Selective Gene Transcription in Bacteriophage T4 by Putrescine¹

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Messenger ribonucleic acid (mRNA) preparations from T4-infected cells formed in the presence or absence of putrescine have been characterized and compared. Hybrid competition experiments indicate that these mRNA molecules are derived from distinct genetic loci. The results are consistent with the hypothesis that putrescine might control differential transcription of the phage genome during morphogenesis. The data are also in accord with previously observed changes in the population of mRNA formed at different times after infection.

The naturally occurring diamine, putrescine, leaks out of host cells immediately after T4 phage infection (6). If putrescine is added to the growth medium during infection, it differentially affects the synthesis of "early" and "late" proteins (21). Phage deoxyribonucleic acid (DNA) synthesis is markedly decreased in the presence of putrescine, and late functions, e.g., synthesis of phage-induced lysozyme and appearance of mature phage, are inhibited. This inhibition starts immediately after infection and is completely reversible. On the other hand, early functions are enhanced by the diamine (21). The aim of the present study was to elucidate the mechanism of putrescine action on phage-directed protein synthesis. The results demonstrate that differential transcription of the phage genome takes place in the presence of the diamine.

MATERIALS AND METHODS

Chemicals. Thymine-2-¹⁴C (specific activity 40.2 mc/mmole) and ³H-uridine (1.44 c/mmole) were purchased from the Radiochemical Centre, Amersham, England. Pronase and putrescine dihydrochloride were supplied by Calbiochem, Los Angeles, Calif.

Bacterial strains. *Escherichia coli* B was obtained from F. W. Stahl. *E. coli* B thy⁻ (a thymine-requiring mutant) was obtained from D. Freifelder.

Bacteriophage strains. T4 wild type and T4BeJ 42, a lysozymeless mutant, were kindly supplied by G. Streisinger. The mutant was used to avoid lysis of infected bacteria during the experiment.

Phage stocks were made by a procedure suggested by G. Streisinger (22). For phage assay, the standard procedures described by Adams were used (1).

Media. M9 and Tris-glucose media were the same as described in the preceding paper (21).

¹ A preliminary account of this work has previously been published (20).

Preparation of T4BeJ 42 specific ribonucleic acid (RNA). For preparation of "early" messenger RNA (mRNA), 100 ml of infected *E. coli* B cells was given a pulse of ³H-uridine (1 μ c/ml; specific activity, 1,44 c/mmole) 3 min after infection. For preparation of "late" mRNA, the infected cells were given a pulse of ³H-uridine 17 min after infection. The duration of the pulse was 2 min, and the temperature of incubation was 37 C. The pulse was ended by pouring the infected cells onto 40 ml of frozen SSC (0.15 M NaCl, 0.015 M Na citrate) containing 0.01 M sodium azide. Unlabeled RNA was prepared by pouring infected cells onto frozen SSC at 5 min for "early" RNA and at 19 min for "late" RNA. After centrifugation, the pellet was suspended in 10 ml of SSC and frozen in dry ice-acetone. After thawing at 20 C in the presence of 1 mg of pronase per ml, the cells were lysed by 0.2% sodium dodecyl sulfate (SDS) and incubated at 37 C for 7 hr, as in a recently described procedure for DNA isolation (24). The solution was then extracted twice with phenol and dialyzed against 0.01 M phosphate buffer (pH 6.7). The resulting solution was then loaded onto hydroxyapatite columns, and a linear PO₄ gradient (0.01 to 0.35 M) was applied to separate the assorted micromolecules, RNA and DNA. Alternatively, stepwise elution was applied. The labeled RNA was then concentrated by reloading on hydroxyapatite and eluted by a small volume of 6 $\times$ SSC, which quantitatively removes it from the column (C. Shalitin and C. Thomas, Jr., unpublished data).

Preparation of bacteriophage DNA. A thymine-requiring strain of *E. coli* B was grown overnight in M9 minimal medium supplemented with 5 μ g of thymine per ml. A 1-ml amount of the overnight culture was transferred into 100 ml of fresh M9 medium containing 5 μ g of thymine per ml. The culture was aerated at 37 C for 3.5 hr, until a concentration of 2 $\times$ 10⁸ cells per ml was attained. The culture was infected by phage T4D (wild type) in the presence of tryptophan at a multiplicity of infection of three phage particles per bacterium. At 10 min after infection, thymine-2-¹⁴C (final concentration, 0.1 μ c/ml; specific

activity, 40.2 mc/mmole) was added; the culture was further aerated at 37 C for 3 hr and was then lysed by chloroform (11). Phage was purified by two cycles of differential centrifugation. After phenol extraction (23), the DNA was finally loaded onto a hydroxyapatite column for purification. The DNA was eluted from the column by small volumes of $6 \times \text{SSC}$.

Preparation and operation of hydroxyapatite columns for purification of DNA and RNA was carried out according to Bernardi (3) and Miyazawa and Thomas (18).

Hybridization experiments. The hybridization experiments were done by the procedure of Gillespie and Spiegelman (8).

Native ^{14}C -DNA preparations (260 counts per min per μg) or nonlabeled DNA preparations were denatured in alkali. The denaturation was done by 2-min exposure of the DNA to 0.3 N NaOH in SSC at 0 C, followed by neutralization with 0.3 N HCl in $6 \times \text{SSC}$. Denatured DNA, 0.33 to 1.0 $\mu\text{g}/\text{ml}$, was immobilized on nitrocellulose membrane filters (type B-6, coarse, 25 mm; Schleicher & Schuell Co., Keene, N.H.). The DNA filters were subsequently dried at room temperature and at 80 C for an additional 2 to 4 hr in a vacuum oven. ^3H -RNA- ^{14}C -DNA hybrids were formed by immersing the DNA filters in vials containing 4 ml of ^3H -RNA in $6 \times \text{SSC}$. Annealing was carried out for 4 to 24 hr at 60 C without shaking (as specified in the legends to the figures), after which the vials were chilled in an ice bath. Filters without DNA or with heterologous DNA were immersed in each vial as a background control. RNA not complexed with DNA was destroyed by immersing the thoroughly washed filters for 1 hr at room temperature in 2 ml of $2 \times \text{SSC}$, containing 2.5 μg of pancreatic ribonuclease per ml (heated for 10 min at 80 C to destroy residual deoxyribonuclease activity). After ribonuclease treatment, the filters were washed thoroughly on both sides with $2 \times \text{SSC}$ buffer. Finally, the filters were dried overnight at room temperature and counted in a Packard Tri-Carb scintillation counter. The channels were set for simultaneous counting of ^3H and ^{14}C . Corrections for spill-over between the channels were made. The approximate counting efficiencies were 20% for ^3H and 65% for ^{14}C .

RESULTS

Characterization of T4 mRNA synthesized in the presence of putrescine. If regulation of the sequential events following phage infection occurs at the level of transcription, the sequential appearance of "early" and "late" proteins should be paralleled by the sequential production of "early" and "late" mRNA species (9, 10, 12). A convenient and reliable method for estimating the fraction of DNA functioning in transcription at a given time after infection is the hybridization technique. When increasing amounts of RNA are added to a constant amount of DNA, a saturation plateau is attained. This plateau is a measure of the number of different DNA sites to which RNA binds, and from this it is possible

to estimate the fraction of DNA involved in transcription.

The ability of T4 mRNA made in the presence of putrescine to form hybrids with complementary strands of DNA was tested by use of immobilized T4 DNA.

The hybridizability of mRNA preparations formed at "early" or "late" times after infection was compared to the hybridizability of the corresponding mRNA preparations, formed in the presence of putrescine. As shown in Fig. 1, early mRNA formed in the presence of putrescine reaches a low saturation plateau which contains about half of the normal early ^3H RNA. Furthermore, it is clearly shown in Fig. 1 that the same saturation plateaus are attained by "early"

FIG. 1. Results of an attempt to saturate DNA with various radioactive RNA preparations. Increasing amounts of ^3H -RNA were added to 0.33 μg of T4 ^{14}C -DNA. The specific activity of DNA was 260 counts per min per μg . Hybridization was carried out in 2 ml of $6 \times \text{SSC}$ as described (4 hr at 60 C). The results are plotted as $^3\text{H}/^{14}\text{C}$ hybridized versus the amount of input radioactivity. Symbols: Δ , ^3H -RNA prepared by a ^3H -uridine pulse 3 to 5 min after infection at 37 C ("early" mRNA); $\blacksquare$, ^3H -RNA prepared by a ^3H -uridine pulse 3 to 5 min after infection at 37 C in the presence of 100 μmoles of putrescine per ml, added to the bacterial suspension simultaneously with infecting phage; $\circ$, ^3H -RNA prepared by a ^3H -uridine pulse 17 to 19 min ("late" mRNA); $+$, ^3H -RNA prepared by a ^3H -uridine pulse 17 to 19 min in the presence of 100 μmoles of putrescine added at time-zero of the experiment. The experimental background (radioactivity bound to "blank" filters lacking DNA) varied from 0.003 to 0.1% of input radioactivity. This background value has been subtracted from the recorded data.

FIG. 2a. Hybridization of "early" ^3H -mRNA with T4 DNA in the presence of "early" and "late" unlabeled RNA. *Escherichia coli* B was infected by T4 BeJ 42 (multiplicity of infection = 10). ^3H -RNA labeled between the 3rd and 5th min after infection at 37 C was purified on hydroxyapatite columns. In these experiments, increasing amounts of unlabeled homologous and heterologous mRNA were added to a series of hybrid annealing mixtures containing 10 μg of immobilized T4 DNA and a constant amount of labeled mRNA (^3H -RNA input = 11 μg ; specific activity = 500 counts per min per μg). The annealing mixtures were incubated for 24 hr at 60 C, and the amounts of labeled hybrid remaining were assayed as described in Materials and Methods. Unlabeled RNA was prepared as described

mRNA and by "late" mRNA formed in the presence of putrescine. However, in the absence of putrescine, the saturation plateau of "late" mRNA contains 1.5 times more ^3H hybridizable RNA. This result indicates that there are many more sites on the DNA complementary to normal "late" mRNA than to "late" mRNA made in the presence of putrescine or to "early" mRNA.

It is noteworthy that the same efficiency of annealing is displayed by all mRNA preparations tested in the range of excess DNA. (See initial slopes of curves in Fig. 1.)

Competition experiments. The normal pattern of competition between labeled "early" and "late" mRNA preparations and unlabeled homologous and heterologous RNA is shown in Fig. 2a and 2b.

It can be seen in Fig. 2a and 2b that the homologous unlabeled RNA preparations competed more effectively with the corresponding labeled mRNA preparations tested. Thus, hybrid competition experiments could be exploited to

in Materials and Methods, and included all types of RNA extractable from the cell. Symbols: $\circ$, unlabeled RNA isolated 5 min after infection ("Early"); $\square$, unlabeled RNA isolated 19 min after infection ("Late"). The results are plotted as relative amounts of ^3H retained by the DNA filters. A value of 1.0 is equal to 37% of input ^3H .

FIG. 2b. Hybridization of late ^3H -mRNA with T4 DNA in the presence of early and late unlabeled RNA. *Escherichia coli* B was infected by T4 BeJ 42 (multiplicity of infection = 10). ^3H -RNA labeled between the 17th and 19th min after infection at 37 C was purified on hydroxyapatite columns. Annealing mixtures containing 10 μg of immobilized T4 DNA and 15.7 μg of ^3H RNA (specific activity = 294 counts per min per μg) were incubated for 24 hr at 60 C, and the amounts of labeled hybrid retained by filters was assayed as described. Symbols: $\circ$, unlabeled RNA isolated 5 min after infection ("Early"); $\square$, unlabeled RNA isolated 19 min after infection ("Late"). A value of 1.0 = 30% of input ^3H .

FIG. 2c. Hybridization of "late" ^3H -mRNA formed in the presence of putrescine with 10 μg of T4 DNA in the presence of "early" and "late" unlabeled RNA formed in absence of putrescine. *Escherichia coli* B was infected by T4 BeJ 42 (multiplicity of infection = 10) in the presence of 100 μmoles of putrescine per ml. ^3H -RNA labeled between the 17th and 19th min after infection at 37 C was purified on hydroxyapatite columns. Annealing mixtures containing 10 μg of immobilized T4 DNA and 12.5 μg of ^3H -RNA (specific activity = 233 counts per min per μg) were incubated 24 hr at 60 C, and the amounts of labeled hybrid retained by filter was assayed as described. Symbols: $\circ$, unlabeled RNA isolated 5 min after infection; $\square$, unlabeled RNA isolated 19 min after infection. A value of 1.0 = 17% of input ^3H .

determine the nature of mRNA formed in the presence of putrescine. The pattern of competition between labeled 19-min RNA formed in the presence of putrescine and unlabeled 5-min RNA is shown in Fig. 2c. Both unlabeled "early" and "late" preparations compete effectively with "late" mRNA formed in the presence of putrescine.

Comparing Fig. 2c with Fig. 2a and 2b shows that the population of mRNA made between 17 and 19 min in the presence of putrescine is different from the normal population of "late" mRNA. The identity of "late" RNA formed in the presence of putrescine is further elucidated in a competition experiment showing its poor ability to compete with normal ^3H "late" RNA for binding sites on the DNA (see Table 1). Table 1 substantiates the finding that 19-min RNA made in the presence of putrescine contains early mRNA and lacks any detectable amounts of "late" messenger species. It was of interest to test whether all the RNA species transcribed at early times are transcribed also at late times in the presence of putrescine.

Table 2 illustrates the results of a competition experiment in which ^3H early RNA was annealed to T4 DNA in the presence of various nonlabeled RNA preparations. As shown in Table 2, 19-min RNA made in the presence of putrescine was an effective competitor for "early" mRNA, the competition being similar to normal 5-min RNA. These results imply that similar genetic loci are transcribed during 3 to 5 min after infection, and during 17 to 19 min after infection in the presence of putrescine. Furthermore, it is demonstrated in Table 2 that early

TABLE 2. *Relative efficiency of annealing ^3H early RNA to T4 DNA in the presence of various nonlabeled RNA preparations^a*

Vial no.	Nonlabeled RNA	Relative ^3H retained on filter ^b
1	None	1.00
2	5 min, 350 μg	0.18
3	5 min, 350 μg , + putrescine	0.41
4	19 min, 340 μg	0.53
5	19 min, 340 μg , + putrescine	0.18

^a Hybridization mixtures containing a constant amount (11 μg) of ^3H early (5 min) mRNA (input, 35×10^3 counts per min) were annealed to 10 μg of T4 DNA in the presence of various unlabeled RNA preparations for 24 hr at 60 C. Unlabeled early RNA was extracted 5 min after infection. Unlabeled late RNA was extracted 19 min after infection. Unlabeled early or late RNA formed in the presence of 100 μmoles of putrescine per ml was extracted 5 or 19 min after infection, respectively. All RNA preparations were purified on hydroxyapatite columns as described.

The values have been corrected for background radioactivity bound to filters lacking DNA. The precision of these values is approximately $\pm 5\%$, based on the reproducibility of the point.

^b A value of 1.0 = 53% of input radioactivity.

TABLE 1. *Relative efficiency of annealing ^3H late RNA to T4 DNA in the presence of various nonlabeled RNA preparations^a*

Vial no.	Nonlabeled RNA	Relative ^3H retained on filter ^b
1	None	1.00
2	5 min, 355 μg	0.48
3	19 min, 200 μg	0.30
4	19 min, 340 μg , + putrescine	0.57

^a Hybridization mixtures containing a constant amount (15.7 μg) of ^3H late mRNA (input 40×10^3 counts per min) were annealed to 10 μg of immobilized T4 DNA in the presence of various unlabeled RNA preparations for 24 hr at 60 C. Unlabeled RNA preparations were the same as described in the footnote to Table 2.

^b A value of 1.0 = 41% of input radioactivity.

RNA formed in the presence of putrescine is lacking some RNA species (see also Fig. 1).

As demonstrated in the preceding paper (21), there was a significant inhibition of DNA synthesis in the presence of putrescine. Further studies on DNA synthesis under the present experimental conditions were undertaken, to test whether DNA replication is required for the shift from "early" to "late" mRNA synthesis. It is obvious from the results in Fig. 3 that, at the time (19 min at 37 C) of "late" mRNA preparation in the presence of putrescine, DNA synthesis was 90% inhibited. On the other hand, it is clearly demonstrated that, very late after infection (50 min at 37 C) in the presence of putrescine, DNA was synthesized at a normal rate. To test whether DNA synthesis was sufficient for the switch to "late" RNA synthesis, phage-infected complexes grown in the presence of putrescine were pulse-labeled between 50 and 52 min at 37 C. RNA was extracted and analyzed by competition experiments. The results of a typical experiment are shown in Fig. 4. When normal 50 to 52 min ^3H -RNA was tested under the same experimental conditions, the same results were obtained. It is thus demonstrated that "late" mRNA species are synthesized in the presence of the

FIG. 3. ^{14}C -Thymine incorporation into *Escherichia coli* B infected by phage T4 BeJ 42 (multiplicity of infection = 44). *E. coli* B cells were grown in Tris-glucose medium supplemented with 0.05% vitamin-free casein hydrolysate (Nutritional Biochemicals Corp.) up to a concentration of 2×10^8 cells per ml. After washing, the cells (2×10^8 /ml) were infected in the presence of tryptophan for 2 min at 26 C with T4 BeJ 42 (multiplicity of infection = 44). Infected cells were then diluted 10-fold into two parallel Tris-glucose growth tubes, and aerated at 37 C (time-zero). Growth tubes contained ^{14}C -thymine (0.1 $\mu\text{C}/\text{ml}$) and deoxyadenosine (250 $\mu\text{g}/\text{ml}$). Symbols: $\circ$, putrescine-free control; $\triangle$, growth tube containing putrescine, 100 $\mu\text{moles}/\text{ml}$.

diamine as soon as DNA synthesis proceeds normally.

DISCUSSION

Hybridization and competition techniques make it possible quantitatively to detect messenger RNA preparations homologous to specific phage genomes, thus enabling us to detect various categories of T4 mRNA. It is clear that there are at least four different patterns of proteins synthesized after T4 infection (14): very early proteins, intermediate early, and late early proteins; in addition, there is another class of late proteins. The same patterns were shown for the early mRNA species (W. Salser, *personal communication*). The experiments reported here agree with those of other investigators (2, 7, 9, 10) in showing that the RNA species synthesized immediately after infection are present throughout the latent period, and that at least some of them continue to be synthesized throughout the eclipse period (Fig. 2b shows that about 66% of late RNA seems to be identical to early RNA). On the other hand, some early mRNA species are lacking in late RNA preparations (Fig. 2a shows that about 63% of early RNA is lacking in late RNA). Khesin et al. (13) concluded that all early mRNA

species are lacking in late RNA preparations. These discrepancies are probably due to the different hybridization technique used by these authors. The addition of putrescine soon after infection seems to inhibit the synthesis of some category of early mRNA species (see Fig. 1 and Table 2). This result is in accord with the immediate inhibition of RNA synthesis in phage-infected bacteria in the presence of putrescine, as demonstrated in the preceding paper (21).

Results of competition experiments with mRNA formed at late times in the presence of putrescine show a marked relative increase in the abundance of early mRNA species at late times. Furthermore, preferential inhibition of late mRNA synthesis is observed (see Table 1). However, it seems likely that some category of "late" mRNA is formed at 19 min in the presence of putrescine. This may be the reason for the strikingly greater relative competition of unlabeled late RNA with this ^3H late preparation, compared to its competition with ^3H early RNA (Fig. 2c compared to Fig. 2a).

We may suggest, on the basis of the data presented, that putrescine, or a natural metabolite

FIG. 4. Hybridization of 52-min ^3H -RNA with 10 μg of T4 DNA in the presence of early and late unlabeled RNA. *Escherichia coli* B was infected by T4 BeJ 42 (multiplicity of infection = 10) in the presence of 100 μmoles of putrescine per ml. ^3H -RNA labeled between the 50th and 52nd min after infection at 37 C was purified on hydroxyapatite columns. Annealing mixtures containing 10 μg of immobilized T4 DNA and 16.7 μg of ^3H RNA (specific activity = 584 counts per min per μg) were incubated for 24 hr at 60 C, and the amounts of labeled hybrid retained by filters were assayed as described. Symbols: $\circ$, unlabeled RNA isolated 5 min after infection; $\square$, unlabeled RNA isolated 19 min after infection. A value of 1.0 = 30% of input ^3H .

derived from it, might be the factor controlling the synthesis of "early" and "late" mRNA during the latent period.

If late mRNA synthesis is controlled through the inhibition by putrescine, then the rate of late mRNA synthesis should be determined by the intracellular concentration of the diamine. Thus, immediately after infection, when adequate amounts of putrescine are present, "early" proteins are synthesized preferentially (4). Late proteins, on the other hand, are detectable only after maximal leakage (6) of putrescine out of infected cells has occurred. We assume that the differential action of putrescine on mRNA synthesis may be the result of its inhibition of DNA synthesis. The hypothesis that DNA replication acts by quantitatively and qualitatively changing mRNA synthesis has been put forth (17, 19). Our data might offer further support to this basic concept. Furthermore, at early times after infection (3 to 5 minutes at 37 C), when no quantitative change in the amount of DNA has yet occurred, the inhibition of DNA synthesis seems to lead to the inability to transcribe part of the genome. It has been recently shown that T4 mutants which fail to make DNA do not synthesize any of the late mRNA species (W. Salser, *personal communication*). Since the DNA of phage T4 is relatively rich in adenine-thymidine (AT) base pairs, its sensitivity to polyamines which have a special affinity to AT base pairs (15, 16) is not surprising.

The addition of putrescine to T4-infected bacteria, which inhibits DNA synthesis, enables us to stretch out the time scale of sequential mRNA synthesis. Thus, mRNA species normally synthesized 5 min after infection are synthesized 19 min after infection, and those normally synthesized 19 min after infection are synthesized 52 min after infection.

It would be of interest to test the possibility of the existence of some T4 mutants unable to synthesize DNA because of a defect in their polyamine leakage mechanism.

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