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Isolation of Mutants of Bacteriophage T4 Unable to Induce Thymidine Kinase Activity

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New mutants of T4 have been isolated by using a strain of *Escherichia coli* lacking thymidine kinase activity. These T4 mutants, designated *tk*, are able to grow on this *E. coli* strain under light on plates containing 5-bromodeoxyuridine and were all found to be unable to induce thymidine kinase (ATP: thymidine 5'-phosphotransferase, EC 2.7.1.21). All of these *tk* mutants fall into one complementation group which maps just to the right of *rI* on the standard T4 genetic map, far from most other genes coding for enzymes involved in pyrimidine metabolism. The *tk* mutants grow as well as wild-type T4, indicating that thymidine kinase is a non-essential enzyme.

Thymidine kinase catalyzes the phosphorylation of thymidine to produce dTMP. Hiraga et al. (9) showed that T4 bacteriophage induces thymidine kinase activity after infection of a mutant of *Escherichia coli* lacking this activity. Because the activity induced after phage infection is much more heat sensitive than is the activity present in wild-type, uninfected *E. coli*, it probably represents a new enzyme synthesized from the phage genome. It should be possible to isolate mutants of T4 unable to induce this enzyme (*tk* mutants) and to find the position of the thymidine kinase locus on the T4 genetic map. Several genes specifying enzymes involved in thymidylate metabolism (6, 21) and the metabolism of other pyrimidine nucleotides (7, 8, 24) map in one region of the T4 genome. Three of these genes, coding for dihydrofolate reductase (6), thymidylate synthetase (21), and ribonucleotide reductase (24), like thymidine kinase, code for enzymes whose activities are normally present in uninfected cells. It would be of interest to see whether the gene for thymidine kinase, which is related to these genes in function, maps near them.

DNA which contains 5-bromodeoxyuridine (BUdR) is light sensitive (13, 20). Therefore, if cells or phage are unable to incorporate BUdR into their DNA, they should be able to grow better in the presence of BUdR and light than cells or phage which do incorporate BUdR into their DNA. BUdR is a good substrate for the thymidine kinase purified from *E. coli* (16), and its phosphorylation by this enzyme is presumably the first step involved in its incorporation into DNA. Cells lacking thymidine kinase activity should be resistant to BUdR. This has been shown to be true in the case of mouse fibroblasts (11) as well as *E. coli* (9). If *E. coli* cells lacking thymidine kinase are grown in the presence of BUdR and light and are infected with T4, phage lacking the ability to induce thymidine kinase should be able to form progeny more readily than can phage which can induce the enzyme. To insure than enough BUdR enters the DNA of phage that can induce thymidine kinase, the size of the pool of thymidine precursors to DNA can be lowered by inhibiting the enzyme thymidylate synthetase, which synthesizes dTMP from dUMP, by adding 5-fluorodeoxyuridine (FUdR) (15). Uridine must be added in the presence of FUdR to permit growth of cells (1), presumably by preventing incorporation of 5-fluorouracil into RNA. Deoxyadenosine (dAdo) inhibits the conversion of thymidine to thymine (23) and should act in the same way to decrease the conversion of BUdR and FUdR to 5-bromouracil and 5-fluorouracil, respectively. The addition of dAdo should also facilitate incorporation of BUdR into the DNA of phage that induce thymidine kinase. BUdR, FUdR, dAdo, and uridine were used to select mutants of T4 unable to induce thymidine kinase.

MATERIALS AND METHODS

Bacterial strains, phage, and media. Phage stocks were prepared on *E. coli* S/6. The *tk* mutants were isolated by plating on *E. coli* KY895, an isoleucine, valine-requiring strain which lacks thymidine kinase activity, isolated from *E. coli* E3110 by Igara-

shi et al. (10). *E. coli* KY895 was obtained from G. R. Greenberg. *E. coli* OK305, which was obtained from O. Karlström, was used for some mapping studies. It is a derivative of *E. coli* B which requires pyrimidine for growth and is deficient in cytidine deaminase activity (8).

T4Do, an osmotic shock-resistant derivative of T4D, was the standard strain of bacteriophage used. Two of the *tk* mutants were isolated from *td8*, a mutant unable to induce thymidylate synthetase which was isolated from $T4BO_1$ and backcrossed to T4D (21). Mapping was done with *r48*, an *rI* mutant of T4D obtained from W. B. Wood, and with *nrdC19*, a mutant of T4D unable to induce ribonucleotide reductase that was derived from an *nrdC19-frd1* double mutant obtained from I. Tessman (J. R. Johnson and D. H. Hall, Virology, in press). 3XD, the glycerol-Casamino Acid medium of Fraser and Jerrel (4), prepared without gelatin, was used to prepare phage stocks and enzyme extracts. Broth medium, synthetic medium, and agar plates containing about 30 ml of broth or synthetic medium were prepared as described by Goscin and Hall (5). *E. coli* OK305 was grown on synthetic medium supplemented with uracil (20 μg/ml) and plated on synthetic agar plates containing cytidine (20 μg/ml). *E. coli* KY895 was grown on synthetic medium containing thiamine-hydrochloride (5 μg/ml), and plated on synthetic medium containing 2 μg of the same chemical per ml.

Chemicals. Cytidine, uridine, uracil, thymidine, BUdR, dAdo, dCTP, and dTMP were purchased from Calbiochem. ATP was purchased from P-L Biochemicals. FUdR was obtained from Hoffman-LaRoche, Inc. Thymidine-*methyl*-^3H (15.4 Ci/mmol) and dTMP-*methyl*-^3H (39 Ci/mmol) were purchased from Schwarz/Mann. Pyrimethamine was obtained from James J. Burchall of Burroughs Wellcome and Co.

Selection of tk mutants. Thiamine-hydrochloride (50 μg), BUdR (5 mg), FUdR (100 μg), uridine (500 μg), dAdo (100 μg), and about 2×10^8 *E. coli* KY895 cells were added to synthetic top agar (2.5 ml) together with phage and plated on synthetic medium. The plates were allowed to stand at room temperature (23 C) overnight under a 5-W fluorescent desk lamp (General Electric F8T5-W) at a distance of 1 cm from the plate. Plaques that formed under these conditions were picked and purified.

Preparation of extracts for enzyme assays. Extracts were prepared in a manner similar to that of Hiraga et al. (9). Cells were grown in 3XD medium at 37 C to a concentration of 2×10^8 cells/ml. 3XD medium was supplemented with thiamine-hydrochloride (2 μg/ml) when *E. coli* KY895 was used to prepare extracts. L-Tryptophan (20 μg/ml) was added to the cells, and immediately afterward phage were added. The infection was stopped by rapidly chilling the cells on ice, and the cells were concentrated 20- to 40-fold by centrifuging 5 min at $6,000 \times g$ and resuspending in 0.05 M Tris-hydrochloride buffer, pH 7.8, containing bovine serum albumin (BSA; 200 μg/ml). The resuspension was sonically treated with a Branson sonifier, and the crude extract was used for enzyme assays.

All protein assays were performed by the method of Lowry et al. (14) with bovine serum albumin as a standard.

Thymidine kinase assay. The thymidine kinase assay was similar to that used by Hiraga et al. (9). The incubation mixture contained in a total volume of 50 μliters: Tris-hydrochloride buffer (pH 7.8, 3.5 μmol), $MgCl_2$ (0.14 μmol), $MnCl_2$ (0.035 μmol), ATP (0.28 μmol), dCTP (0.05 μmol), [*methyl*-^3H]thymidine (0.042 μmol; 60 Ci/mol), BSA (14 μg), and crude extract (25 to 100 μg of protein). This mixture was incubated at 30 C and the reaction was stopped by adding 50 μliters of ice-cold thymidine (10 mg/ml) dissolved in 0.1 N HCl.

Isolation of products of thymidine kinase assay. The isolation of products was based on the method of Randerath and Randerath (19). The incubation mixture was centrifuged at $1,000 \times g$ for 15 min, and 5 μliters of the supernatant fluid was spotted on polyethylenimine-cellulose coated plastic sheets (MN300, Brinkmann Instruments, Inc.) together with cold carrier dTMP. A known amount of ^{3}H-dTMP was also spotted to determine recovery of dTMP after chromatography and counting efficiency. Before chromatography, the sheet was soaked in methanol for 15 min to remove salts and thymidine. After drying, ascending chromatography was first carried out in water to a height of 4 to 5 cm and then in 1 M LiCl for an additional 8 cm. The dTMP spots, detected by using UV light, were cut out, soaked in a 10-ml beaker of water for 15 min, dried, and counted by using liquid scintillation spectroscopy. Soaking in water washes off any thymidine contaminating the dTMP spot but does not affect dTMP recovery.

The dCTPase assays were performed by the method of Price and Warner (18). Phage crosses were performed on *E. coli* B as described by Hall et al. (8).

RESULTS

Isolation of mutants. When about 2×10^6 T4D*o* phage were plated on *E. coli* KY895 under the conditions described in Materials and Methods, 30 to 50 plaques formed. Three of these were picked and purified and named *tk1*, *tk2*, and *tk3*. These phage plated as efficiently under the conditions used to select them as under any other conditions. When plated on B cells growing on broth agar plates, the *tk2* and *tk3* mutants made large, clear plaques similar to the plaques made by rapid lysis (*r*) mutants. When *tk2* and *tk3* are crossed to T4D*o*, the *r* phenotype can not be separated from the *tk* phenotype. We believe these are deletion mutants covering the *tk* cistron and an *r* cistron. *td8* phage (10^7) were plated under the selection conditions on *E. coli* KY895, and two plaques were picked from about 10 that formed. These two mutants were purified and called *tk4* and *tk5*. These phage also plated as efficiently under the conditions used to select them as under any other conditions.

Thymidine kinase activity of tk mutants. All

tk mutants lack the ability to induce thymidine kinase activity after infection of *E. coli* KY895 (Table 1). Since dCTPase activity was normal in the extracts prepared from the cells infected with the mutants, it is clear that the infection was successful. Figure 1 compares the kinetics of induction of thymidine kinase and dCTPase in cells infected with *tk1* and T4D*o*. The *tk1* mutation apparently does not affect the production of dCTPase. *E. coli* KY895 infected with any one of the *tk* mutants contains normal levels of T4-induced dihydrofolate reductase and deoxycytidylate deaminase activities at 10 min after infection (unpublished data).

If the lack of thymidine kinase activity in extracts of cells infected with *tk* mutants were due solely to the presence of an inhibitor, this inhibitor would also inhibit the thymidine kinase activity in extracts of cells infected with tk^+ phage. If the inhibitor is present in excess, a mixture of the two extracts would yield activity less than the sum of the activities of the single extracts. A mixture of extracts prepared from cells infected with *tk1* and T4D*o* gives an amount of thymidine kinase actvity greater than or equal to that expected if the activities present in tk^+ and *tk1* extracts are additive (Table 2). This indicates that there is no excess of inhibitor of thymidine kinase present in the *tk1* extract.

Effect of dCTP on thymidine kinase activity. Okazaki and Kornberg (17) found that dCTP stimulates thymidine kinase activity in extracts prepared from uninfected *E. coli*. We find that it also stimulates the enzyme induced after T4 infection (Table 3).

Mapping of tk mutants. Since two *tk* mutants seemed to be deletions including an *r* locus, we suspected that *tk* was closely linked to one of these loci. The *tk1* mutant was crossed to the *rI* mutant, *r48*, and progeny from the cross were plated under the conditions used to select *tk* mutants. Plaques that formed under these conditions were picked and spotted on *E. coli* B growing on broth agar plates to see how many showed the *r* phenotype. This cross showed that *tk1* maps 4 ± 1 map units from *r48*. Because *nrdC* maps near *rI* (22), *tk1* was then crossed to *nrdC19*, and progeny phage which formed plaques under the conditions used to isolate *tk* mutants were spotted on *E. coli* OK305 cells growing on synthetic agar plates containing

TABLE 1. *Thymidine kinase and dCTPase activities induced by tk mutants*[a]

Phage	Specific activity	
	Thymidine kinase[b]	dCTPase[c]
tk^+ (T4D*o*)	20.8	33
tk1	1.0	41
tk2	2.6	41
tk3	<1.0	39
tk4	<1.0	52
tk5	<1.0	52
Uninfected cells	2.5	<10

[a] *E. coli* KY895 was infected at a multiplicity of 5 phage per cell for 15 min at 37 C.

[b] Specific activity expressed in nanomoles of dTMP formed per hour per milligram of protein.

[c] Specific activity expressed in nanomoles of dCMP formed per minute per milligram of protein.

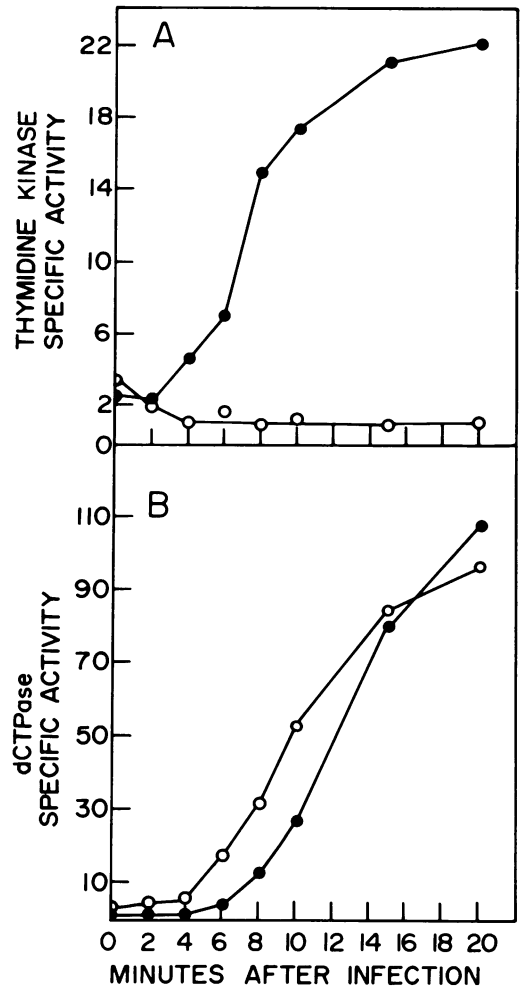

FIG. 1. *Induction of enzyme activities at 37 C after infection of E. coli KY895 with tk^+ (●) and tk1 (○). Thymidine kinase specific activity is expressed in nanomoles of dTMP formed per hour per milligram of protein, and dCTPase specific activity is expressed in nanomoles of dCMP formed per minute per milligram of protein.*

TABLE 2. *Mixing of tk⁺ and tkl extracts*[a]

Phage	Amount of extract (μliters)	Thymidine kinase activity[b]	
		Found	Expected
tk⁺ (T4Do)	25	6.3	
*tk*1	25	0.3	
tk⁺	5	1.9	1.5
*tk*1	20		
tk⁺	10	3.1	2.7
*tk*1	15		
tk⁺	15	4.9	3.9
*tk*1	10		
tk⁺	20	5.2	5.1
*tk*1	5		

[a] *E. coli* KY895 was infected at a multiplicity of 5 phage per cell for 15 min at 37 C.

[b] Expressed as nanomoles of dTMP formed per hour.

TABLE 3. *Effect of dCTP on T4-induced thymidine kinase activity*[a]

dCTP concentration (mM)	Thymidine kinase activity[b]
0.0	0.7
0.1	0.9
1.0	1.1
10.0	1.4

[a] *E. coli* KY895 was infected with T4Do at a multiplicity of 5 phage per cell for 15 min at 37 C.

[b] Expressed as nanomoles of dTMP formed per hour. Enzyme assays were performed as described in Materials and Methods except that the amount of dCTP was varied.

cytidine (20 μg/ml) and pyrimethamine (400 μg). Under these conditions, wild-type phage form a plaque with a white halo of rapidly growing cells, but *nrd* mutants form plaques with no halos (J. R. Johnson and D. H. Hall, Virology, in press). The distance between *tk1* and *nrdC19* is 25 ± 4 map units. Next, *nrdC19* was crossed to *r48*, and progeny were plated on *E. coli* OK305 growing under the conditions used to identify *nrd* mutants as described above. Under these conditions, *rI* mutants make a very thin halo which can be easily distinguished from a wild-type halo. This cross indicated that *r48* maps 18 ± 2 units from *nrdC19*, suggesting that *tk1* maps just to the right of *rI* on the T4 genetic map (Fig. 2).

To verify this map position, a three-factor cross was performed, crossing an *r48-tk1* double mutant obtained from one of the crosses above, to *nrdC19*. Progeny plaques that grew under the conditions used to isolate the *tk* mutants were picked and tested for the *r* and *nrd* phenotypes as described above. Of eight phage picked that showed the *r*⁺ phenotype, all were *nrd*. This is consistent with the *tk1* map position to the right of *rI*, because if *tk* mapped to the left of *rI*, most *tk-r*⁺ progeny would be *nrd*⁺ and only a rare double recombinant would be *tk-r*⁺*-nrd*. The four other *tk* mutants were crossed to *tk1* and were found to map within 5 map units. No *tk*⁺ recombinants could be found when *tk2* or *tk3* were crossed to *tk1*, adding further evidence that *tk2* and *tk3* are deletions.

Complementation studies. Complementation studies of pairs of *tk* mutants were performed in vivo by mixedly infecting *E. coli* KY895, preparing extracts, and assaying for thymidine kinase activity. No evidence was obtained that any of the *tk* mutants complements *tk1* (Table 4). This and the fact that all *tk* mutants map near each other suggest that all *tk* mutants isolated at this time map within the same cistron.

DISCUSSION

It is clear that the plating conditions used strongly select T4 mutants defective in the ability to induce thymidine kinase activity, because all phage examined that grow under the selection conditions fail to induce the activity. The *tk* mutants may not be in the structural gene for phage-induced thymidine kinase, but in a gene that codes for a product necessary for

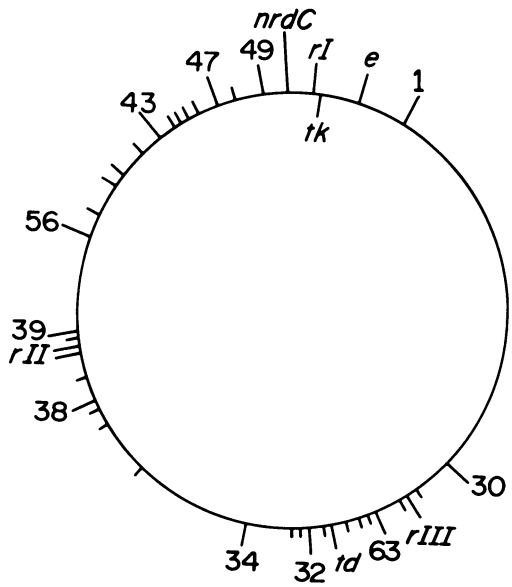

FIG. 2. *Genetic map of T4, modified from Edgar and Wood (2), showing the location of the tk mutants.*

TABLE 4. *Complementation studies in vivo of tk mutants*[a]

Phage	Multiplicity of infection	Specific activity	
		Thymidine kinase[b]	dCTPase[c]
tk$^+$ (T4Do)	8	33.1	122
*tk*1	8	2.8	90
*tk*1	4	3.3	104
*tk*2	4		
*tk*1	4	2.2	120
*tk*3	4		
*tk*1	4	1.5	135
*tk*4	4		
*tk*1	4	1.5	133
*tk*5	4		
Uninfected cells		2.2	<10

[a] *E. coli* KY895 was singly or mixedly infected with phage for 15 min at 37 C.

[b] Specific activity is expressed as nanomoles of dTMP formed per hour per milligram of protein.

[c] Specific activity is expressed as nanomoles of dCMP formed per minute per milligram of protein.

synthesis or activation of either a cell or a phage thymidine kinase. But because the phage-induced enzyme is more heat-sensitive than is the enzyme present in uninfected cells (9), T4 probably does have a structural gene for thymidine kinase. We do not feel that T4 merely modifies the *E. coli* enzyme, because T4-induced activity can be seen in a mutant of *E. coli* lacking thymidine kinase activity and because heat-stable thymidine kinase activity remains constant in *E. coli* B infected with T4, whereas heat-sensitive activity increases (unpublished data). Because all *tk* mutants isolated so far are in the same cistron, this is most likely the cistron coding for the enzyme. This can not be proved until temperature-sensitive or amber *tk* mutants are isolated.

Although the time course of thymidine kinase expression shows that it is an early enzyme, *tk* mutants do not map near genes coding for any related early enzymes except *nrdC*. This indicates that not all T4 genes coding for early enzymes map in the same area of the genome even though they may be functionally related. This is most clearly seen in the case of the genes for ribonucleotide reductase. The gene *nrdC* maps far from *nrdA* and *nrdB* (22), both of which map near the gene (*td*) for dTMP synthetase (Fig. 2). Although T4 early enzymes are induced together and often have related functions, the fact that genes coding for some of them map far from each other indicates that they do not form a simple operon. Some other form of regulatory control must operate to induce early enzymes.

The reason T4 induces thymidine kinase is unclear. Total thymidine kinase activity only doubles after infection of *E. coli* B (unpublished data), although the increase in activity in vivo may be greater than that observed in extracts since the phage-induced enzyme is extremely unstable under our extraction conditions, whereas the enzyme present in uninfected *E. coli* B is quite stable. T4 has two other sources of dTMP besides phosphorylation of thymidine. It can made dTMP from dUMP (3) or break down host DNA (12). The effect of losing thymidine kinase should be small, and indeed, *tk1* grows as well as does wild-type T4 on broth or synthetic media and even on cells which have no thymidine kinase activity. We assume that the ability to induce thymidine kinase benefits T4 under some conditions, but these conditions must be different from our standard laboratory conditions.

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