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The Temporal Expression of T2r+ Bacteriophage Genes *In Vivo* and *In Vitro*

(template/initiation/enzyme synthesis/ chloramphenicol RNA)

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ABSTRACT **The kinetic order of synthesis of deoxycytidylate deaminase (EC 3.5.4.12), deoxycytidylate hydroxymethylase (EC 2.1.2.b), dihydrofolate reductase (EC 1.5.1.3), 5-hydroxymethyldeoxycytidylate kinase (EC 2.7.4.4), and thymidylate synthetase (EC 2.1.1.b) after infection of *Escherichia coli* with T2r+ bacteriophage was found not to correlate with their order of synthesis in an *in vitro* protein-synthesizing preparation. The *in vivo* and *in vitro* synthesis of enzyme-specific messenger RNA measured in the protein-synthesizing preparation preceded each enzyme by about 1 min. Through the use of sheared DNA, it was shown that the thymidylate synthetase gene was most susceptible to a loss in template activity, which suggests that this gene is further removed from its promoter than the other genes are from theirs. With a DNA segment of 2.5 × 10^6 daltons, the synthesis of dihydrofolate reductase alone was obtained, but at a much reduced rate. Translation of the RNA from phage-infected cells treated with chloramphenicol yielded amounts of dihydrofolate reductase and deoxycytidylate hydroxymethylase activities similar to those obtained with RNA from untreated infected cells. These results suggest that the chloramphenicol RNA, which consists primarily of immediate-early RNA, may contain most, if not all, of the information required for the synthesis of phage dihydrofolate reductase and deoxycytidylate hydroxymethylase.**

The prereplicative events in T-even bacteriophage have been divided into immediate-early, delayed-early, and late stages, each responsible for the elaboration of specific proteins that contribute to the formation of phage DNA and the completion of the virion. Immediate-early synthetic events are apparently host-mediated and are prescribed by a specific phage mRNA formed in the presence of chloramphenicol (1, 2). The impairment of DNA synthesis as a consequence of the deletion or restriction of a crucial enzyme prevents the transition from delayed-early to late transcription, an effect that promotes the enhanced production of delayed-early enzymes (3). It appears that the orderly transition from one event to the next in phage development is controlled by specific regulatory agents (4–8).

DNA–RNA hybridization has been useful for clarification of the order of events after phage infection (4, 9). However, hybridization does not distinguish between the existence of an RNA and its capacity to act as a functional unit. Recent studies (10, 11) suggest that an *in vitro* protein-synthesizing preparation that uses DNA and RNA templates might be useful in determining the relative location of genes on an isolated transcriptional unit, since the order of appearance of immediate-early proteins, a single delayed-early enzyme, and a late enzyme was identical to that *in vivo*. To determine if the *in vitro* preparation could be used to reconstruct the events that occur *in vivo*, we asked whether the genes for delayed-early enzymes are expressed with the same fidelity *in vitro* as they are *in vivo*. The answer, as will be shown, is no.

Abbreviations: HMdCMP, HMdCDP, HMdCTP, 5-hydroxymethyldeoxycytidine 5′-mono-, di-, and triphosphosphate, respectively; FH_2, dihydrofolate.

MATERIALS AND METHODS

Microbiological Procedures. Escherichia coli B, maintained on nutrient agar slants, was the parent strain we used. An *E. coli* mutant (BT_1^-), lacking thymidylate synthetase, was isolated from the parent strain by the trimethoprim selection procedure (12). This organism was maintained on M9 (13) agar slants containing 0.4% glucose and a 40 μg/ml supplement of thymine. *E. coli* BT_1^- was checked periodically for revertants by plating on unsupplemented M9 agar. No revertants were evident after 6 months.

Bacteriophage T2r+ and T4r+ were propagated in *E. coli* B and titered (14). For phage infection, either strain of *E. coli* was grown at 37° in M9 medium containing 0.4% glucose, but for *E. coli* BT_1^-, 40 μg/ml of thymine was included. When the cultures reached a density of 4 × 10^8 cells/ml, the T-even phage was added at a multiplicity of 5 with thorough mixing. The infected cells were aerated vigorously on a rotary shaker at 200 rpm. At specified times, infection was terminated by pouring 320-ml aliquots of the culture over 1.5 volumes of chipped ice. The infected cells were isolated by centrifugation and resuspended at a concentration of 2.5 × 10^{10} cells/ml in a solution of 10 mM Tris·HCl (pH 7.5)–10 mM NaCl–5 mM $MgCl_2$. One ml of this suspension was frozen at −56° and assayed subsequently for phage-induced enzyme activities.

Nucleic Acid Extraction. RNA was isolated by a modification (15) of a hot-phenol extraction procedure (9), and adjusted to a final concentration of 5 mg/ml based on an A_{260nm} for RNA of 1 mg/ml equal to 25. DNA was isolated from either T2 or T4 bacteriophage by a phenol extraction and dialysis procedure (16). The final DNA concentration was 300 μg/ml. RNA and DNA were stored unfrozen in ice.

In Vitro Protein Synthesis with DNA and mRNA as Templates. Incubated, dialyzed, cell-free extracts (S-30 fraction) were prepared from uninfected *E. coli* B and BT_1^- grown to 3 × 10^8 cells/ml as described (17). Reaction mixtures for DNA-directed protein synthesis contained in 0.2 ml: 50 mM Tris-acetate (pH 8.0), 1.25 mM dithiothreitol, 11 mM magnesium acetate, 50 mM potassium acetate, 100 mM ammonium chloride, 5 mM calcium chloride, 0.2 mM of each of 20 amino acids, 2 mM ATP, 0.5 mM each CTP, UTP, and GTP, 20 mM phosphoenolpyruvate, 0.2 mM *N*-5-formyltetrahydrofolic acid (folinic acid), 15 μg of phage DNA, and 1.4 mg of S-30 protein.

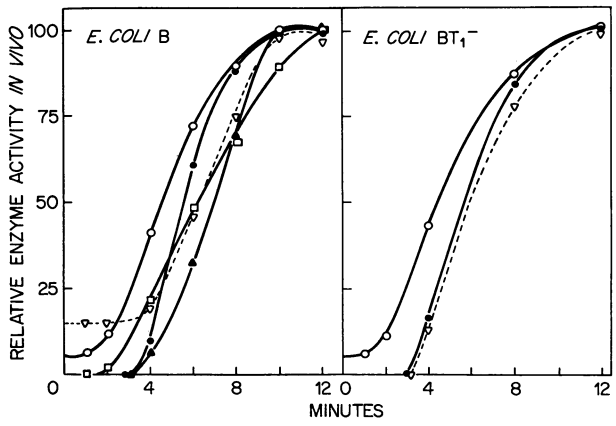

FIG. 1. *In vivo* appearance of T2r⁺-induced enzymes after infection of *E. coli* B and BT_1^-. Maximum activities, in milliunits/mg of protein, were: (○) FH_2 reductase, *E. coli* B, 84; *E. coli* BT_1^-, 65; (□) dCMP hydroxymethylase 15; (▽) dTMP synthetase, *E. coli* B, 6.5; *E. coli* BT_1^-, 8.0; (●) dCMP deaminase, *E. coli* B, 125; *E. coli* BT_1^-, 144; (▲) HMdCMP kinase, 100.

The reaction mixtures for RNA-directed protein synthesis were as described for the DNA-directed preparation, except that the concentrations of magnesium acetate, ammonium chloride, GTP, and phosphoenolpyruvate were reduced to 9, 50, 0.2, and 5 mM, respectively, calcium chloride, CTP, and UTP were omitted, and 200 μg of RNA replaced DNA.

For both the RNA and DNA preparations, all components except S-30 and folinic acid were mixed together and incubated at 37° for 3 min (17). Protein synthesis was initiated by the addition of a 50-μl mixture of S-30 and folinic acid in the same concentrations as described above. At specified intervals, chloramphenicol (200 μg/ml final concentration) was to terminate added protein synthesis.

Enzyme Assays. For determination of phage-induced enzyme activities *in vivo*, the cell suspensions frozen at −56° were thawed and diluted with 1 ml of 10 mM Tris·HCl (pH 7.5) containing 0.08 mM dCTP to stabilize dCMP deaminase. These suspensions were disrupted by a 1-min sonic treatment, and the debris was removed by centrifugation at 30,000 × *g* for 30 min (S-30). The supernatant fraction was decanted and assayed at 37° for the enzymes. Specific activities are reported in milliunits/mg protein, with a milliunit equivalent to the conversion of a nmol of substrate to product in 1 min under the conditions of assay.

For measurement of the *in vitro* synthesis of dCMP deaminase and dCMP hydroxymethylase, the removal of residual ATP was essential to prevent the phosphorylation of dCMP, a reaction that interferes with the assay of both enzymes (18). ATP was trapped by addition of 0.01 ml of a solution containing 3.2 μmol of glucose and 1.3 units of hexokinase to each 0.1 ml of the terminated cell-free synthesis mixture. After 3 min at 37°, the reaction was cooled in ice.

Deoxycytidylate Deaminase (EC 3.5.4.12). The phage-induced deaminase was measured with a spectrophotometric assay (19). The enzyme synthesized *in vitro* and present in a 25-μl aliquot of the ATP-trapped protein synthetic mixture was added to 25 μl of a substrate solution containing 25 nmol [2-^{14}C]dCMP (6.0 cpm/pmol) and 0.2 μmol of dithiothreitol. After a 30-min incubation, the reactions were terminated by heating in a boiling-water bath for 2 min. Precipitated protein and nucleic acids were removed by centrifugation, and duplicate 2-μl aliquots of the supernatant fraction were chromatographed on polyethyleneimine cellulose thin-layer sheets with 0.2 N sodium formate (pH 3.2) as the developer (18). Deoxyuridylate spots were cut out and counted in a scintillation counter.

Deoxynucleotide Kinase (EC 2.7.4.4). The enzymes synthesized both *in vivo* and *in vitro* were assayed with [^{14}C]-HMdCMP (5-hydroxymethyldeoxycytidine 5′-monophosphate) as substrate by a modification of the method of Lembach and Buchanan (20). After a 30-min incubation at 37°, the reactions were terminated as above. Denatured protein was removed by centrifugation, and the HMdCDP plus HMdCTP formed was determined by chromatography of 2-μl aliquots of the supernatant fraction as described for dCMP deaminase. The HMdCDP and HMdCTP regions, which remained near the origin, were cut out and counted in a scintillation counter.

Dihydrofolate (FH_2) Reductase (EC 1.5.1.3). The enzymes both *in vivo* and *in vitro* were assayed spectrophotometrically (21) in the presence of 100 mM KCl. Trimethoprim (35 nM) was included in the assays of the *in vitro* preparation to suppress the amount of host enzyme since the T2 phage-induced FH_2 reductase is insensitive to the drug at this concentration (21, 22).

Thymidylate Synthetase (EC 2.1.1.b). The enzyme synthesized *in vivo* was assayed spectrophotometrically (23) and that synthesized *in vitro* by tritium release from [5-^{3}H]dUMP (24). The nucleotide was separated from ^3H_2O by passage of the assay mixture through a 0.5 × 2 cm column of charcoal. In most experiments, S-30 preparations from *E. coli* BT_1^- were used in the protein synthesis mixtures, since they contained no measurable synthetase.

Deoxycytidylate Hydroxymethylase (EC 2.1.2.b). The enzyme was assayed *in vivo* and *in vitro* by the displacement of tritium from [5-^{3}H]dCMP in the presence of tetrahydrofolate and enzyme (25). Tritium release was measured as described above for the *in vitro* thymidylate synthetase assays.

Other Methods. Protein was determined by the method of Lowry *et al.* (26). Dihydrofolate and tetrahydrofolate (23) were prepared as described. The HMdCMP was prepared by the method of Pizer and Cohen (27), but on a larger scale.

RESULTS

Time Sequence of Appearance of Phage-Induced Enzymes. The appearance of early enzymes after phage infection can perhaps be distinguished kinetically (28). Of the five enzymes we measured, three map as a closely linked cluster in the sequence FH_2 reductase (*frd*)*, dTMP synthetase (*td*), and dCMP deaminase (*cd*) (29), and if transcribed counterclockwise from the same operator, their synthesis should proceed in this order. While the difference in appearance of dTMP synthetase and dCMP deaminase at 37° is questionable (Fig. 1), measurement of the induction at 30° established a 1-min interval between their appearance. The enzymes first detected after T2 infection were FH_2 reductase, followed by dCMP hydroxymethylase (Fig. 1).

* This gene was referred to previously as *wh* (29).

Time Sequence of Appearance of mRNA Specific for the Phage-Induced Enzymes. That the induction of each of the enzymes in Fig. 1 was preceded in appearance by a specific mRNA was determined by isolating RNA at various times after phage infection and measuring its template activity. Fig. 2 reveals that maximal enzyme-specific mRNA varied from 4 min after infection for FH_2 reductase and dTMP synthetase, to 8 min for HMdCMP kinase. Each RNA then declined rapidly in activity, with a half-life of about 3 min. Similar findings were obtained for HMdCMP kinase by Sakiyama and Buchanan (30). A comparison of the synthesis of enzyme and mRNA (Figs. 1 and 2) for dTMP synthetase in *E. coli* B and BT_1^- infected with T_2 indicates that the sequence of events is probably identical in the two and that extracts from the latter can be used for the *in vitro* synthesis of dTMP synthetase. Although the synthetase mRNA appears to peak earlier than the deaminase mRNA in cells grown at 37° (Fig. 2), the reverse was true in cells grown at 30° (an effect more in accord with the enzyme studies in Fig. 1).

In Vitro Synthesis of Phage-Induced Enzymes Directed by T2r⁺ DNA. In contrast to the kinetics of enzyme appearance after phage infection, the order of *in vitro* enzyme synthesis with T2 phage DNA as template was not that anticipated from the *in vivo* experiments in Fig. 1. In Fig. 3, the sequential order of enzyme synthesis appears to be HMdCMP kinase, FH_2 reductase, dCMP hydroxymethylase, dCMP deaminase, and dTMP synthetase. The initial expression of enzyme activity varied from 2–3 min for HMdCMP kinase to about 10 min for dTMP synthetase, a much broader range than that described for the enzyme synthesis *in vivo*. Because of the high background activity of dTMP synthetase in the host S-30 extracts, *E. coli* B T_1^- was used to establish with certainty the rather late appearance of this enzyme. To determine if the difference between the *in vivo* and *in vitro* syntheses was due to translational discrimination in the expression of mRNA, actinomycin D was added at various times after initiation of cell-free synthesis. The incubation was continued for an additional 20 min to allow the transcripts made

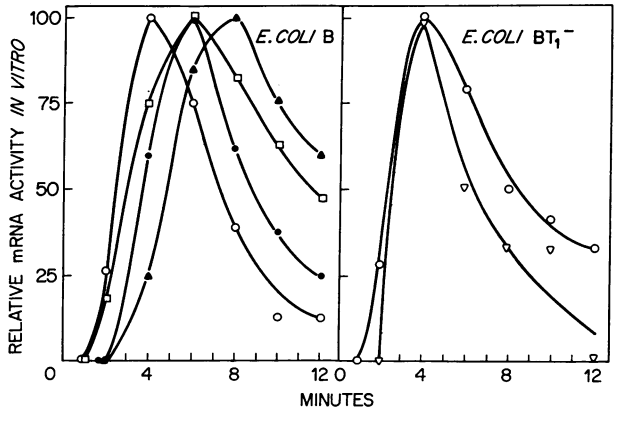

FIG. 2. *In vivo* appearance of enzyme-specific mRNA after T2r⁺-infection of *E. coli* B and BT_1^-. RNA was extracted from aliquots of the infected cells in Fig. 1 at times designated and used as template for *in vitro* enzyme synthesis. RNA-directed cell-free synthesis was conducted for 20 min (see *Methods*). Maximum *in vitro* activities, in milliunits/mg of protein, were: (○) FH_2 reductase, *E. coli* B, 1.2; *E. coli* BT_1^-, 1.1; (□) dCMP hydroxymethylase, 0.04; (▽) dTMP synthetase, 0.02; (●) dCMP deaminase, 0.1; (▲) HMdCMP kinase, 0.25.

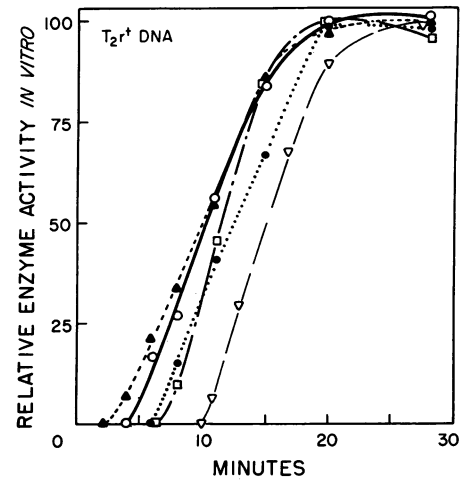

FIG. 3. Kinetics of *in vitro* synthesis of phage enzymes directed by T2r⁺ DNA. The protein synthesis mixture described in *Methods* was increased 15-fold and, at the times indicated, 0.3 ml was added to ice-cold tubes containing 65 μg of chloramphenicol in 20 μl. Aliquots of this solution were assayed for enzyme activity. Maximum activites, in milliunits/mg of protein, were: (○) FH_2 reductase, 5.1; (□) dCMP hydroxymethylase, 0.28; (▽) dTMP synthetase, 0.16; (●) dCMP deaminase, 0.17; (▲) HMdCMP kinase, 0.27. The S-30 fraction from *E. coli* B was used to measure the first four enzymes, while the S-30 fraction from *E. coli* BT_1^- was used for the synthesis of dTMP synthetase.

before addition of actinomycin sufficient time for translation into functional enzyme. An almost exact duplicate of Fig. 3 was obtained when enzyme activity was plotted against time of addition of actinomycin, except for the appearance of each enzyme-specific mRNA 1–2 min before its enzyme product. These results indicate that once the message is available *in vitro*, it will be translated into protein with no discrimination in the order of synthesis. This may not be the case, however, *in vivo*.

Although the kinetics of synthesis of FH_2 reductase, HMdCMP kinase, and dCMP hydroxymethylase were rearranged relative to their appearance *in vivo*, FH_2 reductase, dCMP deaminase, and dTMP synthetase were expressed in the same order in both cases. The kinase was the last enzyme to be expressed *in vivo* (Fig. 1), but was the first enzyme to be synthesized *in vitro*. Thus, as shown in Fig. 3, HMdCMP kinase was detected between 2 and 3 min, even before FH_2 reductase, and was found in the actinomycin study to be preceded by its mRNA by 1–2 min.

The possibility that the sequential synthesis of enzymes *in vitro* (Fig. 3) resulted from a discrimination in the initiation of enzyme-specific mRNA synthesis was eliminated by the use of rifampin. As expected, no enzyme-specific mRNA was synthesized *in vitro* when rifampin was added at zero time, but mRNA for each enzyme was detected when rifampin was added 1 min after initiation. The failure of rifampin to eliminate enzyme synthesis, once initiation has occurred, signified that RNA polymerase attaches to the promoters for the various genes at about the same time *in vitro*. This experiment, however, does not exclude the possibility that the availability of promoters may be more restricted *in vivo*.

Relationship of DNA Size to the Synthesis of Delayed-Early and Immediate-Early Proteins. In an effort to clarify the apparent contradiction between the temporal order of

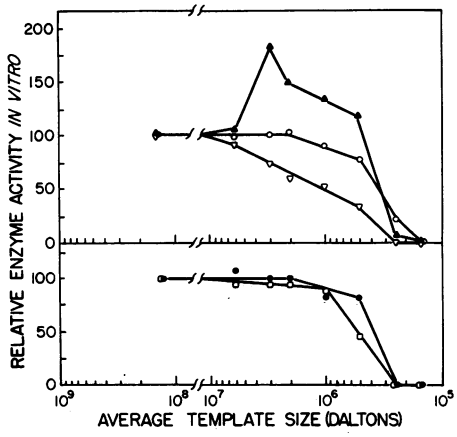

FIG. 4. *In vitro* enzyme synthesis as a function of DNA template size. DNA fragments from 6×10^6 to 5×10^5 daltons were obtained by passing native DNA (1.3×10^8 daltons) solutions containing 300 μg/ml 20 times through 2.5 cm long 26-, 27-, or 30-gauge hypodermic needles. Smaller pieces were obtained by sonication (Biosonik II, microprobe) for 10 sec at minimum intensity or 50 sec at 20% intensity in ice. DNA was measured by sedimentation analysis on linear 4.6 ml, 5–20% alkaline sucrose gradients (31), with 4.5S [^{14}C]DNA as a marker. An activity of 100 corresponded to, in milliunits/mg of protein: (○) FH_2 reductase, 5.2; (□) dCMP hydroxymethylase, 0.23; (▽) dTMP synthetase, 0.18; (●) dCMP deaminase, 0.28; (▲) HMdCMP kinase, 0.42.

genetic expression and the location of the *frd*, *td*, and *cd* genes on the T-even phage genetic map, the functional capacity of DNA sheared to considerably smaller sizes was measured in the *in vitro* protein synthesizing preparation (17). Since the efficiency of genetic expression depends on the distance of each gene from its promoter, if the *frd*, *td*, and *cd* genes were part of a polycistronic message, their location relative to one another might be clarified. Even if they were not part of a coordinate unit, the relative distance of each gene from its promoter should be provided. A clear-cut response of the *td* gene to DNA shearing was obtained, in contrast to *frd* and *cd* (Fig. 4). Thus, as the size of the DNA segment used as a template in the *in vitro* preparation was diminished, so was its capacity to direct the synthesis of dTMP synthetase. With an average DNA size of 1×10^6 daltons, only 50% of the synthetase was made, but at least 90% each of the dCMP deaminase and FH_2 reductase were synthesized. It appears (Fig. 4) that dTMP synthetase is farther from its promoter than the other genes.

The sharp rise in HMdCMP kinase with DNA segments of 3×10^6 daltons is reminiscent of the increase in the immediate-early internal protein I obtained by Brody *et al.* (11) with DNA of a similar size. The only two enzymes that could still be detected with DNA segments of 2.5×10^5 daltons were HMdCMP kinase and FH_2 reductase. If, as the data suggest, the genes for these enzymes are associated with the *immediate-early* DNA segment, mRNA for each should be present in the RNA extracted from phage-infected cells treated with chloramphenicol (1, 2).

Template Activity of Chloramphenicol RNA. A distinctive characteristic of immediate-early RNA is its tendency to accumulate as a 10–11S component in the presence of such inhibitors of protein synthesis as chloramphenicol (1, 2), puromycin, and 5-methyltryptophan (20). Since we could infer from the rather early synthesis of FH_2 reductase and dCMP hydroxymethylase *in vivo* that the templates for these enzymes might be associated with immediate-early RNA, this RNA fraction was isolated and its capacity for enzyme synthesis was tested in the *in vitro* preparation. Both enzymes appeared to be translated by the chloramphenicol RNA (Table 1). Since none of the other enzymes could be detected, a clear-cut delineation between these enzymes and those synthesized at later intervals is established.

The fact that methotrexate completely blocked the activity of the newly synthesized FH_2 reductase, and HMdCMP impaired the hydroxymethylase (data not shown), provided support for the supposition that the activities measured were actually due to these enzymes. Little difference existed in the capacity of normal and chloramphenicol RNA to be translated into FH_2 reductase and dCMP hydroxymethylase. The reductase template, however, lost activity much more rapidly than did the templates for the other enzymes on storage of either RNA. In contrast to the shearing experiment (Fig. 4), where HMdCMP kinase was synthesized like an immediate-early protein, the data in Table 1 suggest that the template for this enzyme is either not present in the chloramphenicol RNA or that it cannot be expressed under the conditions of *in vitro* synthesis.

DISCUSSION

The use of phage DNA and RNA templates to direct the *in vitro* synthesis of proteins known to occur at specific stages of the phage growth cycle provides an important supplement to the earlier RNA–DNA hybridization studies. FH_2 reductase, dCMP deaminase, and dTMP synthetase exist in a closely linked cluster on the T4 phage genome [the *frd*, *td*, *cd* locus (29)]. Although we used T2 in most of our studies, we presume that a similar genetic arrangement exists in both phages, as we obtained identical results with T2 and T4, both *in vivo* and *in vitro*. The suggestion that these genes may be coordinately linked is not supported by the finding that the gene products are expressed in an order

TABLE 1. *In vitro template capacity of chloramphenicol RNA isolated at various times after T2 infection of E. coli B**

RNA isolation (min after infection)	Chloramphenicol	*In vitro* enzyme activity† (milliunits/mg)				
		FH_2 reductase	dCMP hydroxymethylase	dCMP deaminase	dTMP synthetase	HMdCMP (deoxynucleotide) kinase
1.3	+	0.6	0.012	0	0	0
	−	0.6	0.010	0.002	0	0
4	+	2.4	0.023	0	0	0
	−	1.9	0.042	0.044	0.040	0.125
7	+	1.6	0.028	0.003	0	0
	−	0.6	0.053	0.062	0.025	0.280

* 2 Liters of *E. coli* B were grown to 4×10^8 cells/ml and 50 μg/ml of chloramphenicol was added to 1 liter. Both cultures were infected after 5 min with T2r$^+$ phage at a multiplicity of 5. RNA was isolated from 320-ml aliquots at the times indicated.

† Activity was measured (see *Methods*) after 20 min of *in vitro* synthesis with RNA at a final concentration of 1.25 mg/ml.

(*frd, cd, td*) (Figs. 1 and 3) that is not in sequence with their placement in the T4 genome (*frd, td, cd*). Furthermore, FH_2 reductase activity is not enhanced in DO amber mutants (32), in contrast to other phage early enzymes. Similar conclusions were derived by Mathews (28).

The *in vitro* expression of the *frd, td,* and *cd* genes (Fig. 3) appears to complement the order of synthesis *in vivo* (Fig. 1), but with even more clear-cut initiation times. The use of *in vitro* studies, however, as a means of determining the temporal order of genetic expression *in vivo* is invalidated by the finding that the order of synthesis *in vitro* of dCMP hydroxymethylase and HMdCMP kinase, relative to one another and to the reductase and deaminase, is completely different from that *in vivo* (see Figs. 1 and 3). The only realistic explanation that can be presented for this difference is that the *in vitro* preparation lacks specific regulatory agents that occur *in vivo*. Thus, while the *in vitro* preparation might be used to determine the relative locus of various genes (11), caution must be exercised in interpreting the results of such experiments. DNA shearing studies similar to those described in ref. 11 may be useful in specifying the location of genes relative to specific promoters. Thus, the *td* gene appears to be more distal to its promoter than the other genes studied, since its template capacity decreases more sharply than the others (Fig. 4).

The fact that the FH_2 reductase activity is the last to be lost as the size of the DNA is decreased (Fig. 4) suggests that the *frd* gene is the closest of the five genes studied to its promoter. Based on the estimated molecular weight of the DNA segment containing FH_2 reductase, a minimal average transcriptive unit should be about 1500 nucleotides. This would place the FH_2 reductase gene at the immediate early-delayed early junction (33). To determine if this enzyme or any of the others studied might be in the immediate-early region, RNA was isolated from phage-infected cells treated with chloramphenicol. This RNA, classified as the immediate-early RNA (1, 2, 4, 20, 33, 34), is transcribed by unmodified host RNA polymerase after infection and is about 1500–2100 nucleotides long. Further transcription of the contiguous delayed-early genes requires new phage-directed protein synthesis and modification of host RNA polymerase (35), although some question regarding the necessity for these steps was raised recently (11, 36). From the results in Table 1, it appears that aside from promoting the synthesis of three internal capsid proteins (37), the immediate-early RNA may direct the synthesis of FH_2 reductase and at least one-half of the dCMP hydroxymethylase. The refractoriness of the FH_2 reductase (32) to the usual hyperactivation of delayed-early enzymes induced by amber T4 mutants (3) might now be explained by the location of the *frd* gene in a region distinct from that occupied by the delayed-early genes. Similar conclusions have been arrived at (20) with phage-infected spheroplasts treated with 5-methyltryptophan and chloramphenicol. However, because of the possibility that the chloramphenicol RNA may contain some delayed-early sequences (36), more definitive experiments will be necessary to establish with certainty the actual location of FH_2 reductase and dCMP hydroxymethylase.

Peterson, R. F., Cohen, P. S. & Ennis, H. L. (1972) *Virology* **48**, 201–206, using a different method than described here, have verified that chloramphenicol RNA can serve as a template for dCMP hydroxymethylase.

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