Transcriptional Regulation of T4 Bacteriophage-Specific Enzymes Synthesized In Vitro

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In contrast to dihydrofolate reductase and four other phage-specific enzymes, the initiation of deoxynucleotide kinase is essentially prevented if rifampin is added to a culture of *Escherichia coli* B cells within 1.5 min after infection with T4. Deoxynucleotide kinase thus belongs to a group of so-called delayed-early enzymes that is not initiated from an immediate-early promoter site. We prepared crude extracts from infected cells in a manner designed to maintain the integrity of the complexes of native, endogenous T4 DNA with bacterial structural and enzymatic units concerned with RNA synthesis. The initiation of the synthesis of the mRNA for dihydrofolate reductase, an example of an immediate-early enzyme, and deoxynucleotide kinase, a special type of delayed-early enzyme, was studied with these extracts prepared from cells infected in the absence or presence of chloramphenicol. Initiation of transcription of the dihydrofolate reductase gene is immediate when programmed by extracts made either from cells treated with chloramphenicol prior to infection (CM extracts) or from cells 3 min into the normal infection cycle (3-min extracts). However, initiation of transcription of the deoxynucleotide kinase gene programmed by CM extracts is delayed 2 min relative to the immediate initiation of transcription of the deoxynucleotide kinase gene programmed by 3-min extracts. These experiments duplicated in vitro effects of the antibiotics on the synthesis of phage-specific mRNA previously noted only in vivo.

On the basis of the effects of chloramphenicol and rifampin on the development of phage in T4-infected bacteria, several classes of early mRNA have been recognized (4, 11, 14, 21, 25). One class, designated as "immediate-early" RNA, requires only the enzymatic components of *Escherichia coli* for its production. A second group of mRNAs, designated as "delayed-early" is not formed if infection occurs in the presence of chloramphenicol, a potent inhibitor of protein synthesis.

Several investigators have suggested that delayed-early RNA is formed primarily by extensions of immediate-early transcripts (2, 16, 17). However, the synthesis of deoxynucleotide kinase mRNA seems to be an exception to this general premise. By comparison of the size of kinase mRNA to that of a subunit of the kinase enzyme, we have postulated that the kinase gene contains the necessary information for initiation, elongation, and termination of this

segment of the T4 DNA and, therefore, originates from its own promoter site rather than by the extension of the transcription of an adjacent gene (25). There thus seems to be two types of delayed-early RNA, one which can be initiated at immediate-early promoter sites (PE) but which retains a requirement for protein synthesis for complete transcription, and a second at another kind of promoter site that in some way requires the participation of a phage-specific protein during the initiation step. Recent evidence reported by O'Farrell and Gold (21, 22), using rifampin to mark the time of initiation of individual RNAs, strongly supports this conclusion. They have shown that a subset of delayed-early species, designated as "quasi-lates", are initiated at special promoter sites (PQ) that do not become available until approximately 2 min after infection. Addition of rifampin to cultures up to 2 min after infection at 30°C prevents the eventual appearance of the protein products related to these particular phage cistrons.

A perplexing aspect of the search for specific phage proteins concerning the production of

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delayed-early RNAs of either type is the finding that some of these RNA species can be formed in vitro from T4 DNA and highly purified RNA polymerase isolated from uninfected *E. coli* cells (4, 13, 16, 19, 31, 33). It therefore seemed that control systems effected by phage proteins in vivo are lost in vitro.

In our efforts to identify a property of enzyme synthesis in vivo that could be duplicated in vitro, we have turned to the use of rifampin to determine the characteristics of initiation of several early enzymes in vivo, including dihydrofolate reductase, an immediate-early enzyme (14), and deoxynucleotide kinase, a delayed-early enzyme (14, 24). On the basis of the results of these experiments, we have selected these two enzymes as best suited for study in vitro. By use of rifampin and infected extracts prepared in such a way as to maintain physiological structures, we have succeeded in duplicating in vitro an initiation characteristic demonstrated for these enzymes in vivo.

MATERIALS AND METHODS

Chemicals. [2-¹⁴C]uracil, uridine diphosphate-[¹⁴C]glucose, [¹⁴C]formaldehyde, [³H]dCMP, and [¹⁴C]dCTP were purchased from New England Nuclear. Chloramphenicol was a gift from Parke, Davis & Co., and rifampin and egg white lysozyme were purchased from Calbiochem. ATP, GTP, UTP, CTP, dCMP, phosphoenolpyruvate, dithiothreitol, TPNH, dihydrofolate, tetrahydrofolate, and 2-mercaptoethanol were purchased from Sigma Chemical Co.; L-amino acids were obtained from Schwarz/Mann; and no. 29 filters were purchased from Schleicher & Schuell Co.

Preparation of radioactive T4. One liter of *E. coli* B cells was grown in 3 XD medium (9) to a concentration of 5×10^8 cells per ml at 37 C. The cells were infected at a multiplicity of five phage per cell. After 5 min [¹⁴C]uracil (0.5 μ Ci/ml, 1.12 μ g/ml) was added, and incubation was allowed to proceed until cell lysis (2 h). Chloroform (10 ml) was added to break residual unlysed cells, and the crude lysate was centrifuged at $8,000 \times g$ for 10 min at 4 C to remove cell debris. The supernatant fluid was centrifuged at $20,000 \times g$ for 1 h at 4 C, and the phage pellet was suspended overnight at 4 C in 10 ml of buffer containing 10 mM Tris-hydrochloride (pH 7.5), 10 mM KCl, and 5 mM MgCl₂. The phage suspension was then subjected to low-speed centrifugation ($8,000 \times g$) for 10 min at 4 C. The supernatant fluid contained radioactive phage at a concentration of 1.65×10^{11} /ml (specific activity of 2.8×10^6 disintegrations per min per plaque-forming unit). All the radioactivity was shown to be T4 DNA as described previously (6).

Preparation of extracts for the assay of enzymes synthesized in vivo. *E. coli* B was grown at 30 C in 3 XD medium to a concentration of 5×10^8 cells per ml and divided into three portions to which tryptophan (25 μ g/ml) was added. One portion of the culture was

then infected at a multiplicity of five phage per milliliter and incubation continued at 30 C with vigorous aeration. A second portion was initially incubated in the presence of rifampin (200 μ g/ml) for 5 min prior to infection with five phage per cell, whereas the third portion was infected at the same multiplicity, but 1.5 min after infection rifampin at a concentration of 200 μ g/ml was added, and the incubation was continued at 30 C with vigorous aeration. Portions (50 ml) were removed from each infected culture at various times after the addition of phage, and extracts were prepared as previously described (5).

Preparation of extracts containing T4 DNA for enzyme synthesis in vitro. *E. coli* B was grown in 3 XD medium to a concentration of 5×10^8 cells per ml at 37 C. One portion of the culture (30 ml) was incubated for 5 min at 37 C in the presence of chloramphenicol (100 μ g/ml), infected at a multiplicity of five radioactive phage per cell in the presence of L-tryptophan (25 μ g/ml), and incubated with vigorous aeration for an additional 5 min. A second portion of the culture (30 ml) was infected at a multiplicity of five radioactive phage per cell in the presence of L-tryptophan (25 μ g/ml) and was incubated at 37 C for 2 or 3 min. By this procedure greater than 99% of the cells were infected. The infection process was stopped by pouring the culture into 3 volumes of a cold solution containing 15 mM EDTA (pH 7.5) and 150 mM NaCl, and all further steps were carried out at 0 to 5 C. The cells were then sedimented at $2,600 \times g$ for 5 min and suspended at a concentration of 1.2×10^{10} cells per ml in 1.25 ml of buffer containing 50 mM Tris-hydrochloride (pH 8.0), 50 mM NaCl, and 15 mM EDTA. Crude extracts were prepared by a modification of the method of Oleson et al. (23). Lysozyme (0.025 ml of a solution at 10 mg/ml) was added, and the samples were incubated for 30 min. The samples were then frozen in liquid nitrogen, and 15 min later, when partial thawing had occurred, 0.5 ml of cold 80% glycerol was added, and the samples were allowed to thaw completely for an additional 15 min. The samples were then frozen again in liquid nitrogen and thawed for 30 min, after which they were dialyzed for 17 h against 500 volumes of buffer containing 10 mM Tris-hydrochloride (pH 7.5), 5 mM MgCl₂, 50 mM NH₄Cl, and 28% glycerol (vol/vol). Extracts were used immediately after dialysis in all experiments.

Purification of T4 DNA. Radioactive T4 DNA was extracted from radioactive T4 as described by Thomas and Abelson (28).

Cell-free protein synthesis. The S-30 extract was prepared from *E. coli* RNase I⁻ by the method of Nirenberg (20) with the modifications described by DeVries and Zubay (7). The reaction mixture for protein synthesis (0.2 ml) contained 50 mM Tris-acetate (pH 7.8), 7.0 mM magnesium acetate, 50 mM potassium acetate, 100 mM ammonium chloride, 20 mM phosphoenolpyruvate, 2.0 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 0.5 mM UTP, 1.25 mM dithiothreitol, 5.0 mM CaCl₂, 20 amino acids (0.2 mM each), and 0.05 ml of S-30 extract (approximately 1.8 mg of protein). Protein synthesis was directed by purified T4 DNA or T4 DNA contained in extracts prepared from either cells pretreated with chloram-

phenicol prior to infection or cells normally infected for 2 or 3 min. Incubations were carried out at 37 C for 30 min.

Enzyme assays. Deoxynucleotide kinase, dihydrofolate reductase, α - and β -glucosyltransferase, and deoxycytidylate hydroxymethyltransferase were assayed as described by Natale and Buchanan (19). Deoxycytidine triphosphatase was assayed as described by Wiberg et al. (32), except that [14 C]dCTP (180 counts per min per pmol) was used as a substrate in the assay, and the amount of [14 C]dCMP product formed after 30 min of incubation at 37 C was determined by counting the eluant in 15 ml of Bray solution.

Protein determination. Protein was determined by the method of Lowry et al. (15).

RESULTS

Effect of rifampin on the synthesis of six T4-related enzymes in vivo. Rifamycin B and rifampin, inhibitors of the initiation of RNA synthesis (12, 27), have been used to great advantage in studies to determine the sequence of initiation of the synthesis of individual enzymes induced after infection of *E. coli* with T4 (14). One of the most recent reports is that of O'Farrell and Gold (21), who have shown that many of the gene products of the "early" group are formed if rifampin is added no sooner than 2 min after infection at 30 C. However, one group of products, designated by them as quasi-lates, is not formed, at least in significant quantity, if initiation of RNA synthesis is prevented before this time. We have extended this approach to the study of the pattern of appearance of six early enzymes. The enzymes measured were dihydrofolate reductase, deoxycytidylate hydroxymethyltransferase, deoxycytidine triphosphatase, α -glucosyltransferase, β -glucosyltransferase, and deoxynucleotide kinase. In one vessel the normal course of appearance of enzyme activities was followed for 40 min at 30 C in the absence of an inhibitor of RNA synthesis. However, in the second vessel rifampin (at a concentration of 200 μ g/ml) was added 5 min before infection, and in the third vessel rifampin was added 1.5 min after infection. We have allowed 0.5 to 1 min for most of the cells to respond to the inhibitory action of the antibiotic.

In the experiment reported in Fig. 1, the kinetics of appearance of four of the enzymes (dihydrofolate reductase, deoxycytidine triphosphatase, and α - and β -glucosyltransferase) is approximately the same in the control vessel without rifampin as in the vessel to which the antibiotic was added at 1.5 min after infection. The rate of appearance of deoxycytidylate hy-

droxymethyltransferase activity is slower in the inhibited system, but the total amount of enzyme in either vessel reaches the same level at 40 min after infection. However, at this time the level of deoxynucleotide kinase in the vessel receiving rifampin at 1.5 min is only 14% of that of the uninhibited, infected culture. The unique position of the latter enzyme among those studied is thus clearly evident. These experiments illustrate that delayed early enzymes are formed in two ways, those (α - and β -glucosyltransferase) in which the initiation of transcription occurs early in the infection period, possibly at immediate-early promoter sites (PE), and those (deoxynucleotide kinase) in which initiation occurs later when special sites (PQ) become available.

We also have noted that addition of rifampin at 5 min before infection did not completely suppress formation of five of the six enzymes when activity was measured during the 40-min period. However, under these experimental conditions no deoxynucleotide kinase could be detected at any time after infection. This result provides further evidence that the transcription

FIG. 1. Effect of rifampin on early enzyme synthesis. Infection of cells and preparation of extracts for enzyme analysis are described in the text. Symbols: ●, control without rifampin; ×, rifampin (200 μ g/ml) at 1.5 min; ○, rifampin (200 μ g/ml) at -5 min. In the case of all enzymes the activity is reported in terms of nanomoles of products formed per minute per milligram of protein present in the extracts.

of the kinase gene is under constraints that do not apply to the other enzymes studied.

We have chosen dihydrofolate reductase as a representative of an immediate-early enzyme and deoxynucleotide kinase as an example of a delayed-early enzyme of the quasi-late variety for further studies on DNA-directed enzyme synthesis *in vitro*. In experiments to be described below, we added rifampin to these incubation mixtures at prescribed times and continued the incubation for a total period of 30 min to allow translation of all available mRNA. If duplication of conditions *in vivo* can be obtained, one might hope to observe a delay in the initiation of the synthesis of delayed-early mRNA transcribed from a gene with a PQ type promoter. In contrast, one would not anticipate a delay in the initiation for either an immediate-early gene or a delayed-early gene transcribed from an immediate-early promoter.

Course of synthesis of enzymes by a ribosomal preparation and purified T4 DNA. In the experiment shown in Fig. 2, T4 DNA at a level of 2.5 $\mu\text{g/ml}$ was incubated with a ribosomal fraction (S-30) from uninfected *E. coli* cells. At intervals after the beginning of the incubation at 37 C, samples were withdrawn and added to rifampin to make a final concentration of the latter of 40 $\mu\text{g/ml}$. All samples were incubated for a total of 30 min after which the enzymes formed *in vitro* were assayed. The initiation process for the synthesis of the mRNA of both the kinase and dihydrofolate reductase occurs without any perceptible lag. This experimental procedure thus fails to demonstrate the lag in initiation of kinase mRNA observed *in vivo*. Similar experiments at higher concentrations of T4 DNA (25 $\mu\text{g/ml}$) with similar results have already been reported by Natale and Buchanan (18).

In comparing the relative amounts of enzymes synthesized *in vivo* and *in vitro*, we have observed that dihydrofolate reductase is formed *in vitro* with a much greater efficiency than is deoxynucleotide kinase. The reason for this is unknown, but similar observations have been reported using T2 DNA to direct enzyme synthesis (30). It is generally recognized that the efficiency of synthesis of all enzymes is much lower *in vitro* than that found *in vivo*. This is not surprising in view of the complexity of the general system and the amount of information still unknown about the process of enzyme synthesis.

Enzyme synthesis directed by T4 DNA present in crude extracts of cells infected in the presence and absence of chloramphenicol.

FIG. 2. Time course of initiation of mRNA synthesis directed by purified T4 DNA. Initiation was arrested by addition of rifampin (40 $\mu\text{g/ml}$) at the times indicated. All samples were incubated for a total of 30 min before assay of dihydrofolate reductase (●) and deoxynucleotide kinase (○). Background levels, determined by the addition of rifampin at time zero, were subtracted from all experimental values. The concentration of T4 DNA was 2.5 $\mu\text{g/ml}$. Assays for kinase and reductase activities were performed on 0.1- and 0.05-ml portions, respectively, of a 0.2-ml incubation mixture and in either case represent the nanomoles of product formed per minute by the portion used.

In view of the results cited above on the synthesis of enzymes *in vitro* by a system directed by T4 DNA purified from mature phage, we examined the possibility that extracts of infected cells might exhibit some of the characteristics of enzyme synthesis observed *in vivo*. Two types of cell preparations were used. In the first case crude extracts were prepared from *E. coli* cells infected with T4 at 37 C at a multiplicity of five for a period of 2 min (2-min extract). In the second instance a culture of *E. coli* was treated with chloramphenicol (100 $\mu\text{g/ml}$) for 5 min before infection with T4 at a multiplicity of five. At 5 min after infection the cells were collected, and a crude extract (CM extract) was prepared.

Table 1 shows that both CM extracts and 2-min extracts from a normal infection, when employed in an *in vitro* system, can direct the synthesis of the two T4 enzymes, whereas extracts from uninfected cells cannot, since a source of T4 DNA is lacking. At the low level of T4 DNA present in the extracts of the infected cells (approximately 0.5 $\mu\text{g/ml}$) the level of enzyme synthesis was low but reproducible. Apparently the T4 DNA present endogenously in extracts of infected cells can be used more effectively on a weight basis than can purified T4 DNA added to a ribosomal preparation.

The observed synthesis of deoxynucleotide kinase and dihydrofolate reductase is dependent on RNA synthesis (inhibition by actinomycin D and rifampin), protein synthesis (inhibition by chloramphenicol), and the pres-

TABLE 1. *Requirements for synthesis of deoxynucleotide kinase and dihydrofolate reductase and the effects of inhibitors^a*

Incubation medium	Activity of ^b deoxy-nucleotide kinase synthesized in vitro (nmol/min)	Activity of dihydrofolate reductase synthesized in vitro (nmol/min)
Expt I		
Complete with CM extract	0.063	1.05
+ Actinomycin D (250 μ g/ml)	0.037	0.79
+ Chloramphenicol (100 μ g/ml)	0.037	0.79
- CM extract	0.034	0.71
- S-30	0.015	0.22
- S-30 + actinomycin D (250 μ g/ml)	0.013	0.24
Expt II		
Complete with 2-min extract	0.056	
+ Actinomycin D (250 μ g/ml)	0.034	
+ Chloramphenicol	0.034	
- 2-min extract	0.030	
- S-30	0.015	
- S-30, + actinomycin D (250 μ g/ml)	0.015	
Expt III		
Complete with CM extract	0.058	1.06
+ Rifampin (40 μ g/ml)	0.032	0.63
- CM extract + uninfected extract	0.034	0.64

^a The final concentration of T4 DNA contained in the extracts was 0.5 μ g/ml. Uninfected extract was added at the same absorbancy (260 nm) as the CM extract. When inhibitors were used, they were added just prior to the start of incubation. The uninhibited infection cycle was allowed to proceed 2 min rather than the customary 3 min before preparation of extracts.

^b 0.1 and 0.05 ml of a 0.2-ml incubation mixture were used for the kinase and reductase assay, respectively. Values are reported as nanomoles of products formed in the assay by the portions used.

ence of the S-30 preparation (Table 1). Therefore, the enzyme activities observed result from de novo synthesis directed by T4 DNA present in the extracts of infected cells. Since the S-30 fraction is required for the reaction, the possibility that residual, intact protoplasts are responsible for the enzyme synthesis observed is ruled out.

Good agreement was found within each experiment for values obtained in vessels containing actinomycin D, chloramphenicol, and rifampin added at zero time, or when an extract

of uninfected cells was substituted for one prepared from infected cells. Since in each instance de novo synthesis of enzyme in vitro was prevented for one reason or another, the value of the control vessel with rifampin was taken as the blank value and was subtracted from the experimental values reported in Fig. 2 and 3. The control value is a composite of reagent blank and the activities of small amounts of either viral enzyme that may have been formed within 2 or 3 min of the infection period. Furthermore, it should be recalled that neither viral enzyme is formed in cells treated with chloramphenicol, and that dihydrofolate reductase of *E. coli* cells has been selectively inhibited by use of potassium fluoride and trimethoprim in the assay medium (30). In the kinase assay hydroxymethyl-deoxycytidylate was used as the substrate since only the phage-specific enzyme is capable of utilizing this substrate. A complete description of one experiment is shown in Table 1 to give a better understanding of the magnitude of the changes realized in the experimental as compared to control vessels.

Initiation of synthesis of deoxynucleotide kinase and dihydrofolate reductase mRNAs in vitro. The next experiment was designed to determine the rate at which RNA polymerase initiates the synthesis of deoxynucleotide kinase and dihydrofolate reductase mRNAs in CM and 3-min extracts. At various times after addition of either extract to an in vitro enzyme-synthesizing system, 0.2-ml portions were removed and placed in tubes containing rifampin (0.01 ml, 30 μ g/ml, final concentration) to block further initiation of transcription. Each tube was incubated for a total of 30 min to ensure translation of all completed transcripts. Portions were then removed from the tubes for assay of the two enzyme activities.

The time course of initiation of the transcription of the deoxynucleotide kinase and the dihydrofolate reductase genes in vitro by this particular system is presented in Fig. 3. As can be seen, initiation of transcription of reductase mRNA from T4 DNA present in either the 3-min or CM extracts starts immediately and progresses steadily thereafter (Fig. 3A). However, a delay of 2 min is observed before the initial appearance of kinase mRNA in CM extracts, whereas with 3-min extracts the initiation of the kinase gene occurs immediately (Fig. 3B). These results support the contention that in vivo injected T4 DNA undergoes a transition during the first 3 min of infection that makes the kinase gene available for initiation of transcription. This transition is prevented by inclu-

FIG. 3. Kinetics of initiation of phage mRNA synthesis *in vitro* directed by T4 DNA present in infected extracts. Rifampin (40 $\mu\text{g/ml}$) was added at the times indicated; all samples were incubated for a total of 30 min. (A) Initiation of dihydrofolate reductase mRNA synthesis directed by T4 DNA contained in either CM extracts (●) or 3-min extracts (○). The final concentration of T4 DNA was 0.5 $\mu\text{g/ml}$ of reaction mixture. The values shown are the averages of four separate experiments. (B) Initiation of deoxynucleotide kinase mRNA synthesis directed by T4 DNA contained in either CM-extracts (●) or 3-min extracts (○). The final concentration of T4 DNA was 0.5 $\mu\text{g/ml}$ of reaction mixture. The values shown are the averages of four separate experiments. Background levels, determined by the addition of rifampin at time zero, were subtracted from all experimental values. Assays for kinase and reductase activities were performed on 0.1- and 0.05-ml portions, respectively, of a 0.2-ml incubation mixture and in either case represent the nanomoles of product formed per minute by the portion used.

sion of chloramphenicol in the culture medium. Since the procedure for gentle lysis of the cells presumably maintains intact important physiological structures, it is possible to observe an initiation property of the two genes *in vitro* that one normally sees *in vivo*. A further conclusion of these experiments is that, after 2 min of incubation *in vitro* under circumstances permitting protein synthesis, the transition in the complex must occur since initiation of the kinase gene takes place.

DISCUSSION

As indicated earlier, the control of the transcription of T4 delayed-early genes has been a subject of some controversy. In particular, the transcription of gene 1 for deoxynucleotide kinase, classified now by several investigators (Gold et al. [10], H. Witmer [personal communication], and ourselves) as a delayed-early protein of the quasi-late variety, apparently cannot be explained by currently popular hypotheses. For example, the size of the mRNA for deoxynucleotide kinase suggests that it is

transcribed individually from a T4 gene as a unit (25) and not as an extension of an immediate-early transcript, as has been suggested for other delayed-early species (2, 10, 13, 17, 22, 33). Furthermore, the synthesis of kinase mRNA from T4 DNA by enzymatic components derived exclusively from uninfected *E. coli* (19, 31) argues against the participation of a T4 sigma factor (29).

The effect of chloramphenicol in preventing the synthesis of kinase mRNA *in vivo* has been ascribed by some to a polarity effect on transcription (2, 21, 22). This effect, however, has not been duplicated *in vitro* by the systems so far studied (19, 22). The term "polarity" has not been described precisely in this context in terms of causal factors, and thus, so far has not been particularly useful as an explanation of phenomena observed *in vivo*. However, if the controlled, sequential synthesis of enzymes observed *in vivo* could be demonstrated *in vitro* by use of antibiotics such as rifampin or chloramphenicol, an approach might be possible to providing substantial information about the meaning of polarity in gene transcription. We have particularly in mind the formation of transcription complexes (1, 3, 23) and the relationship of transcription to the association of T4 DNA with bacterial membranes (8).

Towards this end we have attempted to examine the transcription of the genes for dihydrofolate reductase and deoxynucleotide kinase by an enzymatic system prepared in such a manner as to preserve as nearly as possible the important structural features of complexes of enzymatic components and DNA formed in *E. coli* after infection with T4 bacteriophage. The cell extracts produced by the gentle lysis procedure described by Oleson et al. (23) were particularly suitable for the study of the two enzymes under consideration. We had previously found that there is a transitory increase in apparent RNA polymerase activity of cell extracts 1 min after infection as compared to the activity of extracts of uninfected cells. Thereafter, a precipitous decline occurs, until at 4 min the specific activity of RNA polymerase is approximately one-third the activity at 1 min. The higher level is maintained, however, if infection is made in the presence of chloramphenicol. This observation indicated that chloramphenicol prevents the changes in transcription complexes that occur during a normal infection. Furthermore, at 1 min after infection *E. coli* DNA is entirely replaced in the complex with T4 DNA, since neither salmon sperm DNA added exogenously to the incubation or *E. coli* DNA are transcribed. The transitory increase in

polymerase activity was ascribed to the greater rate of transcription by the complex of T4 DNA as compared to *E. coli* DNA. Since the changes in specific activity occur between 1 and 4 min, it is likely that they are related to transcriptional regulatory systems under consideration in this study.

When extracts prepared by the gentle lysis procedure were used, substantial differences were noted in the initiation of transcription of the T4 genes for dihydrofolate reductase and deoxynucleotide kinase, depending on whether cells were infected in the presence or absence of chloramphenicol. In our studies the extracts, containing T4 DNA introduced only by the infective step, were incubated with the 4 nucleoside triphosphates, 20 amino acids, and an S-30 fraction from uninfected *E. coli* cells. At indicated intervals portions were removed and added to rifampin, and the incubation was continued for a total of 30 min to translate all mRNA initiated and formed between the addition of the antibiotic. The synthesis of both enzymes by extracts prepared from cells infected for a period of 3 min in the absence of chloramphenicol proceeded without indication of a lag in the initiation of transcription of either gene.

In contrast, we have found that although the initiation of transcription of the dihydrofolate reductase gene proceeds normally when extracts of cells infected in the presence of chloramphenicol were used there was a definite lag of 2 min and sometimes longer in the initiation of the kinase gene.

This lag possibly occurs for the same reason as found in vivo, but further work will be required to substantiate this conclusion. We consider that when extracts of cells infected in the presence of chloramphenicol were used, fundamental changes in the transcription characteristics had not been allowed to take place and that at least 2 min of metabolic activity in vitro in the absence of either antibiotic was required to bring about those transformations that occur within 2 or 3 min in vivo after a normal infection.

At the present time it is uncertain whether the transformation required for initiation of transcription of the quasi-late genes in general or the kinase gene in particular requires the participation of a specific phage protein or the general process of protein synthesis itself. Support for the former alternative may be offered from in vivo experiments on the basis of the reported mutant isolated by T. Mattson (cited in O'Farrell and Gold [21]) which affects only the expression of the quasi-late, delayed-early

genes, and the fact that initiation of the transcription of the two types of delayed-early genes can be distinguished by rifampin.

Delayed-early mRNAs can possibly be distinguished, therefore, on the basis of a need by some (e.g., α - and β -glucosyltransferase) for protein synthesis at a point in the extension of immediate-early transcripts and a requirement by others (e.g., deoxynucleotide kinase) for a phage-specific protein to effect initiation of transcription of genes at PQ promoter sites.

Studies in vivo. The patterns of formation of deoxynucleotide kinase in the presence of rifampin added at -5.0 and 1.5 min after infection are clearly different from those found for the other five enzymes (Fig. 1). The low formation (14%) of deoxynucleotide kinase in the presence of rifampin added at 1.5 min could result from the timing of addition of the antibiotic since no enzyme was formed if rifampin was included in the medium before infection. The findings for the other five enzymes, however, were quite different. In all cases the increase in enzyme production was the same whether rifampin was added at 1.5 min after infection or not.

In agreement with Earhart et al. (8) we have found that the addition of rifampin to cells before phage infection does not completely suppress RNA synthesis after infection. Cells that had received rifampin at -5 min were able to synthesize from 10 to 20% of the normal complement of five of the six enzymes by 40 min. This latter observation was not the result of a rifampin-resistant subpopulation of cells since no deoxynucleotide kinase was formed under these same conditions.

Moreover, we have noted a gradation in the effect of rifampin added before infection in suppressing the appearance of the six enzymes. A small amount of dihydrofolate reductase is seen as early as 10 min, β -glucosyltransferase and deoxycytidylate hydroxymethyltransferase by 20 min, α -glucosyltransferase and deoxycytidine triphosphatase by 30 min, but no deoxynucleotide kinase by the latest time point, 40 min. These data may suggest that rifampin greatly slows the initiation process for the whole population of *E. coli* cells but does not eliminate it entirely. Furthermore, the enzyme activities appear in the expected order under these circumstances but with considerable delay as compared to the course observed in a normal infection.

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