phage pellet was resuspended in 70 mM potassium phosphate (pH 7.5) and, when used as a sedimentation reference, was lysed together with the toluene-treated cells.

Singly nicked minicircular [^3H]DNA from *E. coli* 15 was prepared as described previously (7). *E. coli* DNA from the same preparation of labeled cells was isolated by the method of Marmur (22).

Sedimentation analyses. For velocity sedimentation, the DNA was layered onto 4 ml of a linear 5 to 20% sucrose gradient and sedimented in a Spinco SW56 rotor at 20 C. Neutral sucrose gradients contained 10 mM Tris-hydrochloride (pH 8.0), 1 mM EDTA, and 1 M NaCl, whereas alkaline gradients contained 0.3 M NaOH, 0.8 M NaCl, and 1 mM EDTA. Buoyant densities were determined by isopycnic centrifugation in CsCl solutions ($\rho = 1.70 \text{ g cm}^{-3}$) at 35,000 rpm for 60 h at 20 C in a Spinco 65 rotor. Fractions were collected with a siphon onto Whatman 3MM filters which were washed three times with 5% trichloroacetic acid, once with ethanol, dried, and counted in a liquid scintillation spectrometer.

RESULTS

DNA synthesis by toluene-treated T4-infected cells. No DNA synthesis was observed in T4-infected cells after toluene treatment under the conditions used for *E. coli* (24). However, considerable synthesis was obtained if the infected cells were treated with toluene at 0 C with occasional shaking. The rate of [$\alpha\text{-}^{32}\text{P}$]dTTP incorporation into DNA in cells treated with toluene at various times after infection is shown in Fig. 1. Little synthesis was seen in uninfected cells; 3 to 5 min after infection synthesis reached a minimum and thereafter proceeded with an increasing rate. Maximum incorporation was observed 20 min after infection which, in this experiment, is about 20 times the level in uninfected cells and is equivalent to about 0.5 phage equivalent per min per cell. In some experiments the rate reached three times this value. As also shown in Fig. 1, in the absence of dATP, dCTP, and dGTP, negligible synthesis was observed. None of the incorporated label was found in the supernatant fluid after a low-speed sedimenta-

FIG. 1. Deoxyribonucleoside triphosphate dependence of DNA synthesis in toluene-treated, T4-infected cells at various times of the infection cycle. HR110 cells, 2×10^6 /ml, were infected with amM41 phage at an MOI of 10. Samples (3 ml) of the culture were removed at the indicated times, toluene treated, and assayed as described in Materials and Methods. Symbols: ▲, synthesis in the presence of all four deoxyribonucleoside triphosphates; ○, synthesis without dATP, dCTP, and dGTP.

tion of the chilled reaction mixture, which implies that the cells have not lysed and that the DNA synthesizing activity is intracellular.

The kinetics of DNA synthesis in a preparation of toluene-treated cells is linear for 10 min and continues at a reduced rate for about 20 min at 30 C. Therefore, assays were carried out for 5 min to keep within the linear range of activity.

Requirements of the reaction. All four deoxyribonucleoside triphosphates are required for DNA synthesis in the toluene-treated cells since omission of any one of them reduced activity sharply (Table 1). The deoxyribonucleoside triphosphate requirement could not be replaced by deoxyribonucleosides or deoxyribonucleoside monophosphates, and a high concentration of unlabeled orthophosphate did not diminish the incorporation of $[\alpha\text{-}^{32}\text{P}]\text{dTTP}$. The dCTP requirement could also be met by HMdCTP. Omission of rATP reduced incorporation by only one-half, in contrast to the large effect seen in toluene-treated, uninfected *E. coli* (24). The twofold stimulation by rATP was observed throughout the infection cycle and after various times of exposure to toluene. The addition of rGTP, rCTP, and rUTP to the complete system stimulated DNA synthesis about 10%.

The optimum assay temperature is 30 C. At 20, 25, 37, and 40 C, there was, respectively, 50, 80, 75, and 40% as much activity as at 30 C.

Inhibitors of the reaction. Pancreatic DNase inhibited synthesis in T4-infected, toluene-treated cells (Table 2), as expected for replicative but not repair synthesis (24). Both *N*-ethylmaleimide and ara-CTP were potent inhibitors in accordance with the sensitivity of T4 DNA polymerase to such agents (17, 27). There was a consistent but small inhibition of the in vitro synthesis by rifampin and RNase. The energy poisons cyanide and azide inhibit T4 DNA synthesis in vivo (32), but did not inhibit the synthesizing activity in T4-infected,

TABLE 1. Requirements for DNA synthesis in toluene-treated cells^a

System	Activity (%)
Complete	100
-dATP	14
-dGTP	19
-dCTP	13
-dCTP, +33 μM HMdCTP	104
-3dNTP	5
-3dNTP, +167 μM 3dNMP	11
-3dNTP, +167 μM 3dN	9
-MgCl ₂	16
+16 mM EDTA	1
-rATP	42
+835 μM rCTP, rUTP, and rGTP	110

^a The complete system contained 6×10^8 toluene-treated, infected cells, 70 mM potassium phosphate (pH 7.5), 16.7 mM MgCl₂, 1.67 mM rATP, 167 μM dATP and dGTP, 333 μM dCTP, and 66 μM $[\alpha\text{-}^{32}\text{P}]\text{dTTP}$. Incubations were done at 30 C for 5 min. dNTP, dNMP, and dN stand for, respectively, the deoxynucleoside 5'-triphosphate, deoxynucleoside 5'-monophosphate, and deoxynucleoside derivatives of adenine, guanine, and cytosine.

TABLE 2. Effect of inhibitors on DNA synthesis in toluene-treated cells^a

Addition	Inhibition (%)
Pancreatic DNase, 0.6 μg/ml	45
<i>N</i> -ethylmaleimide, 1.6 mM	94
Ara-CTP, 640 μM	91
Rifampin, 6 μg/ml	29
Pancreatic RNase, 0.6 μg/ml	18
KCN, 5 mM	3
NaN ₃ , 10 mM	7
Nalidixic acid, 16 μg/ml	10
2-mercaptoethanol, 13 mM	28

^a The inhibitors at the indicated final concentrations were added to the standard assay given in the footnote to Table 1.

toluene-treated cells. Nalidixic acid had no inhibitory effect, as also observed *in vivo* (3).

Characterization of the DNA product. Pycnographic analysis of the newly synthesized DNA demonstrates that this product is T4 DNA. T4-infected cells were treated with toluene and incubated for 5 min in the presence of HMcCTP, dATP, dGTP, and [α - 32 P]dTTP. After sedimentation to equilibrium in CsCl, all of the 32 P-labeled product was found at the position of T4 DNA (Fig. 2B) and not at the position of *E. coli* DNA (Fig. 2A). In experiments in which dCTP replaced HMcCTP in the reaction mixture, the native DNA product also had the same density as the reference T4 DNA in both CsCl and Cs₂SO₄ gradients. Since the buoyant density of T4 DNA depends on the degree of glucosylation and the amount of substitution of C for HMC (13), it appears that the newly made DNA in T4-infected, toluene-treated cells remains associated with its glucosylated DNA template and the latter DNA predominates in the hybrid. In another experiment the DNA product was characterized apart from its template. The reaction mixture con-

tained infected, toluene-treated cells, dCTP instead of HMcCTP, dATP, dGTP, and unlabeled dTTP. After 3 min of incubation at 30 C, [α - 32 P]dTTP was added and the DNA product was pulse-labeled for 1 min. The DNA was extracted with alkali and sedimented to equilibrium in Cs₂SO₄. The 32 P-labeled, denatured DNA was less dense than the reference T4 DNA, as anticipated for C-containing DNA.

Size of the DNA product. To compare the size of the DNA product with its template, cells were prelabeled with [3 H]thymidine from 10 min after infection. Fourteen minutes after infection, the cells were harvested, toluene treated, and incubated for 5 min in the presence of HMcCTP, dATP, dGTP, and [α - 32 P]dTTP. The DNA was prepared as described in Materials and Methods and analyzed by sedimentation through a neutral sucrose gradient (Fig. 3A). The 32 P-labeled DNA cosedimented with the prelabeled [3 H]DNA. These results are compatible with the buoyant density experiments which showed that the product DNA was associated with a large amount of template-primer DNA. In a control experiment in which toluene treatment was omitted, the prelabeled DNA sedimented at the same position as the prelabeled DNA after toluene treatment, indicating that no-double-strand breaks are introduced in the template DNA by toluene treatment and subsequent incubation.

To determine the single-strand size of the DNA product, the infected cells, after toluene treatment and 5 min of incubation in the presence of HMcCTP, dATP, dGTP, and [α - 32 P]dTTP, were lysed with alkali and the 32 P-labeled DNA was sedimented through an alkaline sucrose gradient. Most of the incorporated label was found in fragments about one-half the size of mature T4 [3 H]DNA, although strands of equal or larger size were also observed (Fig. 4).

Discontinuous synthesis in toluene-treated cells. The work of Okazaki has shown that T4 DNA is synthesized in a discontinuous fashion involving the synthesis and subsequent joining of small DNA fragments (25, 26). The most recently replicated portion, labeled with brief pulses of [3 H]thymidine, can be isolated as short DNA chains of 8 to 11S in alkaline sucrose gradients. Furthermore, inhibition of polynucleotide ligase results in a marked accumulation of the nascent short chains. The same pattern of DNA synthesis occurs in toluene-treated, T4-infected cells. [3 H]dTTP was added to the standard reaction mixture at 30 C for various lengths of time, and the DNA was analyzed by alkaline sucrose gradient sedimentation (Fig. 5A). With a 1-min pulse, most of the incorpo-

FIG. 2. Buoyant density of DNA synthesized in toluene-treated, infected cells. The amM41-infected ER47 cells were harvested 20 min after infection, toluene treated, and incubated at 30 C for 5 min in the standard incorporation mixture containing HMcCTP and [α - 32 P]dTTP. The DNA was extracted as described in Materials and Methods and added to a CsCl solution; the final density was 1.70 g/cm³. Centrifugation was done in a 65 rotor at 35,000 rpm for 60 h at 20 C, and fractions were collected from the bottom of the tube. Symbols: $\blacktriangle$, 32 P-labeled DNA; $\bullet$, 3 H-labeled *E. coli* DNA; $\circ$, 3 H-labeled mature T4 DNA.

FIG. 3. Neutral sedimentation of DNA synthesized in toluene-treated, T4-infected cells [^3H]thymidine was added 10 min after infection of strain ER47 by amM41, and the cells were harvested 4 min later. After toluene treatment, the cells were incubated in the standard assay mixture containing HMdCTP and [$\alpha\text{-}^{32}\text{P}$]dTTP. The DNA was extracted as described in Materials and Methods, and a 100- μl sample was layered on a 4-ml, 5 to 20% neutral sucrose gradient. Centrifugation was done in an SW56 rotor at 56,000 rpm for 40 min at 20 C. The arrow indicates the position of an external reference of mature T4 DNA. Symbols: $\blacktriangle$, ^{32}P -labeled DNA product; $\circ$, prelabeled [^3H]DNA.

FIG. 4. Alkaline sedimentation of DNA synthesized in infected, toluene-treated cells. Cells of ER47 were harvested 20 min after infection by amM41, toluene treated, and incubated in the standard HMdCTP- and [$\alpha\text{-}^{32}\text{P}$]dTTP-containing incorporation mixture. The DNA was extracted as described in Materials and Methods, and 100- μl sample was layered on a 4-ml alkaline sucrose gradient. Centrifugation was done in an SW56 rotor at 56,000 rpm for 40 min at 20 C. Symbols: $\blacktriangle$, ^{32}P -labeled DNA product; $\circ$, mature T4 DNA reference.

rated label was found in a slowly sedimenting component of about 10S. With longer pulses (3 to 5 min), a peak of small DNA was still present but most of the label was in faster sedimenting fragments.

The role of T4 ligase in joining the nascent fragments was tested with T4 *tsA80*, a ther-

mosensitive ligase mutant (14). Cells were infected by *tsA80* at 30 C, and 20 min later the culture was shifted to 43 C, a restrictive temperature. After 5 min at 43 C, the cells were harvested, toluene treated, and incubated in the standard assay mixture at 30 C. The DNA

FIG. 5. Size of pulse-labeled DNA in toluene-treated cells. A, T4 amM41-infected HR112 cells were harvested 20 min after infection, toluene treated, and incubated in the standard assay mixture at 30 C except [^3H]dTTP (2 μCi) was added after 3 min of incubation. Samples were removed 1 min ($\circ$), 3 min ($\blacktriangle$), and 5 min ($\bullet$) after addition of label and pipetted into 2 volumes of a stop solution containing 75% ethanol, 21 mM sodium acetate (pH 5.2), 2 mM EDTA, and 2% phenol. After centrifugation at 6,000 $\times g$ for 10 min, the cells were lysed by incubation in 0.3 M NaOH, 0.02 M EDTA, and 1% Sarkosyl at 37 C for 30 min. A 100- μl sample of the lysates was sedimented in 5 to 20% alkaline sucrose gradients. Centrifugation was done in an SW56 rotor at 35,000 rpm for 3 h at 20 C. The arrow indicates the position of an external reference (about 13S) of singly nicked *E. coli* 15 minicircular DNA. B, HR112 was infected at 30 C with T4 *tsA80* (gene 30) phage. Twenty minutes later the culture was transferred to 43 C. After 5 min the cells were chilled to 0 C, harvested, treated with toluene, and incubated at 30 C in the standard DNA synthesis mixture except [^3H]dTTP was added 3 min after the start of the incubation as in A. After 1 min ($\circ$), 2 min ($\bullet$), 3 min (Δ), and 6 min ($\square$) in the presence of label, the cells were lysed, and the DNA was analyzed in alkaline sucrose gradients as in A.

was pulse-labeled with [^3H]dTTP and analyzed in alkaline sucrose gradients (Fig. 5B). Almost all of the incorporated label was found in short DNA chains. With an increase of pulse time to 2, 3, or 6 min the label in this region increased but little radioactivity appeared in faster sedimenting material. These data show that two steps of the DNA replication process, the formation of short fragments and their subsequent joining, can occur in toluene-treated, T4-infected cells.

DNA synthesis in T4 amber mutants. The pattern of DNA synthesis in toluene-treated cells after infection by a number of T4 amber mutants (Table 3) conforms well to results obtained *in vivo*. The studies *in vivo* have shown that gene 43, the DNA polymerase structural gene (9), and genes 41, 44, 45, and 62, the functions of which are unknown, are essential for T4 DNA replication (12, 33). This was also observed in toluene-treated preparations of a nonsuppressor host infected with amber mutants in these genes; the incorporation of [α - ^{32}P]dTTP into DNA 20 min after infection was less than 2% of the lysozyme mutant (*amM41*) control. An amber mutant in gene 33, defective in maturation, induced almost as much synthesis *in vitro* as the lysozyme mutant. Some synthesis at early times after infection was observed in our *in vitro* system with an amber mutant in gene 30, the ligase structural gene (14), but it decreased at later times thus indicating the importance of T4 ligase in DNA

replication (25). An amber mutant in gene 46, defective in the ability to degrade host cell DNA (12), induced considerable DNA synthesis in toluene-treated cells (about 37% of the *amM41* control) early in infection but this synthesis was arrested at later times, as also seen *in vivo* (12).

Amber mutants in two other genes necessary for T4 DNA synthesis, genes 1 and 42, also directed little synthesis in toluene-treated cells (Table 3). Since genes 1 and 42 are the structural genes for a deoxyribonucleotide kinase and dCMP hydroxymethylase, respectively, cells infected by mutants in these genes are deficient in deoxyribonucleoside triphosphates, particularly HMdCTP (10, 34, 23). Although this deficiency may account for the inability of gene 1 and gene 42 mutants to induce DNA synthesis *in vivo*, it can hardly explain the lack of DNA synthesis in assays with toluene-treated cells where deoxyribonucleoside triphosphates are supplied. Although these two enzymes may have a second more direct role in DNA replication, it is also possible that infection by the mutants in genes 1 and 42 does not induce *in vivo* a sufficient number of growing points to permit normal DNA synthesis after treatment with toluene.

DNA synthesis with temperature-sensitive T4 mutants. Temperature-sensitive mutants in genes 32 (which codes for a DNA-unwinding protein [1]), 41, and 43 induced DNA synthesis in toluene-treated cells after infection at permissive temperatures but not after infection at restrictive temperatures. As shown in Fig. 6B for a temperature-sensitive gene 32 mutant, the capacity to synthesize DNA *in vitro* developed normally after infection at 30 C but not after infection at 43 C. In contrast, the *in vitro* synthetic capacity developed even more rapidly in the control *amM41*-infected cells after infection at 43 C than at 30 C (Fig. 6A).

In the experiments thus far with DO (DNA negative) mutants under restrictive conditions, our *in vitro* assays employed cells in which active T4 DNA replication had never been initiated. In a temperature-shift experiment, cells were infected with *tsA14* (gene 41) or *amM41* at 30 C, and 20 min later when DNA synthesis was in progress the cultures were shifted to 43 C. The capacity to synthesize DNA *in vitro* decayed rapidly after the shift to the restrictive temperature for the *tsA14*-infected cells (Fig. 7B) but was stimulated for the *amM41*-infected cells (Fig. 7A). DNA synthesis, therefore, requires the continuing function of the product of gene 41.

Absence of restriction of nonglucosylated DNA in T4-infected, toluene-treated cells. The dependence of DNA synthesis on the exoge-

TABLE 3. Effect of T4 amber mutations on DNA synthesis on toluene-treated cells

T4 amber	Picomoles of [α - ^{32}P]dTTP incorporated		
	5°	10	20
Gene e	19	47	205
Gene 43	1.0	0.9	0.9
Gene 41	2.5	3.0	2.1
Gene 44	2.2	1.2	0.9
Gene 45	3.9	1.4	1.2
Gene 62	3.2	3.6	3.9
Gene 33	22	64	150
Gene 30	11	3.3	1.2
Gene 46	13	12	7.0
Gene 1	6.0	7.4	4.8
Gene 42	4.3	2.8	2.4

^a HR110 cells were infected with each of the indicated amber mutants. Samples were removed 5, 10, and 20 min later, treated with toluene, and assayed for DNA synthesis as described in Materials and Methods. The activity of an uninfected control ranged from 3 to 9 pmol.

^b Minutes after infection.

nous concentration of HMdCTP or dCTP in T4-infected, toluene-treated cells that are permissive for nonglucosylated T4 DNA is shown in Fig. 8. The less efficient utilization of dCTP at low concentrations may be the result of the active dCTPase induced by T4 (36).

It has been found (16) that toluene-treated, uninfected *E. coli* cells that are restrictive to nonglucosylated T-even phages (T*) in vivo cease DNA replication when HMdCTP is substituted for dCTP, whereas similarly treated cells permissive to the T* phages incorporate HMdCTP and continue DNA replication. However, we find that T4-infected, toluene-treated cells that are restrictive to T* phages show no discrimination against HMdCTP. The kinetics of incorporation of [α - 32 P]dTTP into DNA in vitro were the same whether HMdCTP (33 μ M) or dCTP (333 μ M) was present in the reaction mixture with T4-infected cells of both restrictive and permissive strains of *E. coli* K-12 and *E. coli* B. In addition, the substitution of HMdCTP for dCTP did not diminish the in vitro rate of DNA synthesis after infection of a UDPG-pyrophosphorylase negative host (strain W4597) which is unable to glucosylate T4 DNA (18). It is unlikely that restriction occurred, although synthesis continued, since the single-strand size of the T4 DNA made in toluene-treated cells of a restrictive host (strain HR110) and a permissive host (strain HR112) was the same whether the reaction was promoted by HMdCTP or dCTP. Therefore, under our con-

FIG. 6. DNA synthesis in toluene-treated cells infected with a thermosensitive mutant in gene 32. A culture of HR110 growing at 30 C was divided into two portions when the density reached 2×10^8 cells/ml. One-half was transferred to 43 C, and the other was maintained at 30 C. After 5 min both cultures were infected with either T4 amM41 (A) or with T4 tsP7 (B). Samples (3 ml) were removed at the times indicated, toluene treated, and assayed for DNA synthesis at 30 C for 5 min.

FIG. 7. Effect of temperature shift on DNA synthesis in toluene-treated cells infected with a thermosensitive mutant in gene 41. HR110 (2×10^8 cells/ml) growing at 30 C was infected with T4 amM41 (A) or with T4 tsA14 (B). Twenty minutes after infection (arrow) the cultures were transferred to 43 C. Samples (3 ml) were removed at the times indicated, toluene treated, and assayed for DNA synthesis at 30 C.

FIG. 8. Dependence of DNA synthesis in toluene-treated, T4-infected cells on HMdCTP and dCTP concentration. T4 amM41-infected ER47 cells were harvested 20 min after infection. Toluene treatment and assay conditions are described in Materials and Methods, except HMdCTP or dCTP were added at the indicated final concentrations.

ditions we do not observe restriction of nonglucosylated HMC-containing T4 DNA.

DISCUSSION

Toluene-treated, T4-infected cells appear to maintain the in vivo replicative processes. The rate of DNA synthesis in toluene-treated cells at various times after infection is proportional to the in vivo rate. Buoyant density analysis shows that the DNA product is T4 DNA and that host

cell DNA is not replicated. Amber mutants of T4 in genes 1, 30, 41, 42, 43, 44, 45, and 62 induce little or no DNA synthesis in toluene-treated cells, as also observed in vivo (12). Conversely, mutants blocked in processes other than DNA synthesis, such as lysozyme production and maturation, induce DNA synthesis. No DNA synthesis is observed in toluene-treated cells infected with temperature-sensitive DO mutants at a restrictive temperature. Even after DNA replication has been initiated at a permissive temperature, a shift to a restrictive temperature causes a rapid decay in DNA synthesis after toluene treatment, which implies that the product of the thermosensitive gene is essential for continuous synthesis in this system.

T4 DNA polymerase is required for DNA synthesis in toluene-treated cells. No synthesis is observed after infection by amber or temperature-sensitive mutants (at nonpermissive temperatures) in gene 43, the DNA polymerase structural gene (9). Also, DNA synthesis is markedly inhibited by ara-CTP and *N*-ethylmaleimide, and T4 DNA polymerase is highly sensitive to the substrate analogue and sulfhydryl-attacking reagents (17, 27).

In our system, DNA appears to be made discontinuously, as occurs in vivo. A short pulse of [³H]dTTP labels small DNA of about 10S in alkaline sucrose gradients, and with longer pulses the incorporated radioactivity is transferred to larger DNA molecules. This transfer is dependent on T4 polynucleotide ligase since mutants in gene 30, the ligase structural gene, accumulate small DNA even after long labeling times.

However, there are some differences between DNA synthesis in vivo and in toluene-treated cells. The rate of DNA synthesis in the permeable system approaches one-fourth of that in intact, infected cells. The single-strand size of the newly synthesized DNA is predominantly one-half as long as mature T4 DNA. Extensive replication does not occur in toluene-treated cells since the buoyant density analysis shows that the label incorporated after toluene treatment is associated with DNA that was made before toluene treatment. Therefore, during the 5-min incubation period, the DNA made in toluene-treated cells does not act as a template for further synthesis; initiation of new rounds of replication may not occur in this system.

Mutants in gene 1, the structural gene for a deoxynucleotide kinase (10), and gene 42, the structural gene for dCMP hydroxymethylase (34), cannot induce DNA synthesis in toluene-treated cells. These gene products are neces-

sary, in vivo, for T4 DNA replication, but were expected to be dispensable in the toluene-treated system where the deoxyribonucleoside triphosphates for DNA synthesis, including HmCTP, are supplied. A direct participation of these gene products in the T4 DNA replication apparatus is possible and has been suggested for the gene 42 product (6). A simpler explanation is that DNA synthesis in vitro is dependent on the number of growing points established in vivo and this number is low after infection by the gene 1 and gene 42 DO mutants.

Toluene treatment does not introduce double-strand breaks in the parental DNA since no difference was found in the sedimentation velocity of prelabeled T4 DNA in neutral sucrose gradients prior and after toluene treatment. The synthesis is intracellular and the DNA product remains associated with its template. The DNA profiles of the template and the product in neutral sucrose gradients are nearly superimposable, suggesting that the in vitro synthesis continues intact the replication at the growing points preformed in vivo.

T4 induces several nucleases which selectively attack C-containing DNA (20). No extensive and endonucleolytic degradation of C-containing DNA was observed in toluene-treated cells, perhaps because synthesis is not very extensive in our system, and C can replace at least 20% of the HMC in vivo, without this DNA undergoing much degradation (21).

Host-controlled restriction of non-glucosylated HMC-containing T4 DNA was not observed in toluene-treated cells of a restrictive host. This is consistent with the finding in vivo that phage particles in a T* lysate that have retained one glucosylated DNA strand were protected from the *E. coli* restriction (5). Also, the absence of restriction of nonglucosylated T4 DNA, in our experiments with toluene-treated cells of a strain defective in the ability to synthesize UDPG, indicates that intracellularly the nonglucosylated T4 DNA is protected from restriction damage. In vivo, it is known that T4 can grow for a single cycle in this mutant and produce full yields of T* phage (18).

DNA synthesis in T4-infected, toluene-treated cells is stimulated only twofold by rATP whereas a 10- to 20-fold stimulation is seen in toluene-treated, uninfected *E. coli* (24). The ATP-independent synthesis in uninfected cells has been attributed to repair rather than replicative synthesis since it is DNA polymerase I dependent, present in mutants unable to replicate DNA, and stimulated by pancreatic DNase (24). In contrast, the ATP-independent synthe-

sis after T4 infection is dependent on T4 DNA polymerase, absent in a number of DO mutants, and inhibited rather than stimulated by pancreatic DNase. It is possible that the endogenous ATP is sufficient to satisfy an ATP requirement of T4 replication or the twofold stimulation may be just a reflection of the need to maintain the terminal phosphates of the exogenous deoxynucleoside triphosphates.

It is clear that this system is permeable to molecules of low molecular weight and can afford access of pancreatic DNase to the DNA within the cells. Whether the system is permeable to larger macromolecules remains to be seen.

The toluene-treated system is a useful tool for the study of the stages in T4 replication. Two steps involved in T4 replication, the formation of Okazaki fragments and their subsequent joining by ligase, were demonstrated in this report. Recently, we have observed RNA attached to DNA in toluene-treated, T4-infected cells. The possibility of RNA involvement in T4 DNA replication is presently under investigation.

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