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Biochemistry of DNA-Defective Mutants of Bacteriophage T4

VI. Biological Functions of Gene 42

THOMAS W. NORTH, MARY E. STAFFORD, AND CHRISTOPHER K. MATHEWS*
Department of Biochemistry, College of Medicine, University of Arizona, Tucson, Arizona 85724

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Bacteriophage T4 gene 1 and 42 amber mutants (defective in deoxynucleoside monophosphate kinase and deoxycytidylate hydroxymethylase, respectively) are able to synthesize DNA in cell-free lysates prepared as described by Barry and Alberts (1972), in contrast to their inability to do so in plasmolyzed and toluenized cell systems. Addition of extracts containing an active gene 1 or 42 product has no effect on synthesis in lysates defective in the respective gene. Thus, if these enzymes do play additional direct roles in replication, these roles are not manifest in the lysed-cell system. The gene 42 mutant *am* N122/m, a double mutant bearing an additional defect in DNA polymerase, is unable to synthesize DNA in these lysates. This inability is overcome by addition of extracts containing an active T4 DNA polymerase. m is a leaky amber mutation which reduces DNA polymerase activity to a very low level. However, this level is high enough to allow positive genetic complementation tests with gene 43 mutants. Two other gene 42 amber mutants contain additional defects: *am* 269 induces only half the normal level of DNA polymerase, and *am* C87 fails to induce a detectable level of thymidylate synthetase. These defects do not result from pleiotropic effects of the gene 42 mutations. In plasmolyzed cells, temperature-sensitive gene 42 mutants fail to synthesize DNA under conditions where replication forks and 5-hydroxymethyl-dCTP are present. This supports the idea that the gene 42 protein is directly involved in DNA synthesis.

Of the two dozen known T-even phage-coded early enzymes, roughly half are involved in synthesis of DNA precursors and the rest participate in DNA metabolism at the macromolecular level. Two enzymes thought for several years to fall strictly into the former category are deoxycytidylate hydroxymethylase (HMase), coded for by gene 42 (25), and deoxynucleoside monophosphate kinase, coded for by gene 1 (7). Both enzymes participate in the pathway responsible for replacement of dCMP residues in phage DNA by 5-hydroxymethyldeoxycytidylate (HM-dCMP), namely

$$\text{dCMP} \xrightarrow[\text{gene 42}]{\text{HMase}} \text{HM-dCMP} \xrightarrow[\text{gene 1}]{\text{kinase}}$$
$$\text{HM-dCDP} \xrightarrow[\text{kinase}]{\text{host}} \text{HM-dCTP} \rightarrow \text{DNA}$$

T4 strains bearing mutations in genes 1 or 42 display a DNA-negative (DO) phenotype (23) which is most readily explained by the resultant deficiency of a required phage DNA precursor. However, Chiu and Greenberg (3) reported that a particular gene 42 mutant, *ts* L13, retained a DO phenotype even though it evidently could synthesize active hydroxymethylase at a nonpermissive temperature. Similar observations have been made by G. Holmes (10; and Ph.D. thesis, University of Arizona, Tucson, 1973). Chiu and Greenberg proposed that the gene 42 product (P42) plays a second, more direct, role in DNA replication. However, when it was later found that neither HM-dCTP (13) nor HM-dCMP (21) accumulates in restrictive infection by *ts* L13, this conclusion appeared less compelling.

Supportive evidence, however, emerged from studies with in vitro DNA synthesis systems from 42$^-$ phage-infected plasmolyzed (4, 28) or toluenized (6) cells. Such cells are unable to synthesize DNA even in the presence of added HM-dCTP. HM-dCTP is similarly unable to allow synthesis by some gene 1 mutants in these systems (4, 6, 28). The inability of HM-dCTP to bypass either block suggests additional defects in DNA synthesis unrelated to precursor formation. In the case of P42, this defect could be in

the proposed second function. Similar arguments could be advanced for P1. However, an alternative explanation has been advanced by Dicou and Cozzarelli, based on the requirement of in vitro DNA synthesizing systems for preexisting DNA growing forks (6). Infections by these mutants may not produce a sufficient number of growing points in vivo to allow an appreciable amount of synthesis in vitro. To evaluate this possibility one must be able to determine the effect of the gene product upon a template of invariant composition (i.e., which has a constant number of growing points). The cell-free DNA synthesis system described by Barry and Alberts (2) has been used to assay several of the gene products involved in T4 DNA replication. This system allows us to add various donor extracts to a lysate of invariant composition. We have used this system to investigate the roles of genes 1 and 42 in DNA synthesis. We have investigated this question further by utilizing temperature-sensitive (*ts*) gene 42 mutants in a plasmolyzed cell system. This enables us to determine whether P42 is required under conditions where we know that replication forks are present.

(This material is taken from a thesis to be presented by the senior author [T.W.N.] to the faculty of the University of Arizona, Tucson, in partial fulfillment of the requirements for the Ph.D. degree.)

MATERIALS AND METHODS

Biological materials. Sources of *Escherichia coli* strains B, CR63, and D110 (*polA*$^-$, *endoI*$^-$, *thy*$^-$) have been described previously (4). *E. coli* D110-Str^R was isolated as a spontaneous mutant able to grow in the presence of 200 μg of streptomycin sulfate per ml. Retention of the *polA*$^-$ mutation has been confirmed by measurement of its UV sensitivity (27).

T4 bacteriophage strains used were T4D (wild type), the amber mutants listed in Table 1, and temperature-sensitive mutants *ts* LB-1, *ts* LB-3 (both 42$^-$) (16), and *ts* L159 (45$^-$) (C. K. Mathews, Arch. Biochem. Biophys., in press).

Media and growth. Preparation, purification, and assay of phage stocks were as reported previously (13, 17). Unless stated otherwise, bacterial growth was always at 37 C with aeration in M9 medium (1) or SM9, which is M9 supplemented with 0.2% Casamino Acids. For growth of *E. coli* D110 and D110-Str^R, SM9 was supplemented with 20 μg of thymine per ml.

Chemicals and enzymes. HM-dCTP was prepared as described previously (4). All other nucleotides were obtained from P-L Biochemicals. Radioactive compounds were all from New England Nuclear Corp. The ^{14}C-labeled amino acid mixture used was from an algal hydrolysate; specific activities of amino acids in this mixture ranged from 100 to 500 mCi/mmol. Brij-58 was from Atlas Chemicals Industries, Inc.

TABLE 1. *T4 amber mutants*

Mutant gene	Strains[a]	Specific function[b]
1	B24 × 3, E957	Deoxynucleotide kinase
30	H39	DNA ligase
32	E315	DNA unwinding protein
41	NO5 × 3	Unknown
42	N122/m × 3, N122 × 3[c]	Deoxycytidylate hydroxymethylase
	269 × 3,[c] NG93	
	E498, 21 × 3[c]	
	N55 × 5,[c] C87[c]	
	E385, NG205	
	NG352	
43	B22 × 3, 4304	DNA polymerase
44	N82 × 3	Unknown
45	E10 × 3	Unknown
62	NG485 × 3	Unknown

[a] The suffix × 3 or × 5 indicates that the mutant was backcrossed to wild-type T4D three or five times.

[b] Identification of mutant gene products is reviewed elsewhere (12).

[c] Kindly provided by J. S. Wiberg, University of Rochester. All other strains were from our laboratory stocks or from the collection of H. Bernstein, University of Arizona College of Medicine.

Egg white lysozyme (9,000 U/mg; 92% protein) was obtained from Worthington Biochemicals Corp.

Cell-free DNA synthesis and complementation assays. Except for the changes noted below, the procedures of Barry and Alberts (2) were followed. Cells were grown and infected as described (2). However, *E. coli* D110-Str^R, which we have confirmed (data not shown) to have a reduced level of translational ambiguity (10), was used as the host strain rather than *E. coli* D110. Receptor lysates were prepared by lysozyme-EDTA treatment followed by gentle lysis with Brij-58. Donor extracts were made from lysates prepared in this way, by centrifugation at 35,000 × *g* for 15 min to remove membrane material and associated DNA, followed by centrifugation at 140,000 × *g* for 45 min to pellet ribosomes. The resulting supernatants were then used as donor extracts.

In vitro DNA synthesis was assayed by adding 25 μl of a lysate (or for complementation assays 25 μl of a mixture containing equal volumes of receptor lysate and donor extract) to 25 μl of a solution containing the four dNTP's (HM-dCTP rather than dCTP) with [^{3}H]dATP (250 Ci/mol) as described by Barry and Alberts (2). Incubations were carried out at 37 C for 20 min. Reactions were terminated with 50 μl of cold 0.2 M EDTA (Na_2). The mixtures were then spotted onto Whatman #3 2.4-cm filter paper disks. Filters were dried, washed three times in cold 5% trichloroacetic acid-1% sodium pyrophosphate, washed twice in acetone, and dried. They were then placed in 5 ml of

Omnifluor (New England Nuclear Corp.) and counted.

Since labeling conditions were the same throughout, ^3H counts per minute are used as a measure of the relative amount of DNA synthesis occurring in vitro. A blank of 100 counts/min has been subtracted in all values reported. This blank was determined by first adding lysates and then nucleotide solutions directly to the termination mixture on ice (with no prior 37 C incubation). This value was 100 ± 10 counts/min regardless of the lysate or donor-receptor mixture assayed.

In situ DNA synthesis. *E. coli* B was grown in GCA medium (15) at 30 C to 5 × 10^8 cells/ml, and then infected with phage at a multiplicity of 6. Infections were stopped at 15 min by placing cultures on ice. Infected cells were harvested by centrifugation and then plasmolyzed by resuspension in 2 M sucrose, 0.04 M Tris-hydrochloride (pH 8.0), and 0.1 M ethyleneglycol-bis-(β-aminoethyl ether)*N,N'*-tetraacetic acid to a final concentration of 10^{11} cells/ml. Plasmolyzed cells (35 μl) were then added to a reaction mixture (190 μl) containing: 64 mM KCl, 42 mM Tris-hydrochloride (pH 8.4), 16 mM $MgCl_2$, 1 mM ATP, 0.1 mM each dATP, dGTP, and [^3H]dTTP (80 μCi/μmol), and 0.033 mM HM-dCTP. The mixtures were then incubated at the temperatures indicated and samples taken at the indicated times were mixed with equal volumes of 0.2 M EDTA (Na_2). A 0.1-ml amount of each mixture was spotted onto a filter paper disk and this was washed with trichloroacetic acid and counted as described above.

In vivo DNA synthesis. DNA synthesis by T4 phage-infected *E. coli* was measured as described previously (11).

Genetic complementation tests. Qualitative complementation tests between pairs of phage mutants were performed by standard spot tests. Equal volumes of phage mutants at concentrations of 5 × 10^7 PFU/ml were mixed. A 5-μl volume of each mixture was spotted onto a plate seeded with *E. coli* B.

Enzyme assays. Deoxycytidylate hydroxymethylase activity was determined as described by Pizer and Cohen (18). T4 DNA polymerase activity was measured by the procedure of Goulian, Lucas, and Kornberg (8). Thymidylate synthetase was assayed spectrophotometrically as previously described (14).

Electrophoretic analysis of T4 proteins. *E. coli* B was grown in M9 to 5 × 10^8 cells/ml and then UV irradiated (2,600 ergs/mm^2). After irradiation, cells were kept in the dark until after infection. Cultures (1 ml) were infected with phage at a multiplicity of 10 PFU/cell and phage were allowed to adsorb for 10 min on ice. A ^{14}C-labeled amino acid mixture (2 μCi/ml) was then added and the cultures were shifted to 37 C. After 8 min a 500-fold excess of casein hydrolysate (sterile 10% solution) was added. Infections were terminated at 10 min by placing cultures on ice. Infected cells were pelleted and resuspended in 0.1 ml of 0.05 M Tris-hydrochloride (pH 6.8), 1% SDS, 1% mercaptoethanol, and 15% glycerol; they were then boiled for 2.5 min. Samples (20 μl) were then applied to 10% polyacrylamide slab gels containing 0.1% SDS, and electrophoresis was carried out as described by Studier (19). Gels were dried and autoradiographed with Kodak NS2T X-ray film.

RESULTS

DNA synthesis in vitro. The ability of various T4 mutants to synthesize DNA in lysates prepared from infections of *E. coli* D110-Str^R, in the presence of HM-dCTP, is shown in Table 2. Although T4D makes less DNA here than in lysates from infections of *E. coli* D110 (2), the levels of DNA synthesis by T4 mutants, relative to wild type, are comparable to those obtained by Barry and Alberts in genes 32, 41, 43, 44, and 45 (2). Our levels for the gene 62 mutant were somewhat higher than theirs. However we used a different gene 62 mutant (NG485) than did they (E1140). In plasmolyzed cells NG485 is also able to make DNA whereas E1140 maintains its DNA-negative phenotype (4).

Table 3 (part A) shows the ability of various gene 42 mutants to make DNA in this system. All of these mutants direct a considerable amount of DNA synthesis in vitro except for N122/m, which has an additional defect in DNA polymerase (as described below). In addition, donor extracts which contain an active gene 42 product are unable to stimulate synthesis in these 42$^-$ lysates (Table 3A). Table 3 (part B) shows that gene 1 mutants are similarly able to synthesize a considerable amount of DNA in vitro and are not stimulated by the addition of donor extracts containing an active gene 1 product. From these data we can conclude that genes 1 and 42 are not directly involved in DNA synthesis in this cell-free system, in contrast to the apparent requirement for these two gene products in plasmolyzed (4, 28) and toluenized (6) cell systems. B. M. Alberts et al. have also found that genes 1 and 42 are not essential for DNA synthesis in this cell-free system (personal communication).

TABLE 2. *Cell-free DNA synthesis by phage-infected cell lysates*[a]

Phage	^3H (counts/min)	% Wild type
T4D	4,423	100
H39 (30$^-$)	1,831	41
E315 (32$^-$)	660	15
NO5 (41$^-$)	1,105	25
B22 (43$^-$)	196	4
N82 (44$^-$)	193	4
E10 (45$^-$)	420	9
NG485 (62$^-$)	891	20

[a] Phage-infected cell lysates were prepared and assayed as described in Materials and Methods.

Characterization of the m mutation of N122/m. N122 as originally isolated contained an additional defect in DNA polymerase termed m (5, 22, 26). The stock of N122 which we obtained from California Institute of Technology in 1965 we thought to be cured of m, since it was able to complement gene 43 mutants as measured by qualitative spot tests (Table 4). We had also backcrossed this mutant to T4D several times. In our early investigations we found this mutant unable to make DNA in vitro, and in addition it could be stimulated by the addition of 42^+ donors, but not 42^+ 43^- donors (Table 5). We felt that this supported the concept of a second function for P42 and suggested an interaction between genes 42 and 43. However, it was suggested to us by J. S. Wiberg that m may produce enough DNA polymerase to allow a positive genetic complementation test, which requires only a low production of progeny phage, yet may not produce quantities of DNA polymerase sufficient to allow an appreciable amount of DNA synthesis in vitro.

TABLE 3. *Cell-free DNA synthesis by 42^- (A) and 1^- (B) phage*[a]

Phage	^3H (counts/min)	% Wild type	Stimulation[b]
			By 42^+ donor
A. N122/m	130	3	2.0–3.5
N122	3,102	71	0.8–1.0
269	1,123	26	1.3
N55	2,607	59	1.1
NG205	2,016	46	1.0
E385	2,306	52	1.2
NG352	1,821	41	1.1
			By 1^+ donor[b]
B. E957	3,406	77	0.9
B24	2,300	52	0.9

[a] Receptor lysates and donor extracts were prepared and complementation assays were performed as described in Materials and Methods.

[b] Stimulation is relative to the level of ^3H incorporation with a 42^- donor (A) or a 1^- donor (B), which is defined as 1.0. Donor extracts were prepared from *am* E10-infected cells. Similar results were obtained with *am* N82 donors.

TABLE 4. *Genetic complementation tests*[a]

Cross	N122/m	B22	4304	N55
N122/m	–	+	+	–
B22	+	–	–	+
4304	+	–	–	ND[b]
N55	–	+	ND[b]	–

[a] As described in Materials and Methods.

[b] Not done.

TABLE 5. *Stimulation of N122/m receptor lysates by various donor extracts*[a]

Phage donor	^3H (counts/min)	Fold stimulation[b]
E957 (1^-)	326	3.1
B24 (1^-)	345	3.3
E315 (32^-)	211	2.0
NO5 (41^-)	263	2.5
N122/m	105	1.0
N122 (42^-)	347	3.3
B22 (43^-)	125	1.2
4304 (43^-)	137	1.3
N82 (44^-)	294	2.8
E10 (45^-)	354	3.4
NG485 (62^-)	231	2.2

[a] Receptor lysates and donor extracts were prepared and complementation assays were performed as described in Materials and Methods.

[b] Stimulation is relative to the level of ^3H incorporation with an N122/m donor extract, which is defined as 1.0.

To investigate this possibility we measured the DNA polymerase activity induced by our stock of N122, to see whether it still contained the m mutation. We found that it was defective in DNA polymerase (Fig. 1), as had been demonstrated for N122/m (5, 22). This ability to induce only a very low level of DNA polymerase accounts for the failure of this mutant to make DNA in vitro as well as the complementation data in Table 5. Thus, our presumed N122 is in reality N122/m. In addition, in a mixed infection of our N122/m with B22 (43^-), a low but detectable level of DNA is made (Fig. 2). This accounts for the positive genetic complementation tests (Table 4). A stock of N122 known to be cured of m (obtained from J. S. Wiberg) was both able to induce DNA polymerase activity (Fig. 1) and able to make DNA in a mixed infection of *E. coli* B with B22 (Fig. 2). Hereafter we shall refer to our stock as N122/m and that obtained from J. S. Wiberg as N122.

Figure 2 also shows that, in a mixed infection of N122/m and E10 (45^-), a normal rate of DNA synthesis is attained. Thus, m does not inhibit DNA polymerase in trans, ruling out the possibility that the m defect produces an inhibitor of DNA polymerase.

Figure 3 shows that N122/m can make DNA in infection of *E. coli* CR63. Since both the gene 42 and the m mutation are suppressed by this host strain, m appears to be an amber mutation. (The high level of suppression makes it

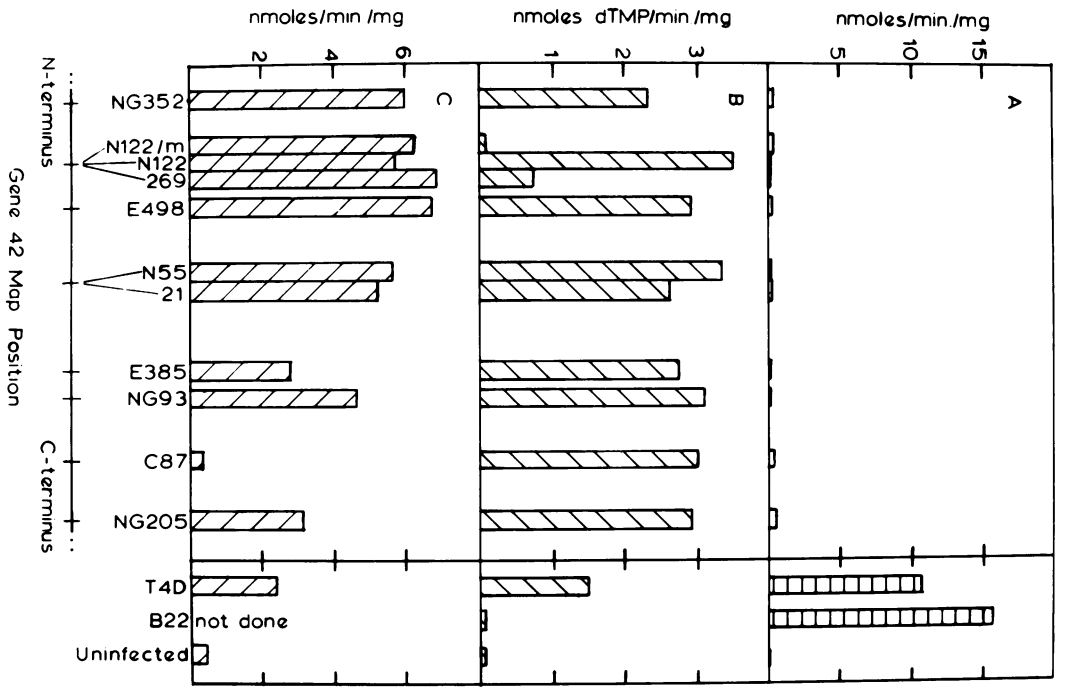
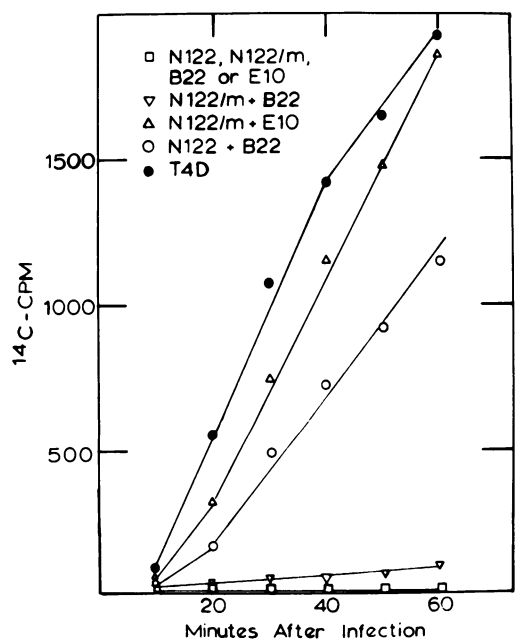


FIG. 1. *HMase (A), DNA polymerase (B), and thymidylate synthetase (C) activities of extracts prepared from phage-infected E. coli D110. Cells were grown at 37 C in SM-9 + 20 μg of thymine per ml to a density of 4 × 10⁸/ml, and then infected with 6 phage per bacterium. Infections were terminated after 30 min by placing the culture flasks in an ice bath. Infected cells were then pelleted, resuspended in 50 mM Tris-hydrochloride (pH 7.4)-10 mM mercaptoethanol, and disrupted by sonic oscillation. Bacterial debris was removed from these sonic extracts by centrifugation at 20,000 × g for 20 min. Enzymes were assayed as indicated in Materials and Methods. The location of mutants within gene 42 has been approximated from the mapping data of G. Holmes (9) and of J.S. Wiberg (personal communication).*



unlikely that m is an ochre or umber mutation.) This was confirmed by slab-gel electrophoretic analysis of early proteins made in infection of *E. coli* B. Figure 4 shows that the DNA polymerase band, identified by its absence in infections with B22 (43⁻), is missing in infections with N122/m. Therefore, the gene 43 protein is not produced by this mutant. The ability of this mutant to induce a low level of DNA polymerase activity could result either from the amber fragment which it produces or a very low production of the gene 43 protein which is not detectable by slab-gel electrophoretic analysis. We have not been able to detect the amber fragment produced by this mutant; this may result either from its co-migration with another band or from its being too small to detect.

FIG. 2. *DNA synthesis in phage-infected E. coli B. Cells at 3 × 10⁸/ml were infected with 6 phage per bacterium in unmixed infections or 3 of each phage per bacterium in mixed infections. DNA synthesis was measured as indicated in Materials and Methods.*

Enzyme activities of other 42⁻ phage. Another gene 42 amber mutant, 269, which maps at the same site as N122 (J. S. Wiberg, personal communication) makes only about half of the wild-type level of DNA polymerase (Fig. 1). This defect was found in the backcrossed stock, 269 × 3, obtained from J. S. Wiberg. Since Tomich et al. (20) have provided intriguing evidence for an interaction between dCMP HMase and DNA polymerase, it was of interest to determine whether this particular mutant had an additional defect in DNA polymerase or was the result of a single gene 42 mutation with a pleiotropic effect.

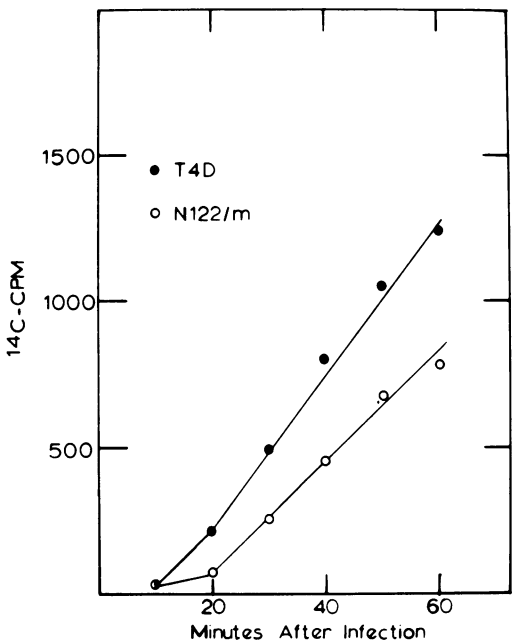


FIG. 3. *DNA synthesis in phage-infected E. coli CR63. Procedures are as indicated in Materials and Methods.*

Figure 1 shows that 269 was the only gene 42 mutant examined which was found to have this defect. All of the other 42⁻ phage overproduce DNA polymerase, as expected for DO mutants (26). To determine whether the polymerase defect resulted from a pleiotropic effect of the gene 42 mutation, we isolated three independent am^+ revertants from different *am* plaques and measured their DNA polymerase activities (Table 4A). Since all of the revertants are low in DNA polymerase activity, the amber gene 42 mutation has segregated from the polymerase defect and thus the defect is an independent mutation. Since slab-gel electrophoretic analysis shows the presence of a polymerase band (Fig. 4), this is presumably a point mutation which decreases the activity of the DNA polymerase. This lower DNA polymerase level also results in a lower level of DNA synthesis in vitro relative to other 42⁻ phage (Table 3A).

Tomich et al. (20) have also provided evidence suggesting an interaction between T4 thymidylate synthetase and dCMP HMase. Of the 10 gene 42 amber mutants examined, we found that one of them, C87, was defective in

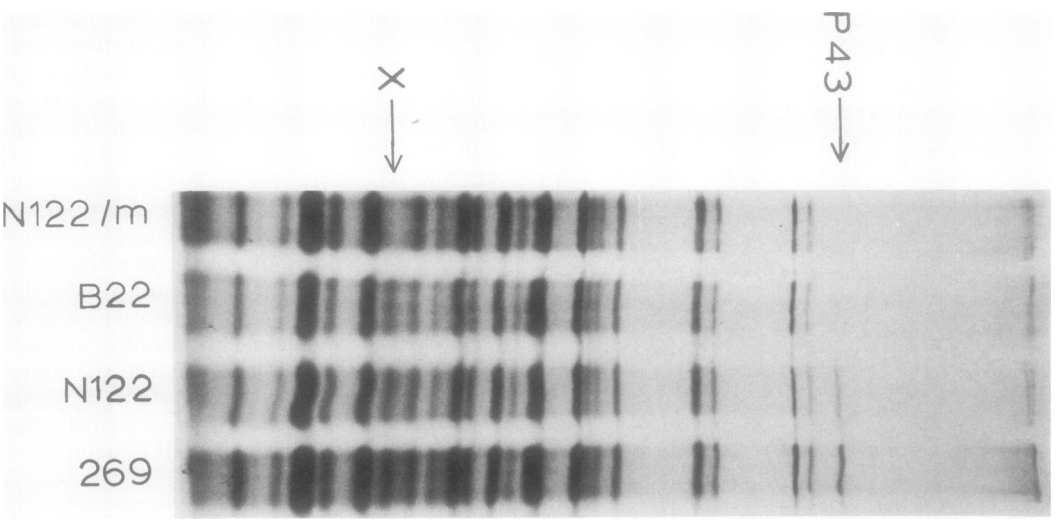


FIG. 4. *Autoradiograms of proteins synthesized in T4-infected E. coli B, resolved on 10% polyacrylamide gels containing 0.1% SDS. The protocol for labeling of proteins and electrophoretic analysis is described in Materials and Methods. P43 is identified by its absence in extracts from am B22. Band X is an unidentified band which is also absent in extracts from am N122/m infections.*

thymidylate synthetase activity (Fig. 1). We used the same approach as above to ask whether this represented a pleiotropic effect of the gene 42 mutation or a second-site mutation. Three am^+ revertants were independently isolated from different am plaques. Table 4 (B) shows that all three revertants were still defective in thymidylate synthetase. Thus C87 is also a double mutant. All of the other 42^- phage had levels of thymidylate synthetase greater than that of wild type.

TABLE 6. *DNA polymerase (A) and thymidylate synthetase (B) activities of extracts prepared from phage-infected E. coli D110*[a]

Phage	Enzyme activity
A.	DNA polymerase (nmol of dTMP incorporated/min/mg)
T4D	1.49
269	0.70
269-R1[b]	0.57
269-R2[b]	0.56
269-R3[b]	0.59
Uninfected	<0.03
B.	Thymidylate synthetase (nmol/min/mg)
T4D	2.40
C87	0.37
C87-R1[b]	0.67
C87-R2[b]	0.30
C87-R3[b]	0.52
Uninfected	0.30

[a] Procedures are as described in the legend to Fig. 1.
[b] Independently isolated am^+ revertants of *am* 269 (A) or *am* C87 (B).

When we began this work we were particularly intrigued by the results of G. Holmes (10; and Ph.D. thesis, University of Arizona, Tucson, 1973), who had found that two gene 42 amber mutants, namely NG93 and E498, produced significant levels of dCMP HMase activity under nonpermissive conditions of infection. If confirmed, this would appear to provide strong evidence in favor of a second role for the gene 42 protein. However, we were unable to confirm Holmes' observations. As shown in Fig. 1, none of the gene 42 amber mutants we tested could induce significant dCMP HMase activity in infection of either *E. coli* D110 or *E. coli* B. We are not sure of the reasons for this discrepancy, but we repeatedly observed the same results depicted in Fig. 1.

DNA synthesis in situ. It has been suggested that the failure of gene 42 mutants to synthesize DNA in plasmolyzed or toluenized cells may reflect a deficiency of DNA chains to extend because of the lack of DNA synthesis in vivo before the infected cells were harvested (6). We have investigated this possibility by utilizing *ts* gene 42 mutants in a plasmolyzed cell system. Both mutants used, *ts* LB-1 and *ts* LB-3, direct

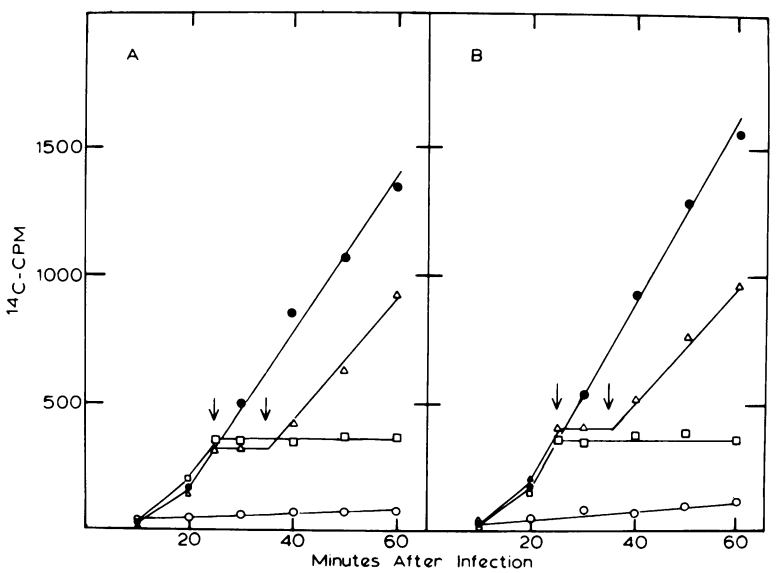


FIG. 5. *DNA synthesis in vivo after infection of E. coli B by ts LB-1 (A) or ts LB-3 (B) at 30 C (●), 43 C (○), 30 C for 25 min followed by a shift to 43 C (□), or 30 C for 25 min, shifted to 43 C from 25 to 35 min, and then shifted back to 30 C at 35 minutes (△). DNA synthesis was measured as described in Materials and Methods. The arrows indicate times of the temperature shifts. For the shift from 43 to 30 C (second arrow), it was assumed that there was no synthesis until the shift, since the parallel culture left at 43 C made no further DNA.*

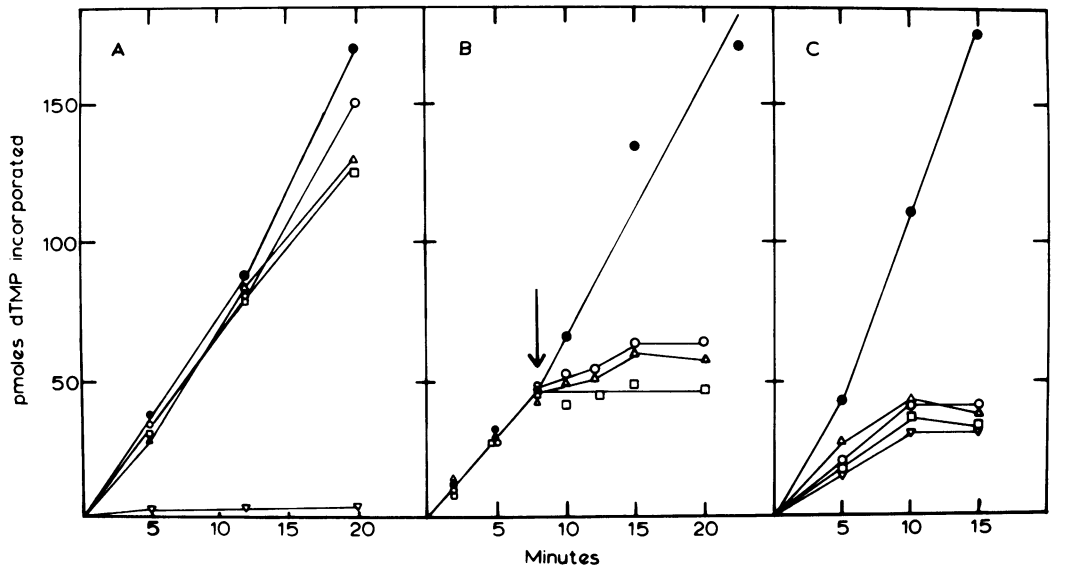


FIG. 6. *DNA synthesis in plasmolyzed cells infected by T4D (●), ts LB-1 (○), ts LB-3 (△), ts L159 (□), or am N122 (▽) at (A) 30 C, (B) 30 C for the first 8 min and then shifted to 43 C (arrow indicates time of shift), and (C) 43 C. Procedures are described in Materials and Methods.*

DNA synthesis in vivo which is rapidly and reversibly halted after a shift from permissive (30 C) to restrictive (43 C) temperature (Fig. 5). With this in mind we can ask whether mutant-infected cells can synthesize DNA in vitro after infection at 30 C in vivo, to ensure the presence of active replication forks. A gene 45 mutant, *ts* L159, was used as a control, since it rapidly and reversibly shuts off DNA synthesis after a temperature upshift in vivo (Mathews, Arch. Biochem. Biophys., in press), and p45 is almost certainly involved in the polymerization step and not the provision of precursors (4; B. M. Alberts, C. F. Morris, D. Mace, N. Sinha, M. Bittner, and L. Moran, *In* M. M. Goulian, P. D. Hanawalt, and C. F. Fox [ed.], *DNA Synthesis and Its Regulation*, in press).

At 30 C in vitro, the two *ts* gene 42 mutants and *ts* L159 all synthesize DNA at the same rate as does T4D (Fig. 6A). However, after a shift to 43 C after 8 min at 30 C, all three *ts* mutants shut off their DNA synthesis, while the rate of DNA synthesis by T4D actually increases (Fig. 6B). The shutoff is not as rapid with the gene 42 mutants as with the gene 45 mutant. Nevertheless, it is complete, and the very fact that the rate of synthesis after shutoff is less than the corresponding wild-type rate is proof that P42 plays a role in this system other than the provision of DNA precursors. Similar results were observed when the temperature upshifts were carried out after 6 or 9 min in vitro at 30 C (data not shown). Further support for a role of P42 in this system was obtained in experiments where plasmolyzed cells, previously infected at 30 C, were tested for DNA synthesis at 43 C, without a prior incubation at 30 C in vitro (Fig. 6C). Again, DNA synthesis with the *ts* mutants was defective with respect to wild type, although significant levels of isotope incorporation were observed. We believe that this represents a process which is not physiologically significant, because comparable levels of incorporation were seen with (i) the *ts* gene 45 control mutant and (ii) a gene 42 amber mutant, N122, which displays a DNA-negative phenotype in vitro at 30 C.

Our data establish that the failure of gene 42 mutants to synthesize DNA in plasmolyzed cells cannot be ascribed solely to a deficiency of growing chains to extend. Thus, one must postulate that P42 interacts directly with the replication apparatus, as proposed by Greenberg et al. (3, 28).

DISCUSSION

We have sought to confirm, by independent approaches, the findings of Greenberg et al. (3, 20, 28) that: (i) dCMP HMase participates directly in replication and (ii) dCMP HMase interacts with other T4 early enzymes, notably DNA polymerase and dTMP synthetase. Originally we felt that the system of Barry and Alberts would provide a route for demonstrating

a direct role in replication. Once we had realized that our N122 stock still contained the m mutation and that other gene 42 mutants were capable of DNA synthesis in this system, this approach lost its appeal. In a similar vein, our discovery that stocks of some gene 42 mutants contain additional defects in DNA polymerase or dTMP synthetase seemed to provide a route for confirming postulated interactions involving dCMP HMase and these enzymes. Again, our finding that these secondary defects involve independent mutations blocked this avenue of approach.

What, then, is the status of the postulated complex metabolic roles for the gene 42 product? The strongest evidence in favor of a direct role in replication is the fact that plasmolyzed or toluenized cells previously infected with 42⁻ mutants cannot incorporate nucleotides into DNA, even when the known metabolic block is circumvented by provision of HM-dCTP. However, as stated earlier, this may simply reflect a deficiency of polydeoxynucleotide chains to extend in vitro because of the failure of replication forks to have been formed in vivo. This question has been explored by the use of temperature-sensitive gene 42 mutants in a plasmolyzed cell system. Such systems are defective in DNA synthesis at restrictive temperature, even when the presence of replication forks is assured by prior infection of the cells at permissive temperature. Thus, we feel that P42 does interact with the replication apparatus.

The evidence supporting interaction between P42 and other virus-coded enzymes is similarly quite strong. Tomich et al. (20) have shown that HMase activity in vivo cannot be detected until 5 min after infection, several minutes after the enzyme activity is discernible in cell-free extracts. Although we were unable to develop a system for direct demonstration of the postulated complex, we are not aware of any evidence challenging the existence of such a complex. Certainly it seems attractive to postulate the existence of some structural organization involving the enzymes of phage DNA precursor synthesis. The greatly increased rate of DNA synthesis after infection makes it reasonable, on both theoretical (B. M. Alberts, personal communication) and experimental (24) grounds, to picture a complex of enzymes acting to "funnel" precursors into DNA. Although there is no direct evidence supporting the existence of such funnels, this may provide an explanation for the difference between lysed and unlysed cell preparations with respect to the requirements for P1 and P42. Perhaps in toluenized or plasmolyzed cells, as in intact cells, the replication apparatus is accessible only via the funnel, and at an earlier stage than that of deoxynucleoside 5'-triphosphates. If lysis of cells by the procedure of Barry and Alberts separates the "funnel" from the replication apparatus, then this apparatus becomes accessible to 5'-dNTP's. Although purely speculative, this explanation accounts for all available data and presents a framework for further experimentation.

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