JOURNAL OF VIROLOGY, Dec. 1973, p. 1195-1203

Vol. 12, No. 6
Printed in U.S.A.

Replication of T4 DNA in *Escherichia coli* Treated with Toluene

R. C. MILLER, JR., D. M. TAYLOR, K. MACKAY, AND H. W. SMITH
Department of Microbiology, University of British Columbia, Vancouver 8, Canada

Received for publication 6 August 1973

Deoxyribonucleoside triphosphates are incorporated into T4 DNA in infected cells treated with toluene. Under the proper conditions the incorporation is controlled by the known T4 DNA polymerase and proceeds by a semiconservative mechanism. Both strands of the phage DNA are replicated into a high molecular weight progeny molecule. The replication system is accessible to extracellular pancreatic DNase added to the reaction mixture. At early times after infection a second replication system, not under control of the gene 43 polymerase, has been detected which synthesizes T4 DNA in toluenized cells.

Until recently, no in vitro system has been shown to faithfully replicate T4 DNA in a sustained manner by the normal semiconservative mechanism (1, 9, 21). Therefore, it has been our objective to establish conditions which allow incorporation of deoxynucleoside triphosphates into replicating T4 DNA. Furthermore, it has been hoped that the system would be permeable to phage-coded proteins for in vitro complementation studies. The development of this system should be very useful in studying nucleotide sequences at initiation sites (3) or "first-step-breakdown product" (FSBP) starts (13), for studying the nature of replicative intermediates (2, 7, 15, 22), and hopefully for studying the role of various enzymes in the replication process.

Moses and Richardson (24) have reported that treatment of *Escherichia coli* with toluene permits the incorporation of deoxynucleoside triphosphates into progeny bacterial DNA. This communication reports the adaptation of this technique to T4-infected cells. The necessary reaction conditions and the nature of the product are discussed. It is shown that under the appropriate conditions deoxynucleoside triphosphates are incorporated into T4 DNA by a basically semiconservative mechanism, that the synthetic system is under the control of phage-coded enzymes, and that the system may possibly be complemented by the addition of enzymes or soluble extracts.

MATERIALS AND METHODS

Deoxynucleoside triphosphates. Deoxynucleoside triphosphates (dNTP) labeled with ^{32}P in the alpha (α) position were the generous gift of the New England Nuclear Corp. They were checked for purity by paper chromatography in system A of Kleppe, Van de Sande and Khorana (12).

^{3}H-dNTP were purchased from the New England Nuclear Corp. The specific activity of dNTP used in the reaction mixtures was generally 40 μCi/mmol.

Preparation of labeled bacteriophage. (i) ^{32}P. Bacteria pregrown in ^{32}P-orthophosphate TCG (specific activity 0.5 to 2 mCi/mg) were infected with bacteriophage; after 120 min the cells were lysed with chloroform, and the phage were purified by differential centrifugation. Occasionally, the phage were banded in CsCl density gradients for further purification. (13).

(ii) ^{3}H. Bacteria were pregrown in TCG with thymidine (5 μg/ml), 5-fluorodeoxyuridine (5 μg/ml), and uracil (25 μg/ml); after infection with phage the culture was supplemented with ^{3}H-thymidine at 2 μCi/μg. The progeny phage were purified as above.

(iii) 5-BUdR. Bacteria were pregrown in TCG containing 5-bromodeoxyuridine (5-BUdR) (200 μg/ml), 5-fluorodeoxyuridine (5 μg/ml), and uracil (25 μg/ml). The bacteria were infected with bacteriophage, and the progeny phage were purified as above.

TCG medium is Tris-Casamino Acids glucose medium (17). 10 × PO_4 TCG contains 250 μg of P per ml (17).

Treatment of cells with toluene. *E. coli* B23 were grown to 3 × 10^8 in 10 × PO_4 TCG and infected with bacteriophage at a multiplicity of infection (MOI) of 10. At various times after infection (usually 8 min, see Results), the cells were ice chilled, centrifuged at 9,000 × *g* for 5 min at 4 C, resuspended in 0.05 M potassium phosphate buffer, pH 7.0, at 5 × 10^9 cells/ml, and incubated with 1% toluene at 23 C for 15 min. The cells were ice chilled, washed free of toluene by centrifugation, and resuspended at 5 × 10^9 cells/ml in 0.05 M potassium phosphate buffer, pH 7.0.

Standard reaction conditions. Treated cells at 3 × 10^9/ml were incubated in a reaction mixture containing 0.05 M potassium phosphate buffer, pH

7.0, 0.0125 M $MgCl_2$ and 100 μM for each of the four dNTP.

Assay for trichloroacetic acid-insoluble material. Samples were pipetted onto 2.5-cm Whatman 3 MM filter disks and washed batchwise in 5% trichloroacetic acid three times for 15 min at 4 C, once in 50% ethanol-50% ether and once in anhydrous ether. The filters were dried, overlaid with toluene-based 1,4-bis-2-(5 phenyloxazolyl)benzene (POPOP) and 2,5-diphenyloxazolyl (PPO) scintillant and counted for radioactivity in an ISOCAP 300 scintillation spectrometer.

SDS-Pronase-phenol extraction. The procedure for extraction of intracellular T4 DNA was that of Kozinski and Lin (16).

Alkaline sucrose gradient sedimentation was conducted as described previously (22).

CsCl density gradients were analyzed according to the procedure of Kozinski, Kozinski, and James (15).

RESULTS

Kinetics of incorporation. The first step in studying phage DNA replication was to establish the kinetics of incorporation of α-^{32}P labeled dNTP into phage DNA. Figure 1 compares the incorporation of the α-^{32}P dNTP into trichloroacetic acid-insoluble material in cells infected with T4 or T7, and noninfected cells. All reaction mixtures contained the same number of cells. Incorporation was linear and continuous for up to 90 min. Frequently, the cells lysed if incubation was continued for longer times. The kinetics of incorporation were the same if the parental phage was uniformly labeled with 5-bromodeoxyuridine.

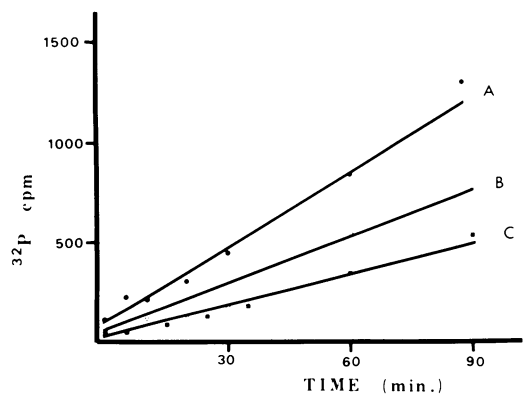

FIG. 1. *Incorporation of α-^{32}P-dNTP by infected E. coli treated with toluene. E. coli B23 was grown to 3 × 10^8 in TCG medium (13) and infected with T4 or T7 bacteriophage at an MOI of 10 and incubated at 37 C for 8 min. The cells were treated with toluene and incubated in the standard mixture as described in Materials and Methods. The cell concentrations and specific activities of the three reaction mixtures were identical. Incorporation into acid-insoluble material was measured in identical samples. A, T4; B, E. coli; C, T7.*

Specificity of template. The DNA being synthesized in the phage-infected cells was predominantly phage specific. DNA labeled during the incubation in the reaction mixture was extracted by the SDS-Pronase-phenol method and tested for hybridization against the appropriate DNA by the method of Denhardt (4). Table 1 shows that the DNA synthesized in T7-infected cells was T7 specific and that DNA synthesized in T4-infected cells was T4 specific.

Reaction conditions: length of toluene treatment. When it was known that incorporation of dNTP was linear, sustained, and specific for the phage DNA, the optimal time of toluene treatment was determined. Infected cells were treated for various times with toluene and then incubated for 30 min in a standard reaction mixture. Figure 2 shows that the optimal time of treatment with toluene was 15 min.

TABLE 1. *Hybridization of newly synthesized DNA*

Source of DNA from cells treated with toluene	Radioactivity associated with DNA		
	T4		*E. coli*
T4-infected *E. coli*[a]	11,150		160
	T7		*E. coli*
T7-infected *E. coli*	5,251		769
	T4 l	T4 r	*E. coli*
T4-infected *E. coli*[b]	476	564	18
	T4		*E. coli*
T4 ts L97 at 42 C in *E. coli*[c] treated with toluene at 6 min postinfection.	734		36

[a] *E. coli* was grown to 3 × 10^8 and infected with bacteriophage T4 or T7. The cells were treated with toluene and incubated in the standard reaction mixture for 30 min. The cells were lysed and the DNA was extracted as described. The newly synthesized DNA was hybridized against DNA from *E. coli*, T4 or T7 as described by Denhardt, 1966. Radioactivity is expressed as counts per 10 min.

[b] DNA as prepared above was hybridized against the separated strands of T4. Radioactivity is expressed as counts per 10 min.

[c] *E. coli* B23 was grown to 3 × 10^8 in TCG and infected with T4 ts *L97* at 37 C. At 6 min after infection the cells were treated with toluene and incubated for 20 min at 37 C in a standard reaction mixture with no radioactive dNTP. The temperature was shifted to 42 C and the reaction mixture was incubated for 5 min at 42 C. ^{3}H-dNTP then were added and the reaction was allowed to incubate 15 additional min at 42 C. The DNA was purified by the SDS-Pronase-phenol method and hybridized against T4 and *E. coli* DNA. Radioactivity is expressed as counts per 10 min.

Concentration of deoxynucleoside triphosphates. The effect of the dNTP concentration on the rate of DNA synthesis was studied for the range 5 μM to 1 mM. Since the kinetics of incorporation were linear up to 30 min after infection, toluenized cells infected with T4 were incubated for 30 min in the standard reaction mixture containing various concentrations of dNTP. Total incorporation was measured. Figure 3 shows that the rate of synthesis increased as the dNTP concentration increased to 1mM. A low level of synthesis was still maintained, if any one of the four dNTP was omitted from the reaction mixture. This was not an unexpected result since the phage-coded kinases are synthesized very early in infection (26), and nucleotides are being released to the intracellular pool from phage-induced degradation of the host DNA. However, a maximal rate of synthesis was attained only when all of the dNTP was at 1 mM. Raising only dCTP to 1 mM did not significantly raise the rate of synthesis if all of the other dNTP were at 40 to 100 μM. Therefore, dCTPase was not playing a major role in controlling the overall rate of synthesis.

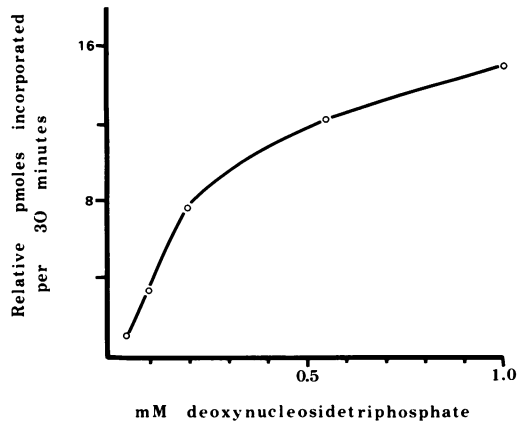

FIG. 3. *Effect of dNTP concentration on DNA synthesis. E. coli B23 was infected with T4, treated with toluene and incubated with identical total amounts of radioactive dNTP in various samples. Each sample varied in the final concentration of dNTP. Therefore, each sample varied in specific activity, also. Portions of each sample were assayed for acid-insoluble radioactivity after 10, 20, and 30 min of incorporation. The incorporation for each reaction mixture was determined, and the graph compares the amount of acid-insoluble material incorporated in 30 min for each dNTP concentration.*

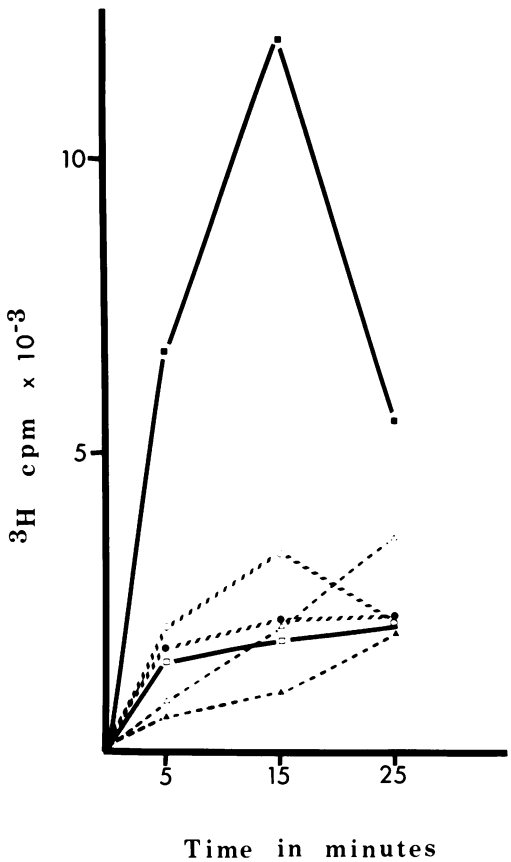

FIG. 2. *Duration of incubation in toluene. E. coli B23 was grown and infected with T4 or T7. Samples of infected cells were treated with 1% toluene for various lengths of time. The treated cells then were incubated with the radioactive dNTP and the total incorporation in identical samples was measured after 30 min. When ATP was added to a reaction mixture, the final concentration was 1 mM. Symbols: —□—, T4 +ATP; —■—, T4 −ATP; ···△···, E. coli +ATP; ···▲···, E. coli −ATP; --○--, T7 +ATP, --●--, T7 −ATP.*

Effect of ATP. Several investigators have shown that ATP stimulates incorporation of dNTP by toluenized cells. This has been linked to the difference between semiconservative and repair synthesis in these systems (19, 24). In toluenized cells infected with T4 the addition of ATP in the range of 5 μM to 1 mM was either inhibitory or had no effect at all. Replication was semiconservative in the absence of ATP in the phage-infected system (see later results).

Mg^{2+} concentration. When triphosphate concentrations are increased to a high level, Mg^{2+} concentrations may become too low to sustain replication. Figure 4 shows that a convenient range of Mg^{2+} could be used in the reaction mixture. Therefore, sufficient Mg^{2+} could be added to overcome potential chelating capacity of the reaction mixture.

Nature of the product: CsCl density gradient analysis. The following experiment was run to show that in the phage-infected system the dNTP were labeling replicating DNA. T4

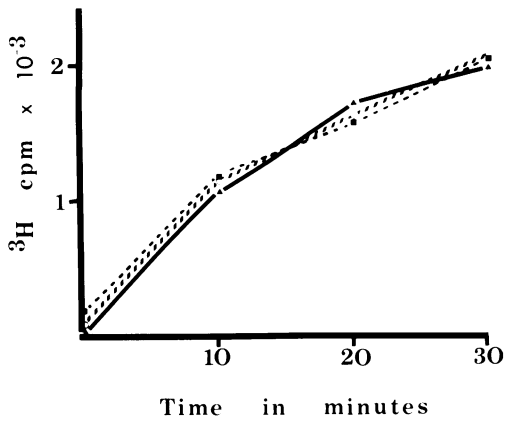

FIG. 4. *Effect of Mg^{2+} on T4-DNA synthesis. E. coli B23 was grown, infected, treated with 1% toluene, and incubated with the standard reaction mixture as described previously. Samples were incubated with varying concentrations of Mg^{2+} (10, 15, and 20 mM), and the incorporation of radioactive dNTP into acid-insoluble material was determined for various times of incorporation. Symbols: --O--, 10 mM; --■--, 15 mM; —▲—, 20 mM.*

phage labeled with ^{32}P and 5-BUdR were used to infect *E. coli*. The infected cells were incubated, harvested, treated with toluene, and incubated in the standard reaction mixture. At 30 min the DNA was extracted by the SDS-Pronase-phenol method and centrifuged to equilibrium in a CsCl density gradient. The progeny DNA labeled in the in vitro reaction was found at the completely light location in the gradient (Fig. 5). Very little ^{3}H label was found at the hybrid location or at the parental location, where label incorporated by repair synthesis would be found. Therefore, the first and most important criterion for semiconservative replication was satisfied; there was no covalent attachment of progeny label to parental DNA in a nonrecombined T4 DNA duplex (8, 14, 25). The same result was obtained regardless of whether the parental ^{32}P, 5-BUdR-labeled phage was purified through CsCl before use in the toluenization experiment. This eliminated the possibility that a small amount of contaminating, unlabeled, light phage was being repaired preferentially.

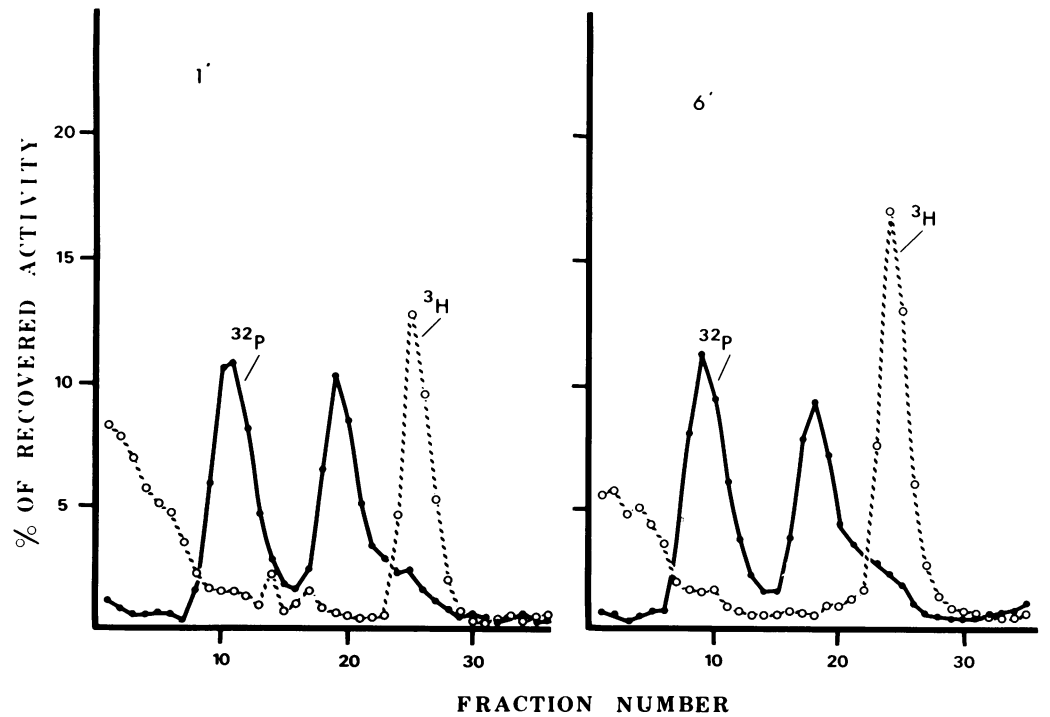

FIG. 5. *CsCl density gradient analysis of progeny DNA. E. coli B23 was grown to 3 × 10^8 in 10 × PO^4 TCG, infected at an MOI of 7 with ^{32}P-T4 labeled with 5-BUdR, treated with toluene, and incubated in a standard reaction mixture containing ^{3}H-dNTP for 1, 6, and 30 min at 37 C. The cells were lysed with SDS, and the DNA was extracted by the Pronase-phenol method. The extracted DNA was banded in a CsCl density gradient, and the distribution of the acid-insoluble material in the gradient was analyzed. The distribution of labels at 30 min was the same as that of the 1- and 6-min samples.*

Essentially the same result was obtained if ^{32}P, 5-BUdR-labeled parental phage were used to infect bacteria grown in 5-BUdR-labeled medium (Fig. 6). Most of the progeny label was found at the completely light location, although some was retained at the hybrid location. This was to be expected in this system, because all of the progeny label must be incorporated initially off of a completely substituted (5-BUdR-labeled) template. Since all of the intracellular DNA is labeled with 5-BUdR before tolueniza-tion in this experiment, the progeny DNA at the light location must have been synthesized on a heavy template. Therefore, this eliminates the possibility that the experiment in Fig. 5 could be explained on the basis of a preferential replication of unlabeled, light, progeny replica-tive DNA.

Two other features of Fig. 5 and 6 should be noted. First, there is a significant amount of radioactivity banding at a position correspond-ing to the RNA-linked DNA molecules found in T4 (2) and T7-infected cells (20). Second, very little additional parental label is transferred to a hybrid location during the incorporation of dNTP. Therefore, no new initiation of replica-tion is taking place in duplexes of parental origin once the cells are treated with toluene; most new initiations are probably being made on progeny DNA molecules in the tolueniz-ed cells.

Control of phage replication. Gene 43 of T4 codes for the known T4 DNA polymerase (5), which is necessary for phage DNA replication in vivo (6). Therefore, the involvement of the gene 43 product in the toluenized cell system was examined. *E. coli* B23 was infected at 37 C with T4 ts *L97* (temperature-sensitive gene 43 prod-uct). The infected cells were incubated at 37 C, and at 8 min after infection the cells were harvested and treated with toluene in the usual way. The treated cells were incubated in the standard reaction mixture at 37 C for 20 min and then were shifted to 42 C. Figure 7 shows that incorporation was stopped almost immedi-ately by the temperature shift. A control reac-tion with cells infected in parallel with wild-type phage continued uptake at the usual rate, as expected. However, if cells were treated with toluene at 6 min after infection instead of 8 min, incorporation was not stopped by the tempera-ture shift (Fig. 8). Incorporation of dNTP into phage DNA in this system was not dependent on gene 43 product. Hybridization studies of DNA synthesized by cells infected with the T4 ts *L97* and treated with toluene at 6 min after infection showed that the DNA being synthe-sized at the restrictive temperature was phage specific (Table 1). The nature of the system responsible for this early temperature-independent synthesis is an interesting question for future study. All further studies reported here were conducted on reaction mixtures con-taining infected cells which were treated with toluene at 8 min after infection. Under these conditions the system was dependent on the

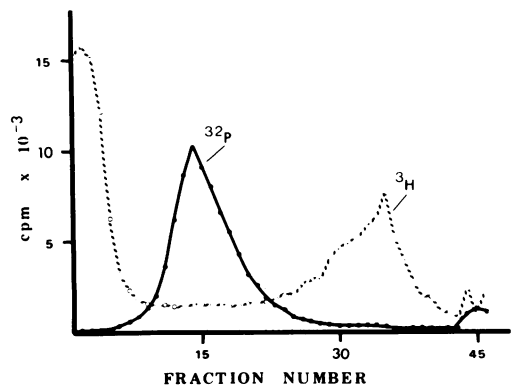

FIG. 6. *CsCl density gradient analysis of progeny DNA. E. coli B23 was grown for two generations in medium containing 5-BUdR (200 μg/ml), FUdR (5 μg/ml), and uracil (25 μg/ml) and was infected with ^{32}P-T4-labeled with 5-BUdR. The cells were treated with toluene, incubated with ^{3}H-dNTP, and analyzed as in Fig. 5.*

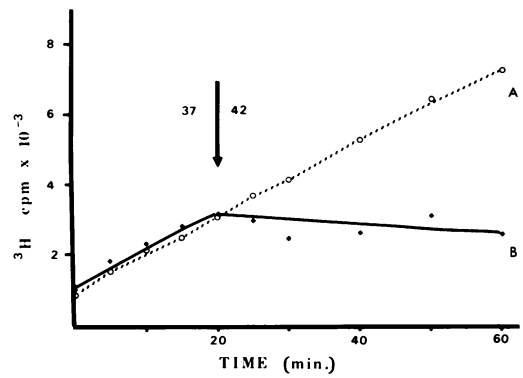

FIG. 7. *DNA synthesis with a temperature sensitive T4-DNA polymerase. E. coli cells were grown to 3 × 10⁸ in TCG and divided into two equal portions. One portion was infected with T4 (MOI = 7.0) and another was infected with T4 ts L97 (MOI = 7.0), a mutant of T4 temperature sensitive for gene 43 product, the T4 DNA polymerase. The cells were treated with toluene and incubated in the standard reaction mixture for 20 min. At this time both reaction mixtures were shifted to 42 C and incubated further. Acid-insoluble radioactivity was measured by incorporation of ^{3}H-dNTP before and after the temperature shift. A, T4⁺; B, T4 ts L97.*

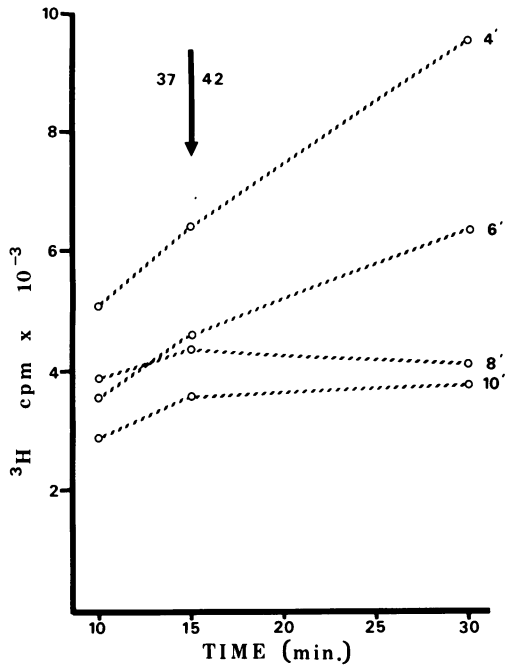

FIG. 8. *DNA synthesis in cells treated with toluene at various times after infection. E. coli B23 was infected with T4 ts L97. At various times after infection the cells were treated with toluene, and DNA synthesis was measured at permissive (37 C) and nonpermissive (42 C) temperatures. The times indicated on the four curves represent the time after infection at which the infected cells were treated with toluene.*

known T4 DNA polymerase, the gene 43 product.

Structure of progeny DNA. The strands of T4 DNA were separated by the procedure of Guha and Szybalski (10) and self annealed. Progeny DNA labeled with ^{3}H-dNTP in the standard reaction mixture was extracted, banded in CsCl and hybridized against the purified, separated strands of T4 by the method of Denhardt (4). Table 1 shows that both T4 strands were present in the progeny DNA.

Chromatography of the isolated progeny DNA on a column of hydroxyapatite (23) indicates that the majority of the DNA is double stranded (data not shown). However, some of the DNA is single stranded, and possibly some exists as covalently cross-linked-complementary, progeny DNA. This data will be the subject of a forthcoming publication (V. Paetkau and R. C. Miller, manuscript in preparation).

Size of the product. T4 infected, toluenized cells were incubated in standard reaction mixtures containing the dNTP at various concentrations: 5, 20, 40, 100, 200, and 1,000 μM. At 30 min the cells were lysed and the intracellular progeny DNA, labeled with ^{3}H, was analyzed by alkaline sucrose gradient sedimentation. The gradients were collected and assayed for the distribution of acid-insoluble ^{3}H-labeled material (Fig. 9). Essentially two size classes of molecules were found in the gradients; one class corresponding to large pieces approximately the same size as reference DNA and a class of smaller molecules. Parental DNA, labeled with ^{32}P, was shown to be the same size before and after treatment with toluene and after uptake of dNTP for 30 min (Fig. 10). These treatments of the infected cells show no effect on the normally nicked (15) intracellular, parental T4 DNA. However, it is obvious that the level of dNTP in the toluenized cells affects the joining of small fragments of progeny DNA into larger fragments (Fig. 9).

Sensitivity to DNase. If the reaction mixture was incubated with pancreatic DNase either during or after the incorporation of ^{3}H-dNTP for 30 min, the product was rendered acid-soluble (Table 2), showing that proteins at least as big as DNase could penetrate the infected toluenized cells. Uninfected, toluenized *E. coli* cells are also permeable to pancreatic DNase (24).

If an isolated product DNA was mixed with heat-denatured ^{32}P-labeled T4 DNA and incubated with exonuclease I, the product was resistant to degradation, whereas the reference DNA was rendered acid soluble. This was consistent with the earlier result that the progeny T4 DNA synthesized in *E. coli* treated with toluene is a double-stranded duplex. Exonuclease I is specific for single-stranded DNA with a free 3′ hydroxyl group (18).

DISCUSSION

This investigation documents the development of a system for labeling intracellular replicating phage DNA with dNTP. It has been shown that DNA synthesis in this system proceeds by semiconservative replication. If infected cells are treated with toluene later than 6 min postinfection, the replication is under the control of the known T4 DNA polymerase. The action of this enzyme in vitro with purified DNA as a template is much different (9, 11) than when the DNA precursors are being incorporated in infected cells treated with toluene. In toluenized cells, the progeny DNA is not covalently attached to the parental DNA, and is replicated from a fully substituted (heavy) duplex to a completed unsubstituted (light) duplex as defined by CsCl density gradient analysis. With purified templates T4 polymer-

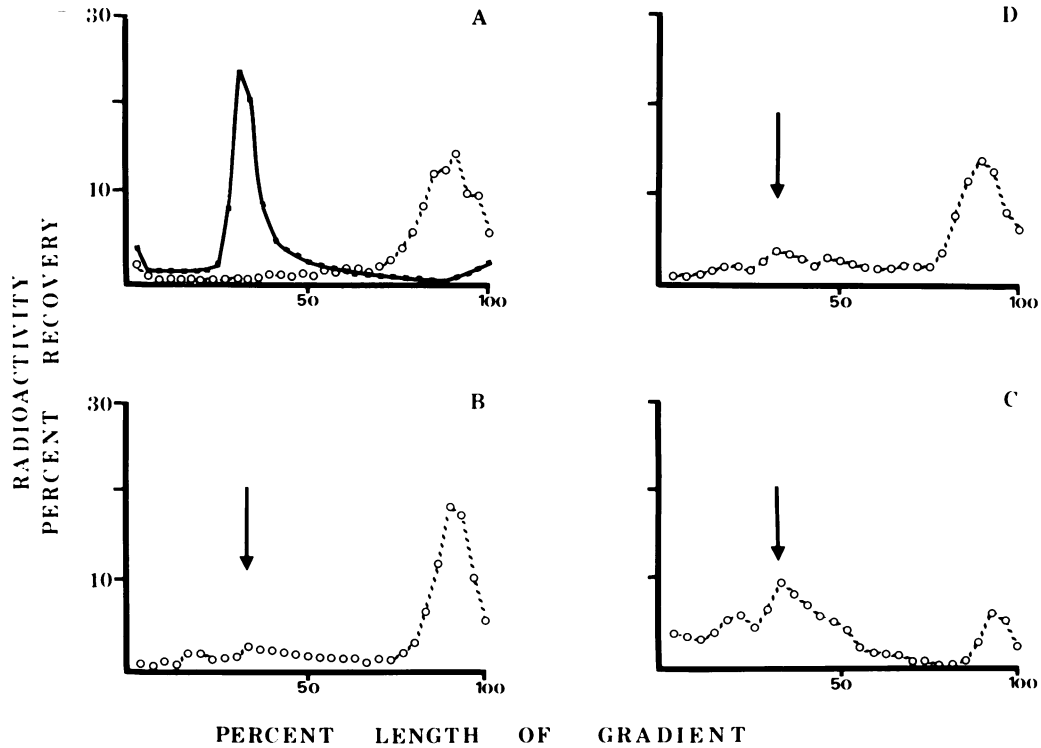

FIG. 9. *Sedimentation of progeny DNA in alkaline sucrose gradients. E. coli B23 was infected with T4, treated with toluene, and incubated in otherwise standard reaction mixtures containing various concentrations of dNTP. At 30 min the infected cells were lysed and the distribution of acid-insoluble material was analyzed in sucrose density gradients.* ^{32}P *reference T4 DNA was added to the gradients. All gradients were analyzed simultaneously in the same centrifuge. Gradients were centrifuged at 28,000 rpm for 3 h in a Beckman SW 50.1 rotor. A, 1 mM; B, 100 μM; C, 20 μM; D, 5 μM.*

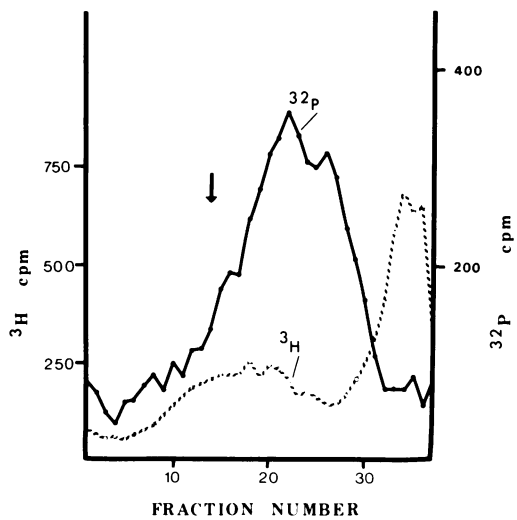

FIG. 10. *Sedimentation of parental and progeny DNA in an alkaline sucrose gradient. An experiment similar to that described in Fig. 9 was conducted with parental T4 phage labeled with* ^{32}P. *The distribution of parental label was determined at (i) 8 min after infection before toluene, (ii) after treating the infected cells with toluene, and (iii) after incubation for 30 min in a standard reaction mixture. The figure describes the distribution of parental and progeny label after incubation in a standard reaction mixture for 30 min. The distribution of parental label after infection and after treating the cells with toluene was identical to the above. Reference DNA was analyzed in a parallel gradient to determine the relative position of mature phage DNA. Gradients were centrifuged as in Fig. 9.*

ase normally catalyzes only a repair synthesis in vitro (9, 11). Since the product of synthesis in toluenized cells is a double-stranded phage DNA of a molecular weight close to the normal size of T4 as determined by alkaline sucrose gradients, it is reasonable to assume that the dNTP are incorporated by a procedure quite similar to, if not identical with, the process which normally operates in vivo. This allows the investigator to label many interesting intermediates in the T4 replication process for nucleotide sequence studies. It should also provide a mechanism for studying the nature of the covalent attachment of RNA to DNA in the RNA-linked

TABLE 2. *Treatment of reaction mixtures or isolated product with deoxyribonuclease*

A. Trichloroacetic acid-precipitable radioactivity (counts/min)

1.	+DNase at 15 min	−DNase
15′	6,027	6,433
30′	2,832	9,515
2.	+DNase at 0 min	−DNase
10′	516	1,789
20′	539	3,203
30′	558	4,005

B. Trichloroacetic acid-precipitable radioactivity (counts/min)

	^{3}H	^{32}P
Before exonuclease I	1,468	3,596
After exonuclease I	1,450	1,633

[a] A.1. *E. coli* B23 was infected with T4, treated with toluene and incubated in a radioactive reaction mixture as before. After 15 min of incubation the trichloroacetic acid-insoluble radioactivity was determined in duplicate reaction mixtures. To one mixture pancreatic DNase was added to 100 μg/ml. Both reactions were incubated for a further 15 min, and the acid-insoluble material was noted. A.2. In another experiment pancreatic DNase was added to one portion of infected toluenized cells at zero time of incubation in the radioactive reaction mixture. Incorporation of radioactivity into acid-insoluble material was determined in aliquots of the two portions for incubation times up to 30 min at 37 C.

[b] B. *E. coli* B23 was infected with T4, treated with toluene and incubated 30 min at 37 C in a standard reaction mixture containing ^{3}H-dNTP. The DNA was extracted by the SDS-Pronase-phenol method and banded in CsCl. The product DNA was pooled, dialyzed, mixed with ^{32}P-labeled reference T4 DNA denatured by heat, and incubated with exonuclease 1. Acid-insoluble material was determined before and after incubation with exonuclease.

DNA molecules found in T4 and T7-infected cells (2, 20).

Results (not documented here) of CsCl density gradient analysis of replicating T7 DNA in toluenized cells indicate that the T7 system behaves similarly to the T4 system and can be studied in a similar fashion, provided cells infected with T7 at 30 C are treated at 6 min after infection with 1% toluene for 15 min at 23 C. No attempt to promote T4 or T7 DNA synthesis has been effective when cells previously treated with toluene are subsequently infected with phage.

Preliminary studies in our laboratory indicate that *E. coli* infected with T4 and treated with toluene at 8 min after infection are permeable to proteins other than DNase. This would allow the future study of complementation of mutants with purified proteins or extracts to test their effect on the replication system.

Studies designed to test the genetic capacity of the product DNA in a T4 phage transformation system are underway.

ACKNOWLEDGMENTS

We would like to thank A. W. Kozinski and R. A. J. Warren for helpful discussions about this work, V. Paetkau and his associates for analyzing the product DNA by hydroxyapatite chromatography, and P. Loewen for the generous gift of exonuclease I.

This investigation was supported by a grant from the National Research Council of Canada.

LITERATURE CITED

1. Barry, J., and B. Alberts. 1972. *In vitro* complementation as an assay for new proteins required for bacteriophage T4 DNA replication: purification of the complex specified by T4 genes 44 and 62. Proc. Nat. Acad. Sci. U.S.A. **69**:2717-2721.
2. Buckley, P. J., L. D. Kosturko, and A. W. Kozinski. 1972. *In vivo* production of an RNA-DNA copolymer after infection of *Escherichia coli* by bacteriophage T4. Proc. Nat. Acad. Sci. U.S.A. **69**:3165-3169.
3. Delius, H., C. Howe, and A. W. Kozinski. 1971. Structure of the replicating DNA from bacteriophage T4. Proc. Nat. Acad. Sci. U.S.A. **68**:3049-3053.
4. Denhardt, D. 1966. A membrane filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. **23**:641.
5. de Waard, A., A. V. Paul, and I. R. Lehman. 1967. The structural gene for deoxyribonucleic acid polymerase in bacteriophages T4 and T5. Proc. Nat. Acad. Sci. U.S.A. **54**:1241.
6. Epstein, R. H., A. Bolle, C. M. Steinburg, E. Kellenberger, E. Boy de la Tour, R. Chevalley, R. S. Edgar, M. Susman, G. H. Denhardt, and A. Lielausis. 1963. Physiological studies of conditional lethal mutants of bacteriophage T4D. Cold Spring Harbor Symp. Quant. Biol. **28**:375-392.
7. Frankel, F. 1966. The absence of mature phage DNA molecules from the replicating part of T-even-infected *Escherichia coli*. J. Mol. Biol. **18**:109-126.
8. Frankel, F. F. 1968. Evidence for long DNA strands in the replicating pool after T4 infection. Proc. Nat. Acad. Sci. U.S.A. **59**:131-138.
9. Goulian, M., Z. J. Lucas, and A. Kornberg. 1968. Enzymatic synthesis of deoxyribonucleic acid. J. Biol. Chem. **243**:627-638.
10. Guha A., and W. Szybalski. 1968. Fractionation of the complementary strands of coli phage T4 DNA based on the asymmetric distribution of the poly U and poly U, G binding sites. Virology **34**:608-616.
11. Kleppe, K., E. Ohtsuka, R. Kleppe, I. Molineux, and H. G. Khorana. 1971. Studies on polynucleotides. XCVI. Repair replication of short synthetic DNA's as catalyzed by DNA polymerases. J. Mol. Biol. **56**:341-361.
12. Kleppe, K., J. H. Van de Sande, and H. G. Khorana. 1970. Polynucleotide ligase-catalyzed joining of deoxyribo-oligonucleotides on ribopolynucleotide templates and of ribo-oligonucleotides on deoxyribopolynucleotide templates. Proc. Nat. Acad. Sci. U.S.A. **67**:68-73.
13. Kozinski, A. W. 1968. Molecular recombination in the ligase-negative T4 amber mutant. Cold Spring Harbor Symp. Quant. Biol. **33**:375-391.
14. Kozinski, A. W., and P. B. Kozinski. 1965. Early intracellular events in the replication of T4 phage DNA. II. Partially replicated DNA. Proc. Nat. Acad. Sci. U.S.A. **54**:634.
15. Kozinski, A. W., P. B. Kozinski, and R. James. 1967. Molecular recombination in T4 bacteriophage deoxyribonucleic acid. I. Tertiary structure of early replicative

and recombining deoxyribonucleic acid. J. Virol. **1**:758-770.
16. Kozinski, A. W., and T. H. Lin. 1965. Early intracellular events in the replication of T4 phage DNA. I. Complex formation of replicative DNA. Proc. Nat. Acad. Sci. U.S.A. **54**:273.
17. Kozinski, A. W., and W. Szybalski. 1959. Dispersive transfer of the parental DNA molecule to the progeny of phage ϕX 174. Virology **9**:260.
18. Lehman, I. R., and A. L. Nussbaum. 1964. The deoxyribonucleases of *Escherichia coli*. J. Biol. Chem. **239**:2628-2636.
19. Matsushita, T., K. P. White, and N. Sueoka. 1971. Chromosome replication in toluenized *Bacillus subtilis* cells. Nature N. Biol. **232**:111-114.
20. Miller, R. C. 1972. Asymmetric annealing of an RNA linked DNA molecule isolated during the initiation of bacteriophage T7 DNA replication. Biochem. Biophys. Res. Commun. **49**:1082-1086.
21. Miller, R. C., and A. W. Kozinski. 1970. Incorporation of deoxyribonucleic acid precursors by T4 deoxyribonucleic acid-protein complexes retained on glass fiber filters. J. Virol. **6**:559-562.
22. Miller, R. C., A. W. Kozinski, and S. Litwin. 1970. Molecular recombination in T4 bacteriophage deoxyribonucleic acid. J. Virol. **5**:368-380.
23. Miyazawa, Y., and C. A. Thomas. 1965. Nucleotide composition of short segments of DNA molecules. J. Mol. Biol. **11**:223-237.
24. Moses, R., and C. C. Richardson. 1970. Replication and repair in cells of *Escherichia coli* treated with toluene. Proc. Nat. Acad. Sci. U.S.A. **67**:674-681.
25. Werner, R. 1968. Initiation and propagation of growing points in the DNA of phage T4. Cold Spring Harbor Symp. Quant. Biol. **33**:501-507.
26. Wiberg, J. S., M. C. Dirksen, R. H. Epstein, S. E. Luria, and J. M. Buchanan. 1962. Early enzyme synthesis and its control in *E. coli* infected with some amber mutants of bacteriophage T4. Proc. Nat. Acad. Sci. U.S.A. **48**:293-302.