Regulation of Early mRNA Synthesis in Bacteriophage T4-Infected Bacteria: Dependence on Bacteriophage-Specific Protein Synthesis

CHRISTINA BOLUND AND OLA SKÖLD

Department of Microbiology, Faculty of Pharmacy, University of Uppsala, Uppsala, Sweden

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The mechanism of the transcriptional turn-off was studied for those species of mRNA in T4 infection which specify early phage proteins. Total synthesis of these early RNA species was determined by short-time labeling with uracil-5-³H after the infection of *Escherichia coli* B with DNA-negative, conditional lethal mutants, which produce no late mRNA under nonpermissive conditions. With both amber and temperature-sensitive mutants a decrease in incorporation into early RNA by more than 90% was observed within 15 min after infection. Analysis by thin-layer chromatography showed uracil-5-³H to enter nucleotide pools throughout the infection cycle. The observed decrease in early RNA synthesis was dependent on protein synthesis, since it could be prevented by chloramphenicol. By varying the time of chloramphenicol addition, the effect of this regulatory protein(s) was found to appear at 3 to 4 min and reached its full extent at 10 to 15 min after infection. The turn-off of transcription of a defined early gene was studied by 5-fluorouracil (FU) rescue of an early, DNA-negative, amber mutant (gene 1, deoxynucleotide kinase) at different times after infection. DNA synthesis could be rescued by FU addition early after infection, but not after 12 min. 5-fluorouracil was efficiently taken up into the nucleotide pools also at late times, as shown by thin-layer chromatography. After incubation in the presence of chloramphenicol from 3 min after infection, DNA synthesis could be rescued as late as 45 min after infection. Thus, when protein synthesis was blocked during the early period of infection, the shut-off of transcription of the studied early gene was prevented.

The development of T-even bacteriophages in sensitive bacteria comprises a temporal sequence of events, which seems to be very precisely regulated (1, 8, 14). A group of enzymes involved in phage-specific DNA synthesis starts to be formed a few minutes after infection, when the host-specific synthesis of macromolecules has been turned off by the phage. The formation of these early enzymes proceeds at a high rate until 10 to 15 min after infection (37°C), when it ceases abruptly (10, 18). This cessation of early enzyme formation takes place a few minutes after the onset of phage-specific DNA synthesis (1) and slightly after the first appearance of structural phage proteins (15). During infection with DNA-negative amber mutants the period of synthesis of early enzymes is extended (8, 10, 18).

In an earlier paper (4) we presented evidence for a regulation mechanism, which shuts off the transcription of early enzyme genes at 9 to 12 min after infection with DNA-negative mutants. The work we describe now is an attempt to further characterize this transcriptional regulation mechanism. Transcription of specified early genes was determined by the phenotypic rescue of phage DNA synthesis by 5-fluorouracil (FU) at different times after DNA-negative amber mutant infection (2, 5).

Determination of total early mRNA formation was achieved by measuring rates of short-time incorporation of radioactive uracil at different times after infection with phage mutants unable to induce late mRNA synthesis. In parallel, the uptake of FU and uracil into the ribonucleotide pools of phage-infected bacteria

was determined by thin-layer chromatography.

When protein synthesis was inhibited by chloramphenicol (CAP) added at 2 to 4 min after infection, the shut-off of early transcription did not take place. This indicates that phage-induced proteins are involved in the regulation mechanism. By systematic variation of CAP addition we could obtain an estimate of the kinetics for the appearance of this protein(s).

MATERIALS AND METHODS

Media. Low-phosphate mineral salts medium, dilution broth for phage, bottom agar, and top agar were described earlier (2).

Chemicals. Thymidine-*methyl*- ^3H , 2,000 to 5,000 mCi/mmol and uracil-5- ^3H , 500 to 1,000 mCi/mmol were purchased from The Radiochemical Centre, Amersham, Great Britain. Deoxycytidine-2- ^{14}C 5'-triphosphate, 20 to 30 mCi/mmol, and formaldehyde- ^{14}C , 10 mCi/mmol, were obtained from NEN Chemicals, Dreieichenhain, W. Germany. The uracil analogue FU was a gift from R. Duschinsky, Hoffman-La Roche, Basel, Switzerland, and 5-fluorouracil-2- ^{14}C , 22 mCi/mmol, was bought from Calbiochem, Inc., Los Angeles, Calif. Chloramphenicol was a gift from Parke, Davis & Company, Detroit, Mich. Tetrahydrofolic acid was a product of Sigma Chemical Company, St. Louis, Mo.

Bacteria and phages. *Escherichia coli* B wild-type was used as nonpermissive host for amber and temperature-sensitive (at 41–43°C) mutants. Suppressor strain CR63 was used for amber mutant plating. Wild-type T4D and the amber mutant *amC42* (gene 1, deoxynucleotide kinase, reference 4) were kindly supplied by J. S. Wiberg. The temperature-sensitive mutants *tsL13* (gene 42), *tsLB3* (gene 42), and *tsL91* (gene 43) were kindly supplied by W. Salser. Phage stocks were prepared as described earlier (2). In no preparation of *amC42* or temperature-sensitive mutant stock did the revertant frequency exceed 10^{-6} .

Infected cells. Cells of *E. coli* B were grown at 37°C to 0.5×10^9 to 0.7×10^9 /ml in the low phosphate mineral salts medium (generation time 50 min). After harvesting by centrifugation or filtration, the cells were cooled and resuspended in the same medium supplemented with L-tryptophan, 50 $\mu\text{g}/\text{ml}$, to a final concentration of 2×10^9 to 4×10^9 /ml and kept on ice until used. At infection (time zero) 5 to 10 phages were added per bacterium. Adsorption was allowed to take place for 1 to 3 min, usually at 37°C. The infected complexes were then diluted 10-fold into warmed medium, containing supplements and radioactive label as indicated for each experiment. Phage adsorption, bacterial survival, and number of infective centers were determined by standard techniques. At the end of the adsorption period more than 99.5% of the bacteria were killed and less than 0.5% of input phage remained unadsorbed. Most of the killed bacteria (about 90%) could be accounted for as infective centers (wild-type infection). When FU was used, thymidine, 20 $\mu\text{g}/\text{ml}$, was always added to neutralize the very strong inhibitory effect of the FU-deoxynu-

cleotide on thymidylate synthetase (3). In experiments with chloramphenicol, a drug concentration of 20 $\mu\text{g}/\text{ml}$ was used. The drug was removed by filtration (Gelman, Metrical GA-6) at the times indicated for each experiment. Filtration and washing three times with 3 ml of warmed medium and final resuspension could be performed in 2 min.

Determination of DNA and RNA synthesis. Synthesis of DNA was determined by the cumulative incorporation of ^3H -thymidine into Cl_3CCOOH -insoluble, alkali-stable material. Synthesis of RNA was studied by short-time incorporation of uracil-5- ^3H into acid-insoluble product. No incorporation of label from this precursor could take place into phage DNA, because both phage DNA pyrimidines are substituted in the 5-position (7). Alkaline hydrolysis was shown to make more than 99.5% of the incorporated activity acid soluble. Samples of 1 ml each were withdrawn at different times from the aerated, phage-infected cultures and precipitated with 1 ml of cold 10% Cl_3CCOOH . For the determination of thymidine incorporation into DNA the precipitates were collected by centrifugation, treated with M NaOH for 15 h at 37°C, neutralized with M HCl, and finally reprecipitated in cold, 5% Cl_3CCOOH and collected on glass fiber filters (Whatman GF/C). For the determination of RNA synthesis, the trichloroacetic acid precipitates were directly collected on filters. The filters were dried, immersed in scintillation fluid, and counted in a Packard scintillation counter.

Uptake of radioactive precursors into ribonucleotide pools. The radioactive precursor was added to the infected cells at different times after phage infection. After 5 and 15 min of incorporation, respectively, 2-ml samples were withdrawn and filtered on a membrane filter (Millipore Corp.). The cells were resuspended in 2.5 ml of 0.3 M perchloric acid. After precipitation for 30 min at 4°C the samples were centrifuged and the pH of the supernatant fluids was adjusted to 5 (indicator paper) by the addition of 6 M KOH minus 0.1 M ethylenediaminetetraacetic acid (EDTA). The precipitating salt was centrifuged off and after the addition of unlabeled uracil and cytosine nucleotide markers, 50 to 100 μl of the supernatant fluids were analyzed for FU, cytosine, and uracil ribonucleotides, respectively, by thin-layer chromatography in one dimension on plastic sheets according to Neuhaud and Thomassen (11). In this way the nucleoside di- and triphosphates of FU, cytosine, and uracil could be well separated. Radioactive spots were localized by autoradiography on X-ray film (exposure for 2 days). The 5-fluorouridine triphosphate was found to run slightly slower than uridine triphosphate at the pH used. The localized spots were cut out and the radioactivity was determined in the scintillation counter. The molar amounts of radioactive precursor incorporated into nucleotide pools was calculated from the known precursor concentration in the incubation medium and the radioactivity of medium samples was spotted on thin-layer chromatography sheets.

RESULTS

Effect of chloramphenicol on FU rescue of early amber mutant function. It is well estab-

lished that certain amber mutations in the early genes of phage T4 can be phenotypically suppressed by FU (2, 5). This suppression is effected by the incorporation of FU into mRNA, where the amber codon UAG is substituted by FUAG, which to some extent can be translated as CAG (glutamine) as demonstrated by Rosen et al. (13). Rescue of phage production by early addition of FU to *E. coli* B infected with the mutant *amC42* (gene 1) is shown in Table 1. It can be seen that the FU-induced rescue of infective centers and of phage production decreased rapidly with time after infection. This time-dependent decrease in FU rescuability could, however, be arrested by the addition of chloramphenicol. In Table 1 it is shown that the presence of chloramphenicol from 3 to 15 min allowed a much later FU rescue of phage production. If chloramphenicol was thus present from 3 min after infection and was then removed by filtration at 15 min, the subsequent addition of FU at 18 min could rescue a substantial phage production, which can be compared to FU addition at 12 min without chloramphenicol, where no phage production at all could be detected.

TABLE 1. Effect of chloramphenicol on the rescuability of *amC42* by FU in *E. coli* B^a

Time of FU ^b addition (min after infection)	Infective centers (% of killed cells ^c)		Burst size ^e	
	-CAP	+CAP ^d 3-15 min	-CAP	+CAP 3-15 min
3	42		30	
6	20		12	
9	8		12	
12	0		0	
18		22		29
24		17		12
-FU	0	0		0

^a Cells were grown and infected as described in the experimental section.

^b Final concentration of FU was 10 μ g/ml; thymidine, 20 μ g/ml, was present from time zero in all experiments.

^c Infective centers were determined at 30 min after the addition of FU. *E. coli* CR63 was used as indicator. Values are corrected for unadsorbed phage.

^d After the 3-min adsorption period the infected cells were diluted into warmed medium containing chloramphenicol 20 μ g/ml. At 15 min chloramphenicol was removed by filtration as described in the experimental section.

^e Lysis with chloroform at 180 min after infection. The values represent number of phage produced per infective center.

The efficiency of FU suppression of early amber mutations can be determined much more precisely by measuring rescue of DNA synthesis as ³H-thymidine incorporation. In earlier work we could show that the FU rescuability of DNA synthesis decreased rapidly with time after infection with several early, DNA-negative amber mutants (2). Also, in this type of experiment the early presence of chloramphenicol prevented the rapid decrease in FU rescuability. It is demonstrated in Fig. 1 that the presence of chloramphenicol from 3 to 15 min after infection with *amC42* allows an FU rescue at 18 min of more than 80% of that DNA synthesis which

FIG. 1. Effect of chloramphenicol on the phenotypic suppression by FU of amber mutant DNA synthesis. Cells of *E. coli* B were grown in the mineral salts medium, harvested, and infected with *amC42* as described in the experimental section. Curve 1: After 3 min of adsorption the infected cells were diluted 10 times into growth tubes containing mineral salts medium (37 C) with ³H-thymidine, 1 μ Ci/ml, 20 μ g/ml. At the indicated times FU was added to the various tubes to 10 μ g/ml. The different amounts of DNA formed between 30 and 60 min after phage infection were plotted versus time of FU addition. These amounts are expressed as percentages of that obtained for FU addition at 3 min. Curve 2: After phage adsorption for 3 min the infected cells were diluted into warmed (37 C) mineral salts medium containing chloramphenicol, 20 μ g/ml. At 4, 15, 30, and 45 min after infection portions of the culture were withdrawn and filtered free of chloramphenicol as described in the experimental section. Three minutes later the infected cells were resuspended into warmed mineral salts medium containing ³H-thymidine, 1 μ Ci/ml, 20 μ g/ml, and FU, 10 μ g/ml. In two experiments (Δ , $\square$) parallel samples were incubated after resuspension in the absence of FU for 5 and 6 min, respectively, and FU was then added to 10 μ g/ml. Values for ³H-thymidine incorporation between 30 and 60 min after the addition of FU were plotted as percentages of the incorporation obtained when FU was added at 3 min (without chloramphenicol).

could be rescued at 3 min. This should be compared to the DNA synthesis rescuable at 12 min in the absence of chloramphenicol, which was less than 10% of the 3-min value (curve 1). In fact, 50 to 70% of that rate of DNA synthesis which was normally rescuable at 3 min could be rescued as late as after 30 to 45 min of incubation in the presence of chloramphenicol (curve 2). It is also indicated in Fig. 1 that the removal of chloramphenicol at 6 and 18 min, respectively, initiated the rapid decrease in FU rescuability normally seen early in infection. These results suggest that the turn-off of early gene transcription is dependent on early protein synthesis.

Since FU rescue is dependent on the incorporation of the analogue into mRNA, the decreasing efficiency of rescue could be due to a decreasing uptake of FU into nucleotide pools and the effect of chloramphenicol could then reflect pool changes only. In earlier work we demonstrated by electrophoretic analysis that FU was incorporated into the nucleotide pool of early mutant-infected cells also at times when FU could no longer rescue any DNA synthesis (2). In order to investigate this important point in more detail the uptake of radioactive FU into the nucleoside triphosphate pool was followed during the infection by thin-layer chromatography and autoradiography. Table 2 demonstrates that FU, when added at different times after infection, is readily incorporated into intracellular nucleoside triphosphate although at a diminishing rate. At 12 min, when FU is no

longer effective in rescuing DNA synthesis (Fig. 1), it is shown that the uptake of FU is about 50% of that at 3 min. It is clear that uptake of FU takes place in good yield as late as 20 min after infection.

Temperature-shift rescue of temperature-sensitive early functions. Phenotypic rescue of early mutations can also be demonstrated by temperature-shift of *ts* mutants. Figure 2 shows an experiment, where temperature-shift rescue of phage DNA synthesis was studied after infection with mutant *tsL13*. This mutant is deficient in gene 42, which codes for the early enzyme deoxycytidylate hydroxymethylase (17). At permissive temperature the mutant produces an enzyme which is irreversibly inactivated at 40 C in vitro (17). At 43 C the infection by *tsL13* did not result in any detectable DNA synthesis (Fig. 2). Early after infection (3–7 min) a shift to 30 C resulted in normal DNA synthesis. Temperature-shifts at later times, however, rescued lower rates of DNA synthesis, the rates decreasing gradually with time after infection. This decrease in temperature-shift rescuability of DNA synthesis after *tsL13* infection was relatively slow compared to the decrease in FU rescuability (Fig. 1). A shift from 43 to 30 C at 12 min after *tsL13* infection could, thus, rescue DNA formation at almost half the rate obtained at 3 min (Fig. 2). The slower decrease could be explained in part by the subsequent translation of that early mRNA present at the time of the shift and in part by a protecting effect of intracellular deoxycytidylate on the enzyme allowing some reactivation at the lower temperature (17).

Effect of chloramphenicol on early RNA synthesis in *E. coli* B infected with early T4 mutants. It is well known that phage mutants which are unable to induce DNA synthesis are also unable to induce late mRNA synthesis (1). Since host-specific RNA synthesis is efficiently turned off by the phage (12), incorporation of radioactive precursor into RNA after the infection of *E. coli* B with a DNA-negative mutant will, thus, reflect only phage-specific early RNA synthesis. By short-time labeling with radioactive uracil it was previously demonstrated that the rate of synthesis of early RNA decreases dramatically between the early and the late period of infection with DNA-negative amber mutants (2, 10). In Fig. 3A are described some experiments employing short-time incorporation of uracil-5-³H into RNA performed with the mutant *tsL13*, which is DNA-negative at the nonpermissive temperature (42.5 C). It can be seen that the rate of early RNA synthesis decreased rapidly with time after infection at

TABLE 2. Uptake of ¹⁴C-FU into the nucleoside triphosphate pool of *E. coli* B after infection with *amC42*

Time of addition ^a (min after infection)	Time of incorporation (min ^b)	
	5	15
3	0.956 ^c	2.615
9	0.633	0.963
12	0.534	1.090
15	0.310	0.538
20	0.211	0.416

^a Cells were grown and infected as described in the experimental section. At the indicated times ¹⁴C-FU was added to 18.7 μM, 0.22 μCi/ml.

^b Samples were withdrawn at 5 min and 15 min, respectively, after the addition of radioisotope, and analyzed by thin-layer chromatography as described in the experimental section.

^c The values are expressed as nanomoles of FU incorporated into FUTP per 10⁸ cells. Specific activity of the added ¹⁴C-FU was 18,700 counts per min per nmol.

FIG. 2. Temperature-shift rescue of DNA synthesis after infection of *E. coli* B with *tsL13* at 43 C. Cells of *E. coli* B were grown and harvested as described in the experimental section. Infection took place at 43 C, and after 3 min of adsorption the infected cells were diluted 10-fold into mineral salts medium at 43 C, supplemented with ^3H -thymidine (1 $\mu\text{Ci/ml}$, 20 $\mu\text{g/ml}$). At the times indicated on each curve, the infected cultures (10 ml total volume) were transferred to an ice bath for 15 s during uninterrupted aeration, and then to a 30 C bath. Values along the ordinate represent counts per minute incorporated per 1-ml sample. The lowest curve ($\square$) was obtained at 43 C without temperature-shift.

42.5 C. The addition of radioactive precursor at 15 min did thus hardly result in any measurable incorporation of isotope. The presence of chloramphenicol, added at 2 min after infection could, however, almost completely abolish this decrease (Fig. 3A). This resulted in a uracil-5- ^3H incorporation at 20 min, which was compa-

FIG. 3. Effect of chloramphenicol on RNA synthesis in *E. coli* B infected with *tsL13* or *amC42*. A, Cells of *E. coli* B were grown and infected with *tsL13* as described in the experimental section. After adsorption at 30 C for 2 min, the infected complexes were diluted 10-fold into mineral salts medium at 43 C with or without chloramphenicol (20 $\mu\text{g/ml}$), and supplemented with uracil (10 $\mu\text{g/ml}$). At 3, 8, 15, and 20 min, respectively, after infection, uracil-5- ^3H (1 $\mu\text{Ci/ml}$) was added to the various experimental tubes and 1-ml samples were withdrawn at 1, 2, 4, and 6 min after radioisotope addition. Samples were treated as described in the experimental section. Values along the ordinate represents counts per minute incorporated per 1-ml sample. $\times$, With chloramphenicol; $\circ$, without chloramphenicol. B, Cells of *E. coli* B were grown and infected with *amC42* as described in Materials and Methods. After adsorption for 2 min at 37 C the infected cells were diluted 10-fold into warmed (37 C) mineral salts medium without or with chloramphenicol (20 $\mu\text{g/ml}$). At 2, 10, 20, and 30 min, respectively, after infection, uracil-5- ^3H (1 $\mu\text{Ci/ml}$, 1 $\mu\text{g/ml}$) was added. Samples of 1 ml each were then withdrawn at 15, 75, 135, and 195 s after radioisotope addition and treated as described in Materials and Methods. Values along the ordinate represent counts per minute incorporated per 1-ml sample. $\times$, With chloramphenicol; $\circ$, without chloramphenicol.

rable in rate to that obtained at 3 min. Experiments analogous to those described in Fig. 3A were performed with the temperature-sensitive, DNA-negative mutants *tsLB3* (gene 42) and *tsL91* (gene 43). Also, with these mutants, a decrease in uracil-5-³H incorporation was observed with time after infection, and, similarly, this decrease could be abolished by chloramphenicol.

Also, with the early amber mutant *amC42*, chloramphenicol was found to prevent the decline in early RNA synthesis (Fig. 3B). In the presence of chloramphenicol from 2 min after infection the rate of incorporation at 30 min was more than 10-fold larger than that observed in the absence of the drug. No effect was seen on the incorporation rate in the period 2 to 5 min.

The rapid decrease in the incorporation of radioactive uracil with time after infection could be due to a decreasing uptake of uracil into the ribonucleotide pools. To check for this possibility ¹⁴C-uracil was added to *E. coli* B at different times after the infection with *amC42*, and extracts were analyzed for the labeling of uridine triphosphate and cytidine triphosphate, respectively. It is demonstrated in Table 3 that the uracil uptake into these nucleotides decreased slowly with time after infection. This decrease was, however, much slower than the decrease in the rate of incorporation into RNA (Fig. 3). At 15 min, when RNA synthesis according to Fig. 3 had almost ceased, the uptake into nucleotides was still 20 to 30% of that obtained at 3 min.

Effect of chloramphenicol on shut-off of host functions and on initiation of early transcription. The early addition of chloramphenicol used in the described experiments might interfere with the very early stages of phage infection. One could ask, for example, if the addition of chloramphenicol to the infected cells as early as 2 to 3 min after infection would allow enough phage-specific protein synthesis to enable the phage to shut off host-specific RNA and DNA synthesis. The experiments described in Fig. 4 demonstrate that the addition of chloramphenicol at 2 min (end of adsorption period) after the infection of *E. coli* B with *amC42*, does not interfere with the phage-induced switch from host-specific to phage-specific RNA synthesis. In the presence of chloramphenicol there was a rapid incorporation of uracil-5-³H into acid-insoluble RNA, and the incorporated radioactivity could be chased with cold uracil after removal of chloramphenicol. The incorporation curves were very similar whether chloramphenicol was present or not. If the drug had interfered with the phage-

TABLE 3. Incorporation of ¹⁴C-uracil into the nucleotide pools of *E. coli* B after infection with *amC42*

Time of addition ^a (min after infection)	Time of incorporation (min) ^b			
	CTP		UTP	
	5	15	5	15
3	0.464 ^c	1.565	0.749	0.766
6	0.320	0.647	0.426	0.417
9	0.332	0.514	0.327	0.323
12	0.132	0.402	0.163	0.258
15	0.173	0.578	0.142	0.447

^a Cells were grown and infected as described in the experimental section. At the indicated times ¹⁴C-uracil was added to 21.1 μM, 0.67 μCi/ml.

^b Samples were withdrawn at 5 min and 15 min, respectively, after the addition of radioisotope and analysed by thin-layer chromatography as described.

^c The values are expressed as nmoles of uracil incorporated per 10⁹ cells. Specific activity of the added ¹⁴C-uracil was 46,200 counts per min per nmol.

induced shut-off of host RNA synthesis, a curve similar to that for uninfected cells, also shown in Fig. 4, would have been obtained. In this case, the incorporated radioactivity could not be chased with cold uracil and is, thus, as expected, mostly located in stable RNA species. The activity located in DNA of uninfected cells was negligible since the incorporation of uracil-5-³H into cytosine of DNA was shown by alkaline hydrolysis to be less than 2%. It was similarly demonstrated that early addition of chloramphenicol did not prevent the phage-induced shut-off of host DNA synthesis. It was shown that by ³H-thymidine incorporation after wild-type T4 infection the addition of chloramphenicol at 2 min after infection completely prevented all DNA synthesis. This means that the drug was added early enough to inhibit the synthesis of those early proteins which are necessary for phage DNA synthesis, but that it did not prevent the phage from turning off host-specific DNA formation (data not shown). If chloramphenicol was added 1 min later (3 min after infection) some DNA synthesis was observed. This slightly longer time period did, thus, allow the appearance of all those early proteins which are necessary for a limited DNA synthesis.

Time-dependence of the chloramphenicol effect on early mRNA synthesis. It was demonstrated above that chloramphenicol could prevent the rapid decrease in early mRNA formation observed after early mutant infection (Fig. 3). In Fig. 5 data are compiled from many similar experiments, in which the time of chlo-

FIG. 4. Influence of early chloramphenicol addition on the shut-off of host RNA synthesis. Cells of *E. coli* B were grown and infected with *amC42* as described. After adsorption for 2 min (37°C), the cells were diluted 10-fold into warmed (37°C) medium supplemented with uracil-5-³H (1 μ Ci/ml, 1 μ g/ml) and without or with chloramphenicol (20 μ g/ml). Samples of 1 ml were withdrawn at 15 s, 2 min and 4 min after the addition of uracil-5-³H. At 4 min (6 min after infection) unlabeled uracil was added to 200 μ g/ml and chloramphenicol and radioisotope were removed by filtration. The cells were resuspended in medium supplemented with cold uracil (200 μ g/ml) and 1-ml samples were withdrawn at the indicated times. Values along the ordinate represent counts per minute incorporated per 1-ml sample. Symbols: $\times$, *amC42*, plus chloramphenicol; Δ , *amC42*, minus chloramphenicol; $\circ$, uninfected *E. coli* B plus chloramphenicol (20 μ g/ml) from 2 to 6 min, unlabeled uracil (200 μ g/ml) added at 6 min.

ramphenicol addition was varied systematically. This was done in order to find out at what time the regulatory mechanism for early mRNA synthesis became insensitive to chloramphenicol inhibition.

The lower curve in Fig. 5A describes the decrease in early mRNA synthesis after *amC42* infection in the absence of chloramphenicol (Fig. 3B). The data are plotted as radioactivity incorporated during short-time-labeling of *E.*

coli B at different times after infection. It can be seen that the addition of uracil-5-³H at 30 min results in an incorporation which is only about one tenth of that obtained at 2 min. The incorporation at 30 min was, however, much larger if chloramphenicol was present from 8 min, and still larger if chloramphenicol was added already at 5 min. In Fig. 5B data are replotted from an array of curves like those of Fig. 5A. The radioactivity incorporated between 30 and 33 min after the infection with *amC42* was plotted versus time of chloramphenicol addition. It can be seen that this value was very small if chloramphenicol was added as early as 1 min after infection, probably because of interference with the initiation of "delayed-early" mRNA synthesis (14). The incorporation increased with later drug addition and reached a maximum with addition at 2 to 4 min. Chloramphenicol addition at 6 min allowed a 30-min incorporation which had already decreased to about half of that obtained with addition at 3 min. Drug addition at 10 and 15 min, respectively, showed the curve to approach the value obtained without chloramphenicol (dashed line). These results demonstrate that the regulatory mechanism which restricts early mRNA synthesis becomes insensitive to chloramphenicol at 10 to 15 min after infection.

DISCUSSION

This investigation aimed at a characterization of that regulation mechanism which restricts the net formation of early enzymes at 10 to 15 min after T-even phage infection (8, 18). In cells infected with DNA-negative early mutants, this shut-off mechanism is deficient in that early enzyme synthesis is extended and exaggerated (8, 10, 18). The regulatory turn-off of early enzyme synthesis has been regarded as dependent on phage DNA synthesis or on late mRNA synthesis, which does not take place in the absence of DNA formation (1). In earlier work we demonstrated the occurrence of a regulation mechanism which restricts the transcription of early genes at about 10 min after infection in the absence of DNA synthesis (2, 16).

A decrease in rescuability of early amber mutant phage production by FU with time after infection was originally demonstrated by Edlin (5). We similarly showed that the FU rescue of DNA synthesis decreased after the infection of *E. coli* B with DNA-negative amber mutants (2). The decrease was quite rapid (Fig. 1). Almost no rescue could be observed at 12 min after infection, e.g., at a time when early enzyme synthesis is normally turned off. The

FIG. 5. Time-dependence of the chloramphenicol effect on early mRNA synthesis. Cells of *E. coli* B were grown and infected with amC42 as described in Materials and Methods. After phage adsorption at 37 C for 2 min, the infected cells were diluted 10-fold

phenotypic FU rescue is effected by the incorporation of FU into mRNA, where the amber codon UAG is substituted by FUAG, which to some extent is translated as CAG (glutamine) (13). The FU rescue is thus dependent on transcriptional activity, and a decrease in rescue would then reflect a decrease in the transcription of that particular gene, which contains the amber mutation. Alternatively, the decrease in FU rescuability could reflect a translational regulation mechanism. It was shown, however, that addition of FU at 16 min would not rescue formation of active enzyme, while FU addition at 2 min allowed synthesis of the rescued enzyme to continue for 25 to 30 min (C. Bolund, *Mol. Gen. Genet.*, 1973, in press). This is not compatible with a translational restriction of FU rescue.

By comparison, the phenotypic rescue by temperature-shift, which is independent of base incorporation, decreased much slower after infection with an early DNA-negative *ts* mutant than FU rescue of early amber mutants. If chloramphenicol was added at the temperature-shift no rescue was seen (unpublished data). Reactivation of heat-sensitive enzyme could thus not explain the slow decrease in rescuability. In this case, the decrease reflects both transcription and translation, since after temperature-shift, it is obvious that at least some of the messenger representing the mutated gene can express itself into active enzyme. The kinetics for the decrease in temperature-shift rescuability is similar to that observed for early enzyme synthesis in the absence of DNA formation (8, 10, 18). The exaggerated enzyme formation in the absence of DNA synthesis could thus be caused by a prolonged translation of those

into warmed (37 C) medium and then immediately distributed into four 1-ml portions for each experimental series. The 1-ml portions were aerated in wide tubes in a shaking water bath. At the indicated times chloramphenicol (20 μ g/ml) was added to each series. Within each series of four tubes, uracil-5- 3 H (1 μ Ci/ml, 1 μ g/ml) was added to each tube at 2, 10, 20, and 30 min, respectively, after infection. After an incorporation period of 3 min, each 1-ml sample was precipitated with trichloroacetic acid and treated as described in the experimental section for determination of RNA synthesis. A, Time of chloramphenicol addition as indicated on each curve. Values along the ordinate represent radioactivity incorporated per 1-ml sample during a 3-min incorporation period starting at the time indicated on the abscissa. B, Replot of data from curves in A and from other similar curves. The radioactivity incorporated per 1-ml sample during the 3-min incorporation period, starting at 30 min after infection, was plotted versus the time of addition of chloramphenicol. The dashed line represents the value obtained in the absence of chloramphenicol.

messengers present at the time of the transcriptional shut-off. And this prolonged translation could be explained by the lack of late mRNA competing for ribosomal translation capacity (1).

The decrease in FU rescuability of DNA synthesis and also the decrease in early mRNA synthesis as measured by short-time labeling was prevented by chloramphenicol. This indicates that the regulatory restriction of early mRNA synthesis is mediated by one or several proteins.

It might be argued that the observed chloramphenicol effect, at least in part, could be explained as a stabilization of the normally labile mRNA by the piling-up of polyribosomes in the absence of protein synthesis (6). The late FU rescuability after chloramphenicol treatment (Fig. 1) requires FU incorporation into RNA, however, and can not be explained by the presence of mRNA formed earlier and protected from degradation.

In a related approach Young and Van Houwe (19) studied the effect of chloramphenicol on the abundance of an early messenger, determined by its ability to direct *in vitro* synthesis of α -glucosyl transferase. In the presence of chloramphenicol from 3 min after infection this messenger activity did not decline in the late period as in normal infection. In a later report (20) this was interpreted to be due to the stabilization of mRNA by chloramphenicol. As discussed above, our experiments strongly suggest continued synthesis of early messengers in the presence of chloramphenicol.

Since a deficient pyrimidine uptake would invalidate our interpretations, the incorporation of uracil and FU, respectively, into ribonucleoside triphosphate pools was determined. At late times after infection the incorporation of uracil-5-³H into RNA decreased to 7 to 8% of that at early times, and yet the uptake of labeled uracil into UTP during the same period only decreased to 20 to 30% of early values. The decrease in RNA incorporation of uracil must then reflect a true decrease in RNA synthesis. These results imply that ribonucleotide pools must expand late in infection in order to account for this late uptake. Such an expansion, especially pronounced for glucosyl uridine diphosphate after DNA-negative mutant infection, was in fact observed (R. Fast and O. Sköld, manuscript in preparation). Variations in UTP-labeling from mutant to mutant could not interfere with our interpretations since only results from *amC42* infection were used to compare the different experimental approaches. The unabated incorporation of uracil into RNA

at late times in the presence of chloramphenicol (Fig. 3) could be seen as a reflection of the continuing RNA synthesis in this case (Fig. 1), draining the UTP pool and thus keeping an unabated entry of uracil into it.

Khesin et al. (9) showed that the addition of chloramphenicol early after T2 phage infection resulted in a continued synthesis of early mRNA as measured by hybridization. From the time dependence of the chloramphenicol effect on the regulation of early mRNA synthesis in our experiments with DNA-negative mutants we want to conclude that the turn-off of early mRNA synthesis is effected by the accumulation of regulatory early protein, which has reached its full activity at 10 to 15 min after infection.

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