DEPENDENCE OF "EARLY" λ BACTERIOPHAGE RNA SYNTHESIS ON BACTERIOPHAGE-DIRECTED PROTEIN SYNTHESIS

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Derepression of the prophage of the bacterial virus λ can be followed by measuring the production of several phage-specific products whose synthesis is low before, but greatly increases at characteristic times after, induction. Viral RNA[1–5] and DNase[6] production begins early, followed by λ DNA synthesis which requires the expression of several λ genes. After λ DNA synthesis and the expression of a maturation gene, lysozyme and the structural proteins of the mature phage appear.[7, 8]

Studies on some of the early genes (defined as those genes expressed before DNA synthesis begins) have revealed that mutations in one of them, gene *N*, not only prevent λ DNA synthesis but greatly depress the level of λ RNA synthesis,[5] and prevent the appearance of λ DNase[9] and an immunologically recognized λ protein.[10]

Recent work indicates that *N* is not the structural gene for λ DNase.[11] Thus it appears that the *N* gene product is necessary for the synthesis of "early" RNA and at least one other "early" protein.

This communication describes the effect of drugs which inhibit protein synthesis, and of mutations in the early genes of the prophage, on viral RNA synthesis. The results are interpreted to mean that a viral protein, or proteins (inducing proteins), of which the *N* gene product is one, is necessary to allow the synthesis of the majority of λ RNA.

Experiments designed to measure the functional stability of these inducing proteins are described and interpreted to indicate a half life of less than three minutes. The relevance of these conclusions to recently proposed models for the control of λ RNA synthesis is discussed.[12–14]

Materials and Methods.—Bacteria: The bacterial strain used is a streptomycin-resistant derivative of *E. coli* K_{12} W3350, which is nonpermissive (Su^-) for suppressible amber mutants.[15]

Bacteriophages: λ C857 was isolated and described by Sussman and Jacob.[16] λ *sus* 96A (in gene *N*), λ *sus* 29 (in gene *O*), and λ *sus* 3 (in gene *P*) were isolated and described by Campbell.[15]

Medium: M56 is a potassium phosphate minimal medium with glucose as the carbon source.[17]

RNA extraction: Five ml of cells at a typical concentration of 2×10^8/ml, which had been exposed to H^3 uracil for a period of time of 0.5–2 min, were diluted into 5 ml of chilled medium containing 2×10^{-2} *M* sodium azide. The cells were centrifuged, resuspended in 2 ml of 10^{-2} *M* tris·HCl, pH 7.5, and 10^{-3} *M* EDTA, and lysed by the addition of 0.1 ml of 10% SDS and incubation for 10 min at 45°C. The protein was removed by extraction with an equal volume of phenol saturated with 0.1 *M* tris·HCl, pH 7.8, and the aqueous phase was freed of dissolved phenol by 4 extractions with 2 volumes of water-saturated ether. The ether was removed by a stream of dry N_2, the nucleic acid extract was then made 10^{-2} *M* in $MgCl_2$, and 20 μg of DNase (Worthington Biochemical Co., electrophoretically purified) was added. After an incubation at 37°C

for 10 min, the DNase was removed by extraction with an equal volume of buffer-saturated phenol, and the aqueous phase containing the RNA was divided into three aliquots. The RNA in one aliquot was precipitated with cold 0.6 *M* trichloroacetic acid to determine the total H^3-RNA present.

RNA-DNA hybridization: The method is essentially that described by Nygaard and Hall.[18] One 0.5-ml aliquot of RNA, isolated as described above, was mixed with 1.0 ml of denatured λ DNA at a concentration of 10 μg/ml, and incubated 1 hr at 76°C. The mixture was cooled slowly and 8.5 ml of H buffer containing 20 μg of RNase was added. After an incubation of 10 min at 37°C, the solution was filtered through a Millipore filter (2.5-cm diameter, 0.45-μ pore diameter), which was washed with five 10-ml aliquots of buffer. As a control, 0.5 ml of each RNA sample was also incubated at 76°C with 1 ml of buffer and treated as above. The radioactivity which bound to this filter (0.03–0.1%) was subtracted from that bound after hybridization with DNA.

The efficiency of hybridization with this procecure was found to be 25% when λ RNA was used that had been synthesized *in vitro* on λ DNA templates by purified *E. coli* DNA-dependent RNA polymerase.

Results.—(a) *The effect of drugs on the synthesis of λ RNA:* Protein synthesis in certain strains of *E. coli* is partially resistant to puromycin; however, depriving the bacterial cells of Mg^{++} ions by treatment with EDTA renders them fully sensitive to the drug, presumably by increasing the permeability of the cell wall.[19, 20]

Figure 1*A* shows the effect of various concentrations of puromycin on the kinetics of induction of β-galactosidase, while Figure 1*B* shows the synergistic effect of EDTA on the inhibition of enzyme synthesis. It is seen here that in this medium 50 μg/ml puromycin + 0.002 *M* EDTA is as effective as 200 μg/ml puromycin in the absence of EDTA.

To ensure inhibition of enzyme synthesis which might be less sensitive to the action of puromycin than the synthesis of β-galactosidase, 200 μg/ml puromycin plus 0.002 *M* EDTA was chosen for the following experiments.

Induction, or derepression, of the genes of the λ prophage was studied by measuring the rate of λ RNA synthesis after heating a mutant which produces a temperature-sensitive repressor or *CI* gene protein. These are, however, two complications to a quantitative analysis. After phage DNA synthesis starts, the number of gene copies is increasing, and thus the total rate of λ RNA synthesis must be divided by the number of λ DNA molecules to obtain the derepression per gene copy (assuming all λ DNA molecules are equally active as templates for RNA synthesis). Furthermore, after λ DNA synthesis begins, a new class of λ RNA expressing those genes needed late in vegetative growth is also synthesized.[2, 14, 21–23]

Both of these complications should be absent during the temperature induction of a mutant prophage producing a temperature-sensitive *CI* gene product and containing a *sus* mutuation in gene *P*, since the product of this gene is necessary for λ DNA synthesis. To the extent that the synthesis of the *P* gene protein is actually blocked, all phage RNA seen in a lysogen of this type would then be "early" RNA.

The kinetics of increase in the rate of RNA synthesis after heating such a mutant, along with the rate of bacterial host messenger RNA (mRNA) synthesis are shown in Figure 2. It can be seen that the rate of λ RNA synthesis greatly

increased within three minutes after the temperature increase, while the rate of bacterial RNA synthesis remained relatively constant. However, treatment of the lysogen with EDTA and puromycin ten minutes prior to the increase in temperature suppressed the induction of λ RNA synthesis by a factor of 10, while leaving the rate of bacterial messenger RNA synthesis unaffected.

Chloramphenicol is another drug which specifically inhibits protein synthesis. Figure 3 shows that induction of λ RNA synthesis is greatly inhibited by the addition of chloramphenicol ten minutes before the increase of temperature.

(b) *Induction of various suppressible mutants of* λ: Figure 4 shows the kinetics of induction of λ RNA synthesis in four different lysogens. All the prophages in these lysogens produce the temperature-sensitive repressor, and three contain in addition a *sus* mutuation in either the *N*, *O*, or *P* gene. The most striking fact to be seen here is that the *sus* mutation in the *N* gene has caused the rate of λ RNA synthesis to be more than ten times lower than the rate in the other

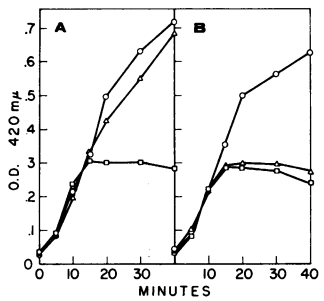

FIG. 1.—Inhibition of enzyme synthesis by puromycin. The induction of β-galactosidase synthesis in cells growing in minimal medium was accomplished at time zero by the addition of 10^{-4} moles per liter of isopropyl-thiogalactoside. Aliquots of about 10^8 cells were taken at the indicated times, mixed with 0.1 ml toluene, and incubated with 2.5 ml of phosphate buffer containing 0.8 gm/liter of o-nitrophenyl β-D-galactoside, for 30 min at 37°C; 1.5 ml of 0.5 *M* Na_2CO_3 was then added and the O.D. at 420 mμ measured.

(*A*) ⊙—⊙—⊙, No puromycin; △—△—△, 50 μg/ml puromycin added at 10.5 min; ⊡—⊡—⊡, 200 μg/ml puromycin added at 10.5 min.

(*B*) ⊙—⊙—⊙, 0.002 Moles/liter EDTA added at 10.5 min; △—△—△, 0.002 moles/liter EDTA + 50 μg/ml puromycin added at 10.5 min; ⊡—⊡—⊡, 0.002 moles/liter EDTA + 200 μg/ml puromycin added at 10.5 min.

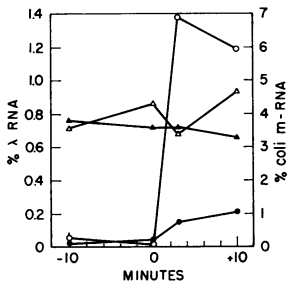

FIG. 2.—The effect of puromycin on the synthesis of host RNA and "early" λ RNA. An aliquot of 1.3 ml of a culture of cells lysogenic for λ C857 *sus P*, growing exponentially in minimal medium at 33°C at a titer of about 3×10^8 cells per ml, was diluted into 3.7 ml of medium at the same temperature but containing 5 μc of H^3 uracil, (at a typical specific activity of 3000 c/mole). Incorporation of the isotope was continued for 2 min, at which time the cells were diluted into 5ml of chilled minimal medium containing 0.02 *M* NaN_3 and the fraction of labeled RNA hybridizable with denatured λ or *E. coli* DNA determined as described in the *Materials and Methods* section.

Puromycin, 200 μg/ml, and 0.002 moles/liter EDTA was added to the remainder of the culture and another 1.3-ml aliquot was taken after 10 minutes. The remainder of the culture was diluted into an equal volume of medium at 57°C (time zero) and incubation continued at 45°C. Aliquots of 2.5 ml were taken at 3 and 10 min after the temperature increase, and added to 2.5 ml of H^3 uracil-containing medium at 45°C and then treated as were the other samples.

△—△—△, *E. coli* mRNA; ⊙—⊙—⊙, λ RNA; ▲—▲—▲, *E. coli* mRNA in the presence of puromycin; ●—●—●, λ RNA in the presence of puromycin.

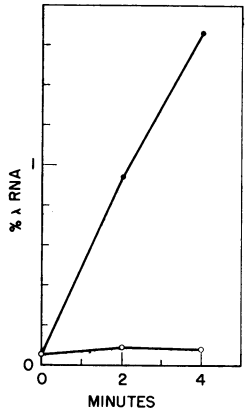

FIG. 3.—The effect of chloramphenicol on the induction of "early" λ RNA synthesis. The temperature of a culture of cells lysogenic for λ C857 *sus P* growing at 33°C was increased to 45°C at time zero. Aliquots were taken just before the temperature increase and after 2 and 4 min at 45°C. The rate of λ RNA synthesis was measured as in Fig. 2.

●—●—●, λ RNA synthesis; ⊙—⊙—⊙, λ RNA synthesis in a culture to which 50 μg/ml chloramphenicol had been added 10 min before the temperature increase.

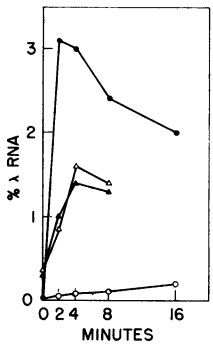

Fig. 4.—The effect of mutations in genes *N*, *O*, and *P* on the rate of λ RNA synthesis. Aliquots of four separate cultures of lysogenic cells were taken just before and at the indicated times after an increase in temperature from 33°C to 45°C and the rate of λ RNA synthesis measured as before.

Cells lysogenic for λ C857, ●—●—●; λ C857 *sus N*, ⊙—⊙—⊙; λ C857 *sus O*, △—△—△; λ C857 *sus P*, ▲—▲—▲.

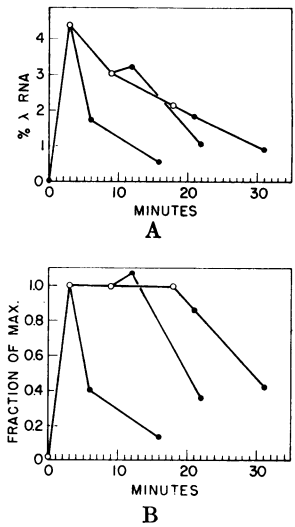

FIG. 5.—The decrease in the rate of λ RNA synthesis after cooling. The temperature of a culture of cells lysogenic for λ C857 was increased from 33 to 45°C. Aliquots were taken at 3, 9, and 18 min and immediately cooled to 33°C by the addition of an equal volume of cool medium. The rate of λ RNA synthesis was measured in each of the cooled cultures 3 and 13 min after cooling.

(*A*) λ RNA synthesis at the high temperature, ⊙—⊙—⊙; RNA synthesis at the low temperature, ●—●—●. (*B*) The relative rate of λ RNA synthesis in the three cooled cultures, with the rate at the time of cooling set at 1.0.

lysogens. Thus it would appear that the *N* gene product is necessary for the synthesis of at least the majority of the "early" RNA.

It is also seen that the presence of *sus O* or *sus P* has reduced the rate of λ RNA synthesis by approximately a factor of 2 from that of the wild type. The effect of these two mutations, while smaller than the effect of the mutation in gene *N*, would, however, indicate that even at early times the lack of the *O* or *P* gene product can diminish λ RNA synthesis.

(c) *The reversibility of heat denaturation of the C857 product:* The reversibility of the induction of λ RNA synthesis in three lysogens all containing the C857 mutation and either the wild-type genes, *sus O*, or *sus P* is seen in Figures 5*A*, 6*A*,

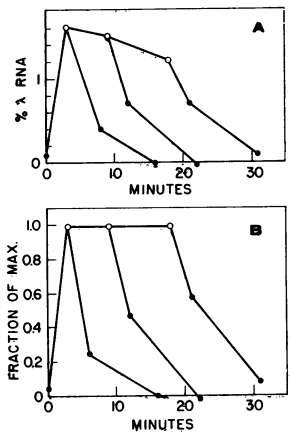

FIG. 6.—The decrease in the rate of λ RNA synthesis after cooling cells lysogenic for λ C857 *sus Q*. The experiment was performed as described in Fig. 5.

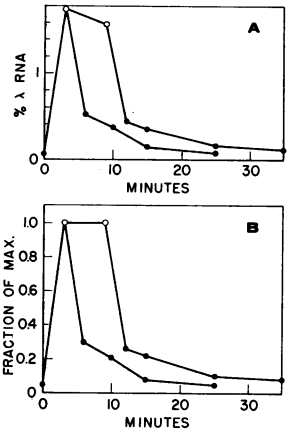

FIG. 7.—The decrease in the rate of λ RNA synthesis after cooling cells lysogenic for λ C857 *sus P*. The experiment was performed as described in Fig. 5, except that two aliquots were cooled at 3 and 9 min after the temperature increase and the rate of λ RNA synthesis followed in each aliquot for a longer time after cooling.

and 7*A*. The temperature of the lysogenic cultures was increased and aliquots of the warm cultures were taken at various times and cooled to the original low temperature. At times throughout this program smaller aliquots of the culture were exposed to H^3 uracil to determine the rate of λ RNA synthesis. It is clear that after the temperature is reduced, the rate of λ RNA synthesis decreases as has been reported previously by other workers.[14] In these experiments as well as those shown previously in Figure 4, the rate of λ RNA synthesis rapidly increases and then decreases, even while the culture remains at the high temperature. In order to focus attention on the decrease due only to the decrease in temperature, the data have been normalized so that the rate of λ RNA synthesis of each aliquot is expressed as the fraction of the rate at the time it was cooled.

(d) *The functional half life of the inducing proteins:* Using the fact shown in the previous section, that the heat denaturation of C857 repressor is readily reversible, an experiment was designed to measure the functional half life of the inducing proteins. The temperature of a culture of lysogens producing the C857 temperature-sensitive repressor and containing a *sus* mutation in gene *P* was increased for a time sufficient to turn on early RNA synthesis maximally. The culture was then cooled to renature the *CI* product and chloramphenicol was added simultaneously to prevent synthesis of additional inducing proteins.

Aliquots of the cooled culture were then taken at various times and again heated (still in the presence of chloramphenicol) to denature the *CI* product, and the rate of λ RNA synthesis was measured. The length of time after cooling during which an increase in temperature could again induce λ RNA synthesis would be a measure of the stability of the inducing proteins. In Figure 8*B*,

where the results of this experiment are presented, it is seen that the half life must be less than eight minutes, since the cool lysogens are not able to resume λ RNA synthesis when heated after this period. The behavior of lysogens subjected to the same temperature program, but not treated with chloramphenicol, is presented in Figure 8*A*, where it can be seen that the second increase in temperature produces the same increase in rate of λ RNA synthesis as the first temperature increase.

If at least one of the inducing proteins is indeed unstable, as the preceding results would suggest, the addition of chloramphenicol to a heat-induced culture would be expected to decrease the rate of λ RNA synthesis even while the culture remains at the elevated temperature, since once protein synthesis is stopped by the drug, the concentration of the unstable proteins should decrease due to their short half lives, causing a progressive decrease in the rate of λ RNA synthesis.

The results of such an experiment are presented in Figure 9, where it is seen that addition of chloramphenicol to heat-induced lysogens does result in a progressive decrease in the rate of "early" λ RNA synthesis.

Discussion.—The results presented here indicate that during the induction of a lysogen at least one λ-specific protein must be made before the major part of "early" λ RNA synthesis can begin. Since a mutation in only one of the three known early genes, gene *N*, depresses RNA synthesis to the same level as do drugs which inhibit all protein synthesis, it seems likely that the protein required is specified by the *N* gene. This same conclusion has been made by other workers;[5] however, the use here of a temperature-sensitive repressor allows it to be inactivated almost instantaneously and the initial kinetics of λ RNA synthesis then reveal the striking difference between the almost absolute requirement for the *N* gene protein on the one hand, and the relatively smaller effect of a defective *O* or *P* gene protein on the other. It remains to be seen whether the lower rate of λ RNA synthesis that is observed with mutations in gene *O* or *P* is due to the failure to make a specific class of RNA, or to a general depression of all the classes of λ RNA synthesis characteristic of this time. Previous work[22, 24] has shown that at late times after infection or induction, less RNA is synthesized by *O* and *P* mutants than by the wild type, but the RNA made by the mutants seems to be of the same class normally made at this late time (complementary to the left half of the strand of DNA rich in poly G binding sites).

This synthesis of late RNA is perhaps not surprising since the block in synthesis of late protein and even mature virus particles seems to be incomplete in these mutants.[24] A mutation in gene *O* or *P* did not affect the initial rate of viral RNA synthesis after infection of sensitive cells.[24]

No proof is offered here that the effect on RNA synthesis of a *sus* mutation in gene *N* might not be due to an extreme polar effect on translation with a subsequent effect on the level of RNA, as has been reported for a few mutations in the *lac* operon.[25] However, it is believed that the *N* gene is transcribed from right to left,[26] and since the mutation used in this study maps in the left half of the gene,[27] it would not be expected to exhibit a polar effect.[28]

Several previous investigations have failed to find an inhibition of "early" λ RNA synthesis when chloramphenicol was added to a culture of lysogens at the

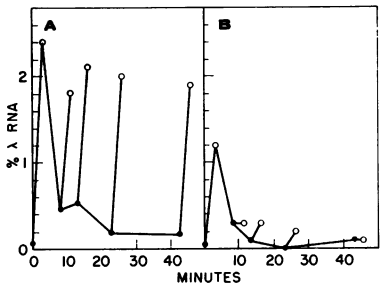

FIG. 8.—λ RNA synthesis when chloramphenicol is present during a second increase in temperature. The temperature of a culture of cells lysogenic for λ C857 *sus P* was increased from 33 to 45°C and kept at the higher temperature 3 min and then immediately cooled to 33°C in the same medium (*A*) or in medium containing 50 μg/ml chloramphenicol (*B*). Aliquots were then taken at 8, 13, 23, and 43 min and immediately brought to 45°C again in minimal medium (*A*) or in medium containing 50 μg/ml chloramphenicol (*B*). After 3 min at the high temperature, the rate of λ RNA synthesis was measured. Cultures at 33°C, ●—●—●; cultures at 45°C, ⊙—⊙—⊙.

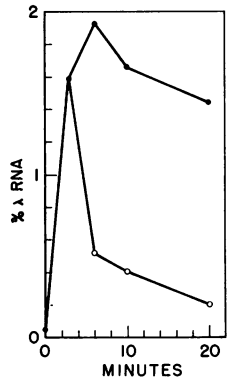

FIG. 9.—The depression of λ RNA synthesis at a high temperature by the addition of chloramphenicol. The temperature of two aliquots of a culture of cells lysogenic for λ C857 *sus P* was increased from 33 to 45°C and after 3 min 50 μg/ml of chloramphenicol was added to one aliquot. The rate of λ RNA synthesis was measured at 0, 3, 6, 10, and 20 min after the temperature increase. Cells in minimal medium, ●—●—●; cells in medium containing50 μg/ml chloramphenicol, ⊙—⊙—⊙.

same time as the temperature was increased.[2, 14, 29] It can only be suggested that events can occur rapidly after inactivation of the repressor, and thus the drug must be added to the cells sometime *before* the temperature increase so that all protein synthesis is stopped before induction is started. On the other hand, Skalka *et al.*[24] have recently observed a depression of "early" RNA synthesis after viral infection of chloramphenicol-treated cells.

The necessity of the *N* gene protein for λ RNA synthesis has suggested that the *CI* product might exert its direct control only on the *N* gene, and indeed evidence has been reported to this effect.[13] The reversible effect of temperature with the mutant used here on λ RNA synthesis would require that the *N* gene product be functionally unstable for the above theory to be maintained. The demonstration that at least one inducing protein has a short half life makes the theory a possible hypothesis again, although of course it does not prove that the *CI* product acts only on the *N* gene.

A model in which the repressor acted only on the *N* gene would be consistent with the observation that the λi^{434} hybrid phage (which has only a small segment of its genome around the neighboring *CI* and *N* genes derived from the similar but heteroimmune phage 434) is insensitive to the λ repressor.[4] Furthermore, recent direct observations indicate the the *CI* product binds to the λ DNA only in this

same small region.[30] However, these last two observations would be just as consistent with the "early" genes being organized into two large operons with their operators in the i^{434} segment, and thus being read in opposite directions away from this region. If the repressor can directly shut off all the early genes, the role of the *N* gene protein might be to multiply the effect of the repressor, such that the rate of RNA synthesis would be inversely proportional to a higher power of the repressor concentration than if the repressor acted alone.

Summary.—Studies on the effect of drugs which specifically prevent protein synthesis, as well as the behavior of mutants, indicate that the *N* gene protein is necessary for the synthesis of λ messenger RNA.

Evidence is also presented to show that the functional half life of this protein is less than three minutes.

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Abbreviations: *sus* = suppressible; tris = tris (hydroxymethyl) aminomethane; EDTA = ethylenediaminetetracetate; SDS = sodium dodecylsulfate.

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