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Characterization of New Regulatory Mutants of Bacteriophage T4

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Plating techniques which eliminate T4 plaque formation on *Escherichia coli* by folate analogue inhibition of dihydrofolate (FH_2) reductase (EC 1.5.1.3) allowed the isolation of folate analogue-resistant (*far*) mutants of T4. One class of *far* mutants overproduces the phage-induced FH_2 reductase. Deoxycytidylate deaminase (EC 3.5.4.12), thymidine kinase (EC 2.7.1.21), and deoxycytidine triphosphatase (EC 3.6.1.12) are also overproduced by 20 min after infection at 37 C. The overproduction of FH_2 reductase by these *far* mutants is not affected by the absence of DNA synthesis. Other types of mutations that affect the synthesis of early enzymes cause overproduction in the absence of DNA synthesis of some of the above enzymes but not of FH_2 reductase. Therefore, overproducing *far* mutants apparently have mutations in previously undescribed genes controlling the expression of the T4 genome. Three of four mutants under study map near gene 56, and one maps near gene 52. All of these mutants show delays in DNA synthesis, phage production, and lysis and appear to show decreased levels of RNA synthesis based on the cumulative incorporation of uridine.

Soon after infection of *Escherichia coli* by bacteriophage T4, six enzymes involved in the biosynthesis of thymidylate are induced (Fig. 1). Two of these enzymes, deoxycytidine triphosphatase (dCTPase) (21, 28) and thymidine kinase (2), are coded for by genes which are unlinked on the genetic map of T4 (Fig. 2). The genes coding for the remaining four enzymes, deoxycytidylate (dCMP) deaminase, dihydrofolate (FH_2) reductase, ribonucleotide reductase, and thymidylate synthetase are clustered between genes 31 and 32 on the genetic map of T4 (Fig. 2), but are unlinked to the genes coding for dCTPase and thymidine kinase (8, 9, 24, 33). We are investigating the possibility that the genes clustered between genes 31 and 32 are coordinately expressed by studying T4 mutants with altered synthesis of FH_2 reductase. To isolate such mutants, folate analogue inhibition of the phage-induced FH_2 reductase was used as a selection technique (12). By use of special plating techniques, wild-type plaque production in the presence of folate analogues was eliminated, and folate analogue-resistant (*far*) mutants were isolated. Some of these *far* mutants overproduce the normal T4 FH_2 reductase. Data characterizing the extensive alterations in T4 gene expression and macromolecular biosynthesis will be presented for three of these mutants, *far*P3, *far*P14, and *far*P85. The mapping of the above mutants has been accomplished, and the existence of two new regulatory genes of T4 has been established.

MATERIALS AND METHODS

Bacteriophage strains. T4D*o*, an osmotic-shock-resistant mutant of T4D, is the standard bacteriophage type from which all *far* mutants were isolated and with which all mutants have been compared. The *far* mutants (12) and the hydroxyurea-sensitive (*hus*) mutants (7) were previously isolated and characterized by this laboratory. The T4 *am* mutants (4) were isolated at the California Institute of Technology and were obtained from C. Levinthal, W. B. Wood, D. P. Snustad, R. B. Luftig, and J. S. Wiberg. The SP62 single mutant, *am*N55 (gene 42) backcrossed five times, and the SP62-*am*N55 double mutant was received from J. S. Wiberg. The mutant *td*H10 (*td*10 of Simon and Tessman, 24 [the H is to indicate that the mutant was induced with hydroxylamine]) were obtained from I. Tessman. The *nrd*B10 single mutant was isolated from an *nrd*B10-*frd*1 double mutant obtained from I. Tessman (12).

Bacterial strains. Enzyme induction studies, extracts, and phage growth studies were performed in *E. coli* B unless indicated otherwise. Phage crosses were performed in *E. coli* CR63. Stocks of all phage were prepared on *E. coli* S/6 except for *am* mutants that were prepared on *E. coli* CR63, the permissive host. A few plate lysates of *far*P85 were prepared with *E. coli* B as the host cell. Bacteriophage plating techniques were done with *E. coli* OK305, a uracil-requiring derivative of *E. coli* B, with very low cytidine deaminase activity and no detectable deoxycytidine

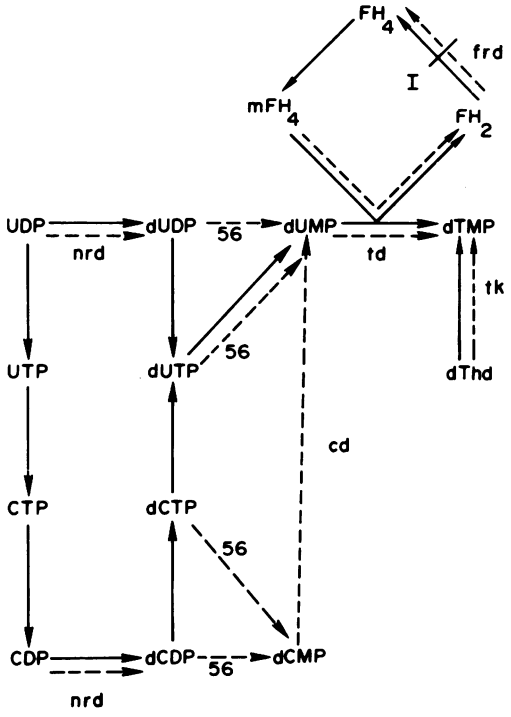

FIG. 1. *Reactions involved in pyrimidine nucleotide interconversions and in the biosynthesis of thymidylate in E. coli before and after T4 infection. Solid lines represent bacterial enzymes. Dashed lines represent phage-induced enzymes for which the gene number or abbreviation is used. The cross bar (/) indicates the target site for folate analogue inhibition. Abbreviations: FH_2, dihydrofolate; FH_4, tetrahydrofolate; and mFH_4, N^5,N^{10}-methylenetetrahydrofolate.*

deaminase activity (15). *E. coli* OK305 was received from O. Karlström. *E. coli* KY895, a strain which lacks thymidine kinase activity (11), was obtained from G. R. Greenberg.

Media. 3XD, the glycerol-Casamino Acids medium of Fraser and Jerrel (5), was used (with the gelatin omitted) in enzyme induction studies, in phage growth studies, and in preparation of phage stocks. Broth medium, synthetic medium (S) and agar plates containing approximately 30 ml of broth or synthetic medium were prepared as described by Goscin and Hall (7). *E. coli* OK305 was grown in synthetic medium supplemented with 20 μg of uracil per ml and was plated on synthetic agar plates supplemented with 20 μg of cytidine per ml (S + Cyd plates).

Chemicals. Hydroxyurea (HU), cytidine, uracil, deoxyadenosine (dAdo), thymidine, dCMP, and deoxycytidine triphosphatate (dCTP) were purchased from Calbiochem. Folic acid was purchased from Sigma Chemical Co. Tetrahydrofolate was purchased from General Biochemicals Corp. Nicotinamide adenine dinucleotide phosphate, reduced form, was purchased from P-L Biochemicals. Norit A was purchased from Pfanstiehl Laboratories. 2,5-Diphenyloxazole (PPO) and dimethyl-1,4-bis-(5-phenyloxazolyl) benzene (POPOP) were purchased from Packard Instrument Co.

[*Methyl*-^{3}H]thymidine (3 Ci/mmol) and [5-^{3}H]dUMP (17.6 Ci/mmol) were purchased from Schwarz/Mann. [5-^{3}H]uridine (28.5 Ci/mmol) and [6-^{3}H]uridine (25.3 Ci/mmol) were purchased from New England Nuclear Corp.

Dihydrofolate was prepared as described by Futterman (6). N^5,N^{10}-methylene tetrahydrofolate was prepared by dissolving 10 mg of tetrahydrofolate in 4.5 ml of 1 M mercaptoethanol, 0.5 M Tris, pH 7.4, which contained 0.2 mg of formaldehyde per ml. The solution was kept in the dark and frozen under nitrogen when not in use.

Bacteriophage plating techniques. The following method was developed by Goscin and Hall (7) as a technique for selecting *hus* mutants. In the plating method, 20 mg of HU and 3×10^8 to 6×10^8 fresh *E. coli* OK305 cells and phage are added to S + Cyd top agar, spread on a S + Cyd plate, and incubated at either 37 or 43 C for approximately 24 h. Only *hus*$^+$ phage form plaques with a good efficiency.

Bacteriophage crosses. Phage crosses were performed as described by Hall et al. (9) with the following modifications: *E. coli* CR63 was used as the host cell and the dilutions of each cross were incubated for 60 min at 37 C before chloroform was added. The *far* × *amber* crosses were usually evaluated by plating on *E. coli* CR63 on broth plates (the permissive condition) for total phage and on *E. coli* B on broth plates (the nonpermissive condition) for wild-type recombinants. The *amber* mutants used, except for a few (*am*E38 and *am*H17 [gene 52] and *am*B5 [*t* gene]), fail to grow on *E. coli* B. Wild-type recombinant progenies were scored on *E. coli* B by their larger plaque morphology in comparison to the *far* mutant

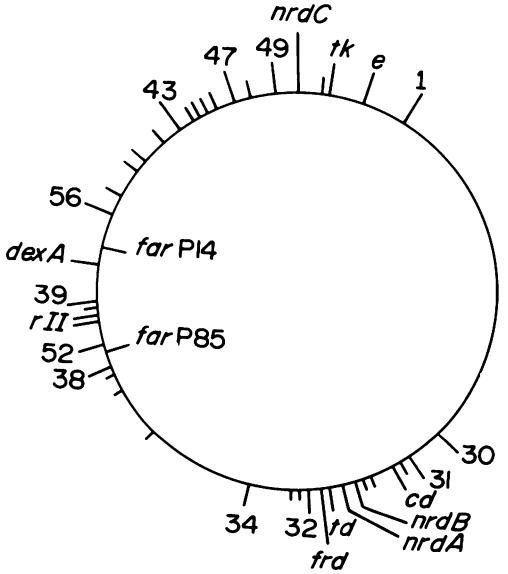

FIG. 2. *Genetic map of T4, modified from Edgar and Wood (3), showing the locations of the far mutants.*

plaque morphology (12). The *far* × *hus* crosses were evaluated by plating on *E. coli* CR63 for total phage and on OK305 on S + Cyd plates with HU for wild-type recombinants; HU prevents growth of *hus* mutants, and *far* mutants are recognized by their smaller than normal plaques. Crosses of *amber* and *far* mutants by *am*B5 (*t* gene) were evaluated by plating on CR63 on broth plates for total phage and on OK305 on S + Cyd + HU plates (nonpermissive for *am*B5) for wild-type recombinants.

Construction of the far-amN55 double mutants. Crosses of the *far* mutants by *am*N55 (gene 42) were performed as described above. Each cross was diluted and plated on *E. coli* CR63 on broth plates, and 29 well-isolated plaques were randomly picked. Each picking was spotted on *E. coli* CR63 and *E. coli* B on broth plates to detect the am^+ pickings. The *am* pickings were plated on CR63 to compare their plaque morphology. Several pickings from each cross that consistently plated with a small plaque morphology were chosen and characterized according to their enzyme induction patterns at 20 min after infection. Those phage that overproduced the FH_2 reductase (one out of one in three cases and three out of four in one case), an enzyme not normally overproduced by *amber* mutants in gene 42 (26) (see Results), were scored as *far-am*N55 doubles.

Preparation of extracts for enzymatic assays. *E. coli* B cells were grown from 25-fold dilutions of fresh overnight stocks to 2×10^8/ml in 3XD media at 37 C with vigorous aeration. The cells were infected at a multiplicity of 6 phage per cell. At the indicated times after infection, samples were taken by: (i) chilling the infected cells in an ice-water bath (single time point extracts), or (ii) by removing a sample and diluting into an equal volume of 3XD chilled to approximately −4 C in a salt-ethanol-ice-water bath (enzyme induction studies). The chilled samples were spun and resuspended at 0.1 to 0.01 the original volume (depending on the enzyme to be assayed) in 0.02 M Tris, pH 7.4, 0.04 M mercaptoethanol. A Branson sonifier equipped with a micro tip adjusted to a setting of 2 was used to disrupt the cells in 5-s bursts. The cells were kept in an ice-water bath at all times. Cell debris was removed by centrifugation at 20,000 × *g* for 20 min. The extracts to be assayed for FH_2 reductase, dCMP deaminase, and thymidine 5′-monophosphate (dTMP) synthetase were dialyzed overnight at 4 C against the resuspension buffer. Extracts for assay of the thymidine kinase and dCTPase were prepared by a similar procedure described by Chace and Hall (2) using *E. coli* KY895. The protein concentrations in extracts were determined by the method of Lowry et al. (17) by using bovine serum albumin as a standard.

Enzyme assays. FH_2 reductase and dCMP deaminase were assayed at 28 and 30 C, respectively, by the method of Warner and Lewis (26) with the modifications of Hall (8). Thymidylate synthetase was assayed as described by Lomax and Greenberg (16) and Kammen (14). T4-induced FH_2 reductase and dTMP synthetase activities were calculated by subtracting the activity found in a control uninfected culture. Thymidine kinase and dCTPase were assayed by the procedure of Chace and Hall (2).

Bacteriophage growth studies. One-step growth curves were performed by using the following procedure. *E. coli* B cells were grown in 3XD to 2×10^8/ml as described for extracts, spun, and resuspended at 5.5×10^8/ml in fresh 3XD. The cells were infected at a multiplicity of 0.1 phage per cell. After 4 min of adsorption at 37 C with minimal aeration, the cells were diluted 10^4- and 10^6-fold in 3XD and aerated in a 37 C shaking-water bath. At the designated times, samples were withdrawn, diluted, and plated on B on broth plates for infective centers or chloroformed, diluted, and plated for intracellular phage.

Procedure for DNA and RNA synthesis measurements. *E. coli* B cells were grown in 3XD to 2×10^8/ml as described for extracts, spun, and resuspended in fresh 3XD at 5×10^8/ml. The cells were infected at a multiplicity of 5 phage per cell (10 phage per cell for complementation experiments). The cells were superinfected 4 min after infection with an additional 5 phage per cell (no superinfection in complementation tests). Label was added 1 min after infection: (i) for DNA synthesis, 5 μCi of [*methyl*-^{3}H]thymidine per ml and 250 μg of dAdo per ml; (ii) for RNA synthesis, 2 μCi of [5-^{3}H]uridine per ml and 250 μg of dAdo per ml. To keep RNA and DNA synthesis experiments comparable, the RNA incubation mixture was 1.7 μM cold thymidine, and the DNA incubation mixture was 71 nM cold uridine. When RNA label was chased, a 50,000-fold excess of cold uridine was added. To determine label incorporation, 0.05-ml samples were withdrawn at the designated times after infection and pipetted onto Whatman no. 3 filter paper disks (2.3 cm diameter). The disks were immediately immersed in ice cold 10% trichloroacetic acid for approximately 20 min, washed twice with 5% trichloroacetic acid with 10-min incubations between washings, and rinsed once with acetone for 10 min. The disks were dried with a stream of warm air for approximately 15 min and counted in toluene-PPO-dimethyl-POPOP scintillation fluid in an Intertechnique scintillation counter. Phage burst size at 60 min, and cell survivors at 20 min after infection were measured for each infected sample in all experiments. The burst sizes were lower than in the one-step growth curves, apparently due to the different procedure, but the relative bursts were the same. Cell survivors at 20 min after infection were always less than 1% of the original concentration.

As an alternative method for measuring DNA synthesis, [6-^{3}H]uridine was used to label both RNA and DNA. Cells were grown and infected as described above. The label was added at 1 min after infection with a final concentration of 5 μCi/ml along with 250 μg of dAdo per ml. No cold uridine or thymidine was present in these experiments. To determine label incorporation, two 0.05-ml samples were taken simultaneously at the designated times. One sample was immediately added to 2.5 ml of ice-cold 10% trichloroacetic acid, while the other was added to 0.2 ml of 0.3 N sodium hydroxide containing 1% sodium dodecyl sulfate (SDS), heated at 80 C for 15 min, chilled, neutralized with 0.2 ml of 0.3 N hydrochloric acid, and precipitated by adding 2.5 ml of cold 10% trichloroacetic acid (31). The trichloroacetic acid-precipitated samples were allowed to stand overnight

at 4 C, collected on glass fiber filters, and counted in toluene-PPO-dimethyl-POPOP scintillation fluid in an Intertechnique scintillation counter. This alkali treatment hydrolyzes greater than 90% of the acid-insoluble material labeled by [5-^{3}H]uridine (RNA) and essentially none of the acid-insoluble material labeled by [*methyl*-^{3}H]thymidine (DNA). Therefore, the alkali-resistant acid-insoluble material labeled by [6-^{3}H]uridine is DNA and was labeled with deoxyribonucleotide derivatives of the ribonucleotide, [6-^{3}H]uridine, which were synthesized in vivo. DNA synthesis of wild-type (T4D*o*) infected cells is similar as measured by cumulative incorporation of alkali-resistant acid-insoluble [6-^{3}H]uridine or by acid-insoluble [*methyl*-^{3}H]thymidine.

RESULTS

Enzyme induction by the far overproducers. Early in the investigation of *far* overproducer mutants, it was established that the FH_2 reductase and at least one additional enzyme, dCMP deaminase, were overproduced relative to wild type by 15 min after infection (12). The kinetics of induction of FH_2 reductase, an immediate early enzyme, and dCMP deaminase, a delayed early enzyme (25), has now been compared for wild-type and the *far* overproducers (Fig. 3). The FH_2 reductase specific activity begins to increase by 1 min after infection and appears to be induced normally for the first few minutes of infection by the *far* overproducers. By 3 or 4 min after infection (3 min for *far*P85 and 4 min for *far*P14 and *far*P3, data not shown for *far*P3), FH_2 reductase specific activity begins to increase more rapidly in *far* mutant-infected cells than in wild-type infected cells. The rapid increase in FH_2 reductase specific activity ceases between 10 and 12 min after infection in both wild-type and *far* mutant-infected cells. In contrast, dCMP deaminase specific activity that is induced between 4 and 12 min after infection in wild-type infected cells is induced at the normal time by *far* overproducers but is still being synthesized 20 min after infection.

The *amber* mutants which affect DNA synthesis, particularly those which do not make any DNA (designated DO), overproduce some early enzymes but not the FH_2 reductase (26, 29). The effect of DNA synthesis on the overproduction of FH2 reductase and dCMP deaminase was evaluated by constructing double mutants containing a *far* overproducer mutation and *am*N55, an *amber* mutation in gene 42.

When induction studies were performed with the *far-am*N55 double mutants (Fig. 4, data for *far*P3 not shown but similar to *far*P14), results were comparable with those obtained with the *far* singles. The *am*N55 mutation has no effect on FH_2 reductase induction by itself and does

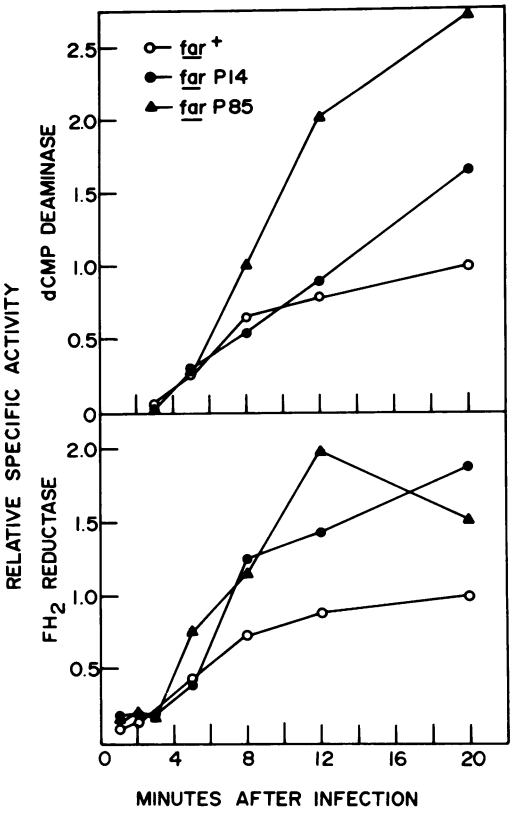

FIG. 3. *Induction of dCMP deaminase (upper panel) and FH_2 reductase (lower panel) by wild type (far$^+$), farP14, and farP85 after infection of E. coli B at 37 C. Extracts were prepared at the designated times, and FH_2 reductase and dCMP deaminase assays were performed as described in Materials and Methods. A wild-type control was prepared in each experiment. Specific activities are calculated relative to that of an extract of cells infected with wild type prepared 20 min after infection. In these wild-type extracts, the FH_2 reductase specific activity was 40 ± 15 (average ± SD) nmol of product formed per min per mg of protein, and the dCMP deaminase specific activity was 50 ± 22 nmol of product formed per min per mg of protein during these experiments. The variability of comparable extracts prepared at the same time is less by at least a factor of two.*

not significantly increase or decrease the FH_2 reductase overproduction in the *far-am*N55 double mutants. However, the *am*N55 mutation results in extended synthesis of dCMP deaminase, and the overproduction of dCMP deaminase by the *far* mutants appears to be additive with the *am*N55 overproduction in cells infected with the double mutants.

In additon to FH_2 reductase and dCMP deaminase, the synthesis of three additional phage-induced early enzymes was studied. Table 1 summarizes the relative specific activi-

ties found in wild-type and *far* mutant-infected cell extracts for dTMP synthetase, thymidine kinase, and dCTPase at 15 and 20 min after infection. At both times, dTMP synthetase is underproduced by the *far* mutant-infected cells relative to wild-type infected cells. Thymidine kinase is overproduced at both time points, but dCTPase is overproduced slightly by the 20-min time point. These results indicate that the time after infection at which extracts are prepared is critical for detection of overproduction, and that the *far* mutations produce a long-lasting and complicated effect on phage gene expression.

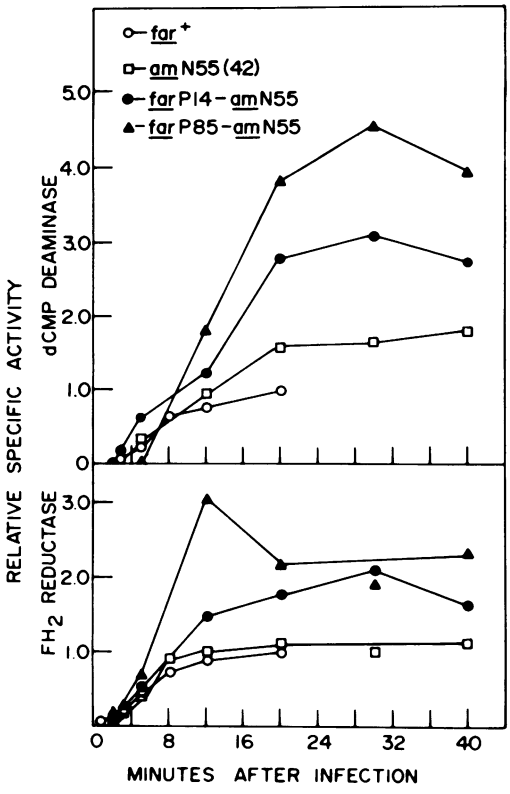

FIG. 4. *Induction of dCMP deaminase (upper panel) and FH_2 reductase (lower panel) by wild type (far+), amN55 (gene 42), and the double mutants farP14-amN55, and farP85-amN55 after infection of E. coli B at 37 C. See legend of Fig. 3 for details.*

Growth characteristics of the far overproducers. One-step growth curves of T4Do and the *far* overproducers are shown in Fig. 5. Both the appearance of intracellular phage and the beginning of lysis are delayed for the *far* mutants. Two of the *far* mutants, *far*P14 and *far*P3 (data not shown), have similar brief delays, whereas *far*P85 has much longer delays. The time between appearance of intracellular phage and lysis is similar to that of wild type for all the *far* mutants, and only *far*P85 shows a significantly reduced burst size 60 min after infection.

Phage DNA synthesis. Phage DNA synthesis was measured by incorporation of [*methyl*-^{3}H]thymidine into acid-insoluble material (Fig. 6). All the *far* overproducers have delays in their DNA synthesis. Two mutants, *far*P14 and *far*P3 (data not shown), appear to initiate DNA synthesis at approximately the same time as wild type but are delayed in achieving a wild-type rate of DNA synthesis. One mutant, *far*P85, is delayed in the initiation of DNA synthesis and does not achieve a wild-type rate of DNA synthesis until late in infection.

Because these *far* mutants overproduce certain of the pyrimidine nucleotide interconversion enzymes, it is possible that the delays seen in their DNA synthesis, as measured by the incorporation of radioactive thymidine, are not real. The size of nucleotide pools in these mutant-infected cells could be altered so that the thymidine entering the cell is diluted and not incorporated into acid-insoluble material as

TABLE 1. *Induction of early enzymes by far mutants at 37 C*

Extract[a]	Relative sp act[b]									
	FH_2 reductase		dCMP deaminase		dTMP synthetase		Thymidine kinase		dCTPase	
	15 min	20 min	15 min	20 min	15 min	20 min	15 min	20 min	15 min	20 min
far+ (T4Do)	0.9	1.0	0.9	1.0	0.8	1.0	0.7	1.0	0.9	1.0
*far*P14	1.6	1.4	2.4	2.8	0.5	0.7	1.1	2.0	0.9	1.3
*far*P85	1.2	1.3	3.1	4.2	0.2	0.4	1.1	1.8	1.0	1.3

[a] Extracts were prepared at the designated times after infection of *E. coli* B or *E. coli* KY895 (last four columns) as described in Materials and Methods.

[b] Specific activity is calculated relative to that of a 20-min extract of cells infected by wild type prepared at the same time. In these wild-type extracts, the specific activities in nanomoles of product formed per minute per milligram of protein were: FH_2 reductase, 30; dCMP deaminase, 80; dTMP synthetase, 7.4; thymidine kinase, 0.52; and dCTPase, 91.

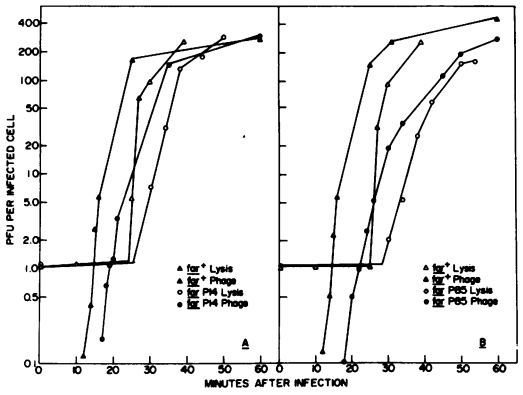

FIG. 5. *A, One-step growth curves for wild type (far⁺) and farP14 after infection of E. coli B at 37 C. Lysis curves were determined by plating for PFU on E. coli B from appropriate dilutions of the infected cells at the designated times. Intracellular phage curves were determined by chloroforming samples from appropriate dilutions of the infection mixtures and then plating for PFU on E. coli B. B, One-step growth curves for wild type (far⁺) and farP85 after infection of E. coli B at 37 C. The procedure is the same as in A.*

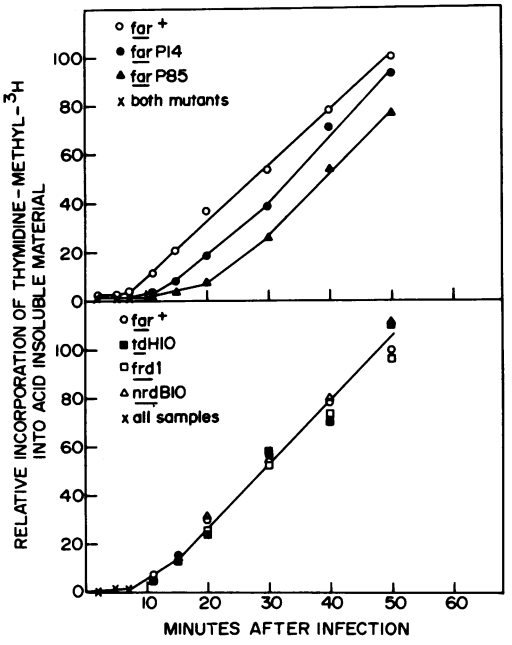

FIG. 6. *Cumulative incorporation of [methyl-³H]thymidine after infection of E. coli B by wild type (far⁺) and several mutants at 37 C. The procedure is described in Materials and Methods. The counts per minute of acid-insoluble material are normalized to the 50-min time point of a wild-type control run at the same time. During these experiments, the counts/min of acid-insoluble material at 50 min were (1.3 ± 0.5) × 10⁵ (average ± SD) for wild type.*

rapidly at the onset of DNA synthesis. An attempt was made to evaluate this possibility by measuring the rate of DNA synthesis in cells infected by mutants defective in the following pyrimidine nucleotide interconversion enzymes: ribonucleotide reductase, *nrd*B10 (33), FH_2 reductase, *frd*1 (8), and dTMP synthetase, *td*H10 (24). The initiation of DNA synthesis and the rate of DNA synthesis by each of these mutants was essentially normal (Fig. 6). Another approach was also used to evaluate the effect of pool size on thymidine incorporation. The incorporation of label from [6-³H]uridine (which enters both HMC and T in DNA) into alkali-resistant acid-insoluble material (Materials and Methods) was measured in cells infected by wild-type *far*P14 and *far*P85. The delays in DNA synthesis observed were comparable to those seen when using [*methyl*-³H]thymidine (Fig. 6). These results lead us to believe that the slight overproduction (about twofold) of certain of the pyrimidine nucleotide interconversion enzymes by *far* mutant-infected cells is probably not enough to mask or perturb phage DNA synthesis measured by the incorporation of a radioactive precursor.

Phage RNA synthesis. Phage RNA synthesis was measured by incorporation of [5-³H]uridine into acid-insoluble material (Fig. 7 and 8). The rates of RNA synthesis in the *far* mutant-infected cells are comparable with wild type only for the first 7 min after infection. Two of the mutants, *far*P14 and *far*P3 (data not shown), have similar profiles of RNA synthesis, whereas *far*P85 has a unique profile with no net RNA synthesis after 7 min. Chase experiments were done with wild-type and the *far* mutant overproducers (data not shown). These experiments indicated that the RNA label was being incorporated into material with a rapid turnover rate in wild-type and *far* mutant-infected cells.

The possibility of RNA label dilution in the *far* overproducer-infected cells was evaluated by measuring the rate of RNA synthesis in cells infected by mutants defective in the ribonucleotide reductase, FH_2 reductase and dTMP synthetase (Fig. 7). In one of these cases, the *frd*1-infected cells, which are defective in FH_2 reductase, a decreased level of label incorporation was seen that indicates RNA label incorporation can probably be perturbed by alterations in the level of FH_2 reductase enzyme. Although the observed RNA synthesis patterns of the *far* mutants might not be real, it should be noted that the pattern or profile of RNA synthesis by *frd*1-infected cells resembles that of wild-type infected cells more than it does any of the *far*

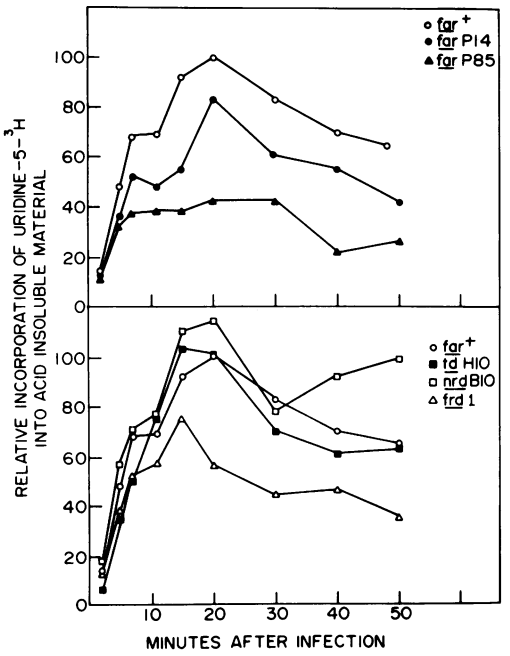

FIG. 7. *Cumulative incorporation of [5-³H]uridine into RNA after infection of E. coli B by wild type (far⁺) and several mutants at 37 C. The procedure is given in Materials and Methods. The counts per minute of acid-insoluble material are normalized to the 20-min time point of a wild-type control run at the same time. During these experiments, the counts/min of acid-insoluble material at 20 min were (1.6 ± 0.2) × 10³ (average ± SD) for wild type.*

mutant-infected cells. The actual amounts of RNA synthesized at each time point may be greater than the measured amount in *far* mutant- and *frd*1-infected cells, but the patterns or profiles of synthesis are probably accurate.

In Fig. 8, the RNA synthesis of the *far-am*N55 doubles is shown. These double mutant-infected cells (*far*P3-*am*N55 not shown) have no net RNA synthesis after 7 min of infection. However, the *am*N55 mutant-infected cells have net RNA synthesis until 15 min after infection. None of the *far-am*N55 doubles or the *am*N55 single have a significant burst size at 60 min after infection (data not shown).

Table 2 summarizes the growth characteristics of the *far* overproducers.

Genetic mapping of the far mutants. The *far* overproducers were mapped to *amber* mutants and to *hus* mutants by two factor crosses. Because these *far* mutants were selected as mutants that will form plaques where wild type cannot (12), it has been extremely difficult to find plating conditions where recombinant wild-type progeny could be scored in large numbers within a reasonable period of time. All 10 *far* overproducers isolated to date are characterized by a small plaque morphology compared with wild type (T4Do) (12). Furthermore, all the *far* mutants have been selected as spontaneous mutants occurring in unmutagenized phage stocks, which greatly reduces the chances of obtaining multiple mutants. Therefore, we assume that the individual *far* overproducers have achieved their folate analogue resistance and enzyme overproducing phenotype because of a single mutation which also causes a small plaque morphology. The procedure used to construct *far-am*N55 double mutants (see Ma-

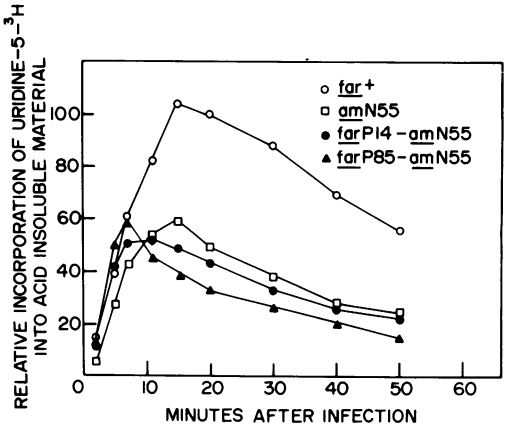

FIG. 8. *Cumulative incorporation of [5-³H]uridine into RNA after infection of E. coli B by wild type (far⁺), amN55 (gene 42), and the double mutants farP14-amN55 and farP85-amN55 at 37 C. See legend of Fig. 7 for details.*

TABLE 2. *Growth characteristics of far mutants[a] at 37 C*

Phage	Min after infection			Relative burst size[b] at 60 min (phage/cell)
	Approx delay in attaining max rate of DNA synthesis[c]	Intracellular phage appear	Lysis begins	
far⁺ (T4Do)	0	15	26	1.0
*far*P14	7	19	31	1.1
*far*P85	14	24	34	0.7

[a] All experiments were performed with *E. coli* B grown to 2 × 10⁸ cells/ml in 3XD at 37 C.

[b] See Materials and Methods for one-step growth curve procedure. The wild-type burst at 60 min was 264 ± 48 (average ± standard deviation [SD]) phage/cell.

[c] DNA synthesis was measured by the incorporation of [*methyl*-³*H*]thymidine into acid-insoluble material as described in Materials and Methods.

terials and Methods) supports this assumption. Apparent map locations for the *far* mutants were obtained by using this small plaque morphology to distinguish *far* mutants from wild-type recombinants.

The data show that the three *far* overproducers analyzed map in two separate sites of the early region of the T4 genome (Fig. 2). Two of the overproducers, *far*P14 and *far*P3 (Fig. 9), map approximately half way between *hus*3 in the *dex*A gene (7, 27) and *am*E51 in gene 56 (3). The two *far* mutants have been crossed to each other and give less than 2% recombination. Preliminary experiments indicate that they do not complement each other and are probably in the same gene. The order given in Fig. 9 for *far*P14 and *far*P3 is purely speculative.

The third overproducer *far*P85 (Fig. 10) maps between *am*B5 in the *t* gene (13) and *am*H17 in gene 52 (3). Because of its close proximity to *ambers* in gene 52 and because of the long delay in its DNA synthesis (Fig. 6), the possibility that *far*P85 might be a point mutant

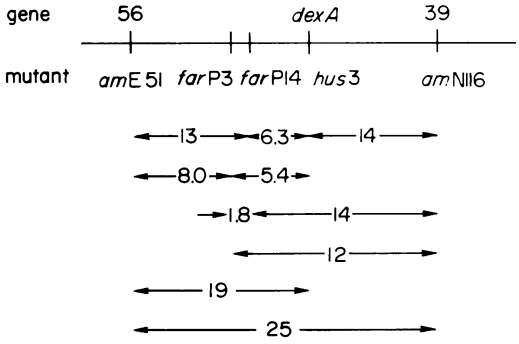

FIG. 9. *The location of farP3 and farP14 on the T4 genetic map. The frequencies of recombinants obtained in two-factor crosses are shown. The order of farP3 and farP14 is not known.*

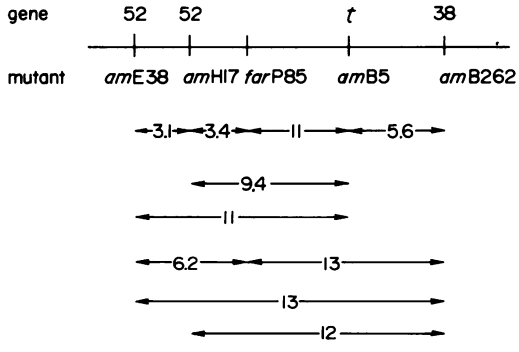

FIG. 10. *The location of farP85 on the T4 genetic map. The average frequencies of recombinants obtained in two-factor crosses are shown.*

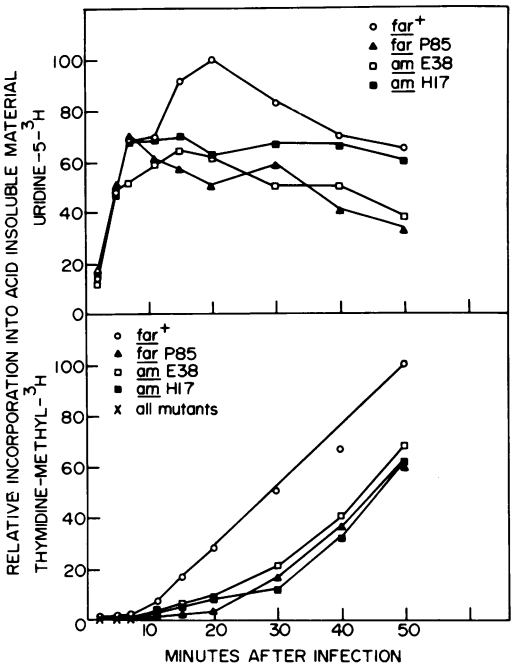

FIG. 11. *Cumulative incorporation of [5-^{3}H]uridine into RNA (upper panel) and cumulative incorporation of [methyl-^{3}H]thymidine into DNA (lower panel) after infection of E. coli B by wild type (far$^+$), farP85, amE38 (gene 52), and amH17 (gene 52) at 37 C. See legends of Fig. 6 and 7 for details.*

in gene 52 was investigated. In Fig. 11, the RNA and DNA synthesis of *far*P85, *am*H17 and *am*E38 (both *ambers* are in gene 52) are compared. The RNA synthesis patterns of the three mutants are very comparable, but *far*P85 appears more delayed in initiation of DNA synthesis than either *amber*. The *far*P85 burst size is about twice that of the *ambers* in these experiments (data not shown). In Fig. 12, the results of complementation tests for DNA synthesis between *far*P85 and the two *ambers* are shown. The complementation for both DNA synthesis and burst size (not shown) are 100% with respect to wild type. As a final check, extracts were prepared 20 min after infection of *E. coli* B with *am*E38 and *am*H17. Neither *amber* overproduces FH_2 reductase, and dCMP deaminase overproduction is minimal compared with *far*P85 (Table 3). These results indicate that this *far* overproducer is not in gene 52.

DISCUSSION

The *far* overproducer mutants were isolated as part of an attempt to probe the regulation of T4 early gene expression, especially the early region between genes 31 and 32 (Fig. 2). Although these mutants do overproduce products

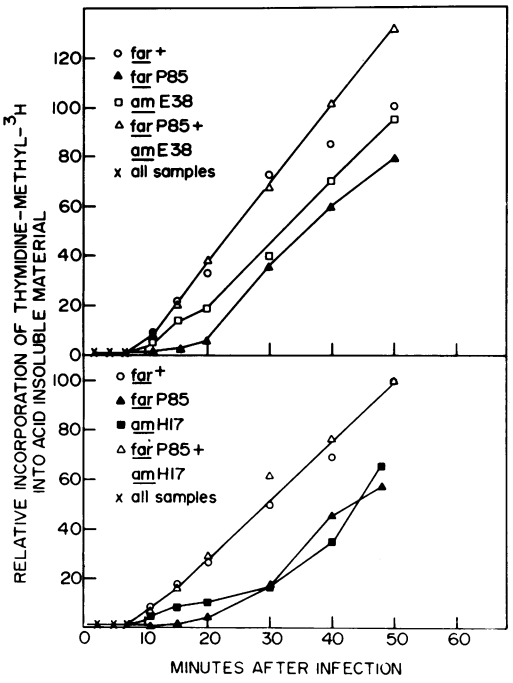

FIG. 12. *Cumulative incorporation of [methyl-^{3}H]thymidine into DNA showing complementation between farP85 and amE38 (upper panel) and between farP85 and amH17 (lower panel) after infection of E. coli B at 37 C. Cells were infected with a multiplicity of 10 phage per cell (in the mixed infections, cells were simultaneously infected with 5 of each phage per cell). The procedure is given in Materials and Methods. The counts per minute of acid-insoluble material are normalized to the 50-min time point of a wild-type control run at the same time. During these experiments, the counts/min of acid-insoluble material at 50 min were (1.2 ± 0.1) × 10^5 (average ± SD) for wild type.*

TABLE 3. *FH_2 reductase and dCMP deaminase activities induced by mutants in gene 52*

Extract[a]	Relative sp act[b]	
	FH_2 reductase	dCMP deaminase
Uninfected cells	0.15	0.0
far$^+$ (T4Do)	1.0	1.0
*far*P3	1.5	2.1
*far*P85	1.4	2.7
*am*E38 (52)	0.90	1.4
*am*H17 (52)	0.90	1.2

[a] Extracts were prepared 20 min after infection of *E. coli* B at 37 C.

[b] See legend of Fig. 3 for details.

of genes mapping in that early region, they also overproduce products of some genes mapping outside of the gene 31 to gene 32 region, such as thymidine kinase and dCTPase. Overproduction of these unlinked gene products coupled with the *far* overproducer map locations, which are far from the gene 31 and 32 area, clearly rule out any possibility that the *far* mutants are promoter type mutants for the early region between genes 31 and 32.

Having ruled out the promoter possibility, the basic problem of how the *far* overproducers exert their effect on T4 gene expression remains. Any mechanism proposed must explain the following observations: (i) The effect on gene expression is exerted early after infection because FH_2 reductase activity begins to accumulate more rapidly in the mutant-infected cells by 3 to 4 min after infection (Fig. 3 and 4). (ii) This effect, by its nature, can cause some gene products to be produced more rapidly than normal (the case for FH_2 reductase) and at the same time can allow overproduction of other gene products mainly by delaying their time of shutoff (the case for dCMP deaminase). (iii) The effect on gene expression must be exerted before and independent of the overproduction due to delay in the shutoff of gene expression in the absence of DNA synthesis (23, 29). This fact is true at least for the dCMP deaminase induction where the overproduction by the *far* mutants is additive with the overproduction by a DO mutant in the *far*-DO double mutants (Fig. 4). (iv) It must be possible for the effect on gene expression to perturb, either directly or indirectly, both phage DNA synthesis and RNA synthesis; the delays in phage production and lysis can probably be attributed to the delay in phage DNA synthesis. (v) At least two phage genes are involved in the regulation of early T4 gene expression, and defects in either of the genes can produce the observed phenomena of overproduction of gene products in a similar manner.

The *far* overproducers are not the only T4 mutants recently isolated which affect expression of genes coding for early enzymes. Wiberg et al. (30) have reported the isolation of a new mutant SP62. They find that double mutants of the type SP62-DO overproduce some early phage-induced enzymes much more than the DO single mutant. We have compared SP62-infected cell extracts with several *far* overproducer-infected cell extracts and found that under our conditions neither SP62 nor a SP62-DO double overproduce FH_2 reductase or dCMP deaminase (unpublished observations).

Furthermore, SP62 maps quite far from either of the *far* overproducer loci, and the SP62 single does not exhibit a delay in its DNA synthesis (30). In all probability, SP62 and the *far* overproducers affect different control functions. The map locations and properties of the *far* overproducers indicate that they define two new genes, one near gene 52 and one near gene 56.

The DNA delays of the *far* overproducers suggest two possible general mechanisms for the alterations in gene expression. The DNA delays could result from a reduced synthesis or delay in expression of some gene product(s) required for DNA synthesis, whereas products normally induced earlier are overproduced. The mechanism for such a delay presumably would involve a defect in the transcription or translation of these essential genes that directly resulted from the mutation causing overproduction. An alternative possibility would require the direct but nonessential functioning of the gene products altered in the *far* overproducers in the synthesis or initiation of synthesis of T4 DNA. The DNA delays in this case would result directly from the alterations of these gene products, and the overproduction would result from a prior effect of the defective gene product.

Other DNA delay mutants, defining several genes, have been found previously (3, 4). The strong similarities between the DNA delays of *far*P85 and the *amber* mutants of gene 52 coupled with their close linkage suggest that the two genes might possess similar or very closely related functions. The mechanism of the DNA delay caused by gene 52 *ambers* and *ambers* in other genes is unknown, but a number of laboratories have investigated and reported on their properties (18, 19, 32). Our studies of enzyme induction in cells infected with *amber* mutants in gene 52 indicate that the synthesis of phage-induced enzymes is not similar to that of *far* mutant-infected cells. Nonetheless, it is possible that understanding the mechanisms involved in the defects of gene 52 mutants or of *far*P85 will be of help in elucidating the effects of the other.

Several laboratories have proposed models for regulation of T4 genes based on data they have obtained concerning the temporal expression of phage mRNA and/or proteins in the presence and absence of various inhibitors of macromolecular biosynthesis (1, 10, 20, 22, 23). All of these models propose, or acknowledge the possibility of the existence, of phage genes that regulate the expression of the phage genome. The known properties of the *far* overproducers could be construed to fit any number of the proposed models, and a resolution of the problem must await further analysis of these new regulatory mutants of T4.

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