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Expression of the *rel* Gene during R17 Phage Infection*

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ABSTRACT: Phage RNA synthesis has previously been reported to be under the influence of the *rel* gene of the host. It has also been suggested that guanosine 5′-diphosphate 3′- or 2′-diphosphate (ppGpp) is an inhibitor of host RNA synthesis, and may inhibit phage RNA synthesis as well. The effect of isoleucine starvation on the accumulation of ppGpp and the relative rate of phage RNA synthesis was compared in a stringent and a relaxed host infected with R17 RNA phage under conditions where host RNA and protein synthesis were inhibited by the addition of rifampicin. ppGpp was accumulated in the infected stringent but not in the infected relaxed cells upon isoleucine starvation. Since in the presence of rifampicin no ppGpp was formed in the uninfected host, the accumulation of ppGpp in the infected host must be phage specific. Phage RNA synthesis was not decreased in either a stringent or a relaxed host under these conditions. Therefore phage RNA synthesis is not influenced by the *rel* gene of the host in the same manner as is host RNA itself, nor is the accumulation of ppGpp dependent upon a decrease in RNA synthesis.

Amino acid starvation or restricted aminoacylation of tRNA causes a severe reduction of RNA accumulation (termed the stringent response) and the rapid accumulation of two guanosine nucleotides (MS I and MS II) in stringent (*rel*$^+$), but not relaxed (*rel*) strains of *Escherichia coli* (Cashel and Gallant, 1969; Cashel, 1969). Cashel and Kalbacher (1970) identified MS I as guanosine 5′-diphosphate 3′- or 2′-diphosphate (ppGpp). MS II, whose structure is not known, is produced in lesser amounts and is not characteristic of all stringent strains.

Travers *et al.* (1970b) have discovered a factor, ψ, which preferentially stimulates the synthesis of *E. coli* ribosomal RNA *in vitro*. This factor is found as a component of Qβ RNA phage replicase. This suggests the existence of similar regulatory mechanisms involving the factor for the synthesis of ribosomal and viral RNA. In fact, Travers *et al.* (1970a) demonstrated that ppGpp inhibits *in vitro* both ψ-stimulated RNA synthesis by bacterial RNA polymerase and poly(G) synthesis by Qβ replicase in a quantitatively similar manner.

Attempts to examine the effect of the *rel* gene on phage RNA synthesis *in vivo* have yielded conflicting results. Friesen (1969) and Khan and Yamazaki (1970) reported that the incorporation of radioactive uracil into phage RNA is influenced by the allelic state of the *rel* gene in a manner similar to that of the host. On the other hand, Siegel and Kjeldgaard (1971), using a spheroplast assay for infectious RNA, reported that the synthesis of Qβ phage RNA continues in a similar manner in both stringent and relaxed hosts deprived of a required amino acid. Therefore, there exists uncertainty concerning the relationships between the *rel* gene, ppGpp formation, and RNA synthesis during RNA phage infection.

In the present work we have examined the effect of amino acid starvation on ppGpp formation during R17 infection of a stringent and a relaxed host, and determined the rates of phage RNA synthesis under these conditions.

Materials and Methods

Bacteria, Phage, and Culture Conditions. Male strains, CP78 F$^+$ (*rel*$^+$) and CP79 F$^+$ (*rel*), were derived from CP78 and CP79 (Fiil and Friesen, 1968) by S. R. Khan in our laboratory. Both require arginine, histidine, leucine, threonine, and thiamine, and are isogenic except at the *rel* locus. F74 (F$^+$, *rel*$^+$, *met*, *his*) and F8 (F$^+$, *rel*, *met*, *his*, *thy*) were kindly provided by J. D. Friesen, York University, Toronto, Canada. A male specific RNA phage, R17, purified as described previously (Enger *et al.*, 1963), was used throughout.

For bacterial growth, Tris–maleate synthetic medium designated as TMM (Paranchych, 1966) was used throughout. Supplementation consisted of each required amino acid (a final concentration of 50 μg/ml) and thiamine (10 μg/ml). Bacteria were grown at 37° on a gyrotory water-bath shaker. Cell density was measured by absorbance at 500 nm (A_{500}) by means of a Bausch and Lomb spectrophotometer.

Chemicals. Rifampicin was purchased from Calbiochem;

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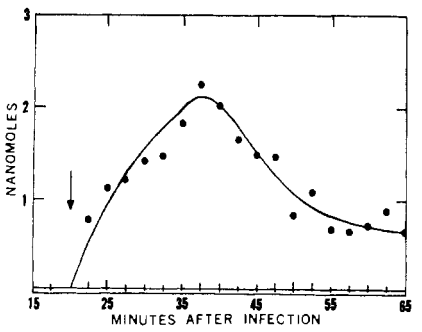

FIGURE 1: Accumulation of ppGpp in R17 infected cells during isoleucine starvation. CP78 F^+ (*rel*$^+$) and CP79 F^+ (*rel*) cultures prelabeled with [^{32}P]phosphate as described in Materials and Methods were infected at A_{500} = 0.4 with R17 phage at a multiplicity of 5 plague-forming units per cell (zero time). At 15-min postinfection, rifampicin was added to a final concentration of 500 μg/ml. At 20-min postinfection, valine was added to a final concentration of 500 μg/ml to cause isoleucine starvation (Leavitt and Umbarger, 1962). Samples were taken at the times indicated and assayed for ppGpp as described in Materials and Methods. The amounts of phosphate incorporated into ppGpp were calculated as nanomoles of phosphate per milliliter of culture at A_{500} = 1.0. (●) CP78 F^+ in the presence of valine (isoleucine starvation); (–) levels of ppGpp produced by CP78 F^+ without valine or with valine plus chloramphenicol (100 μg/ml) and by CP79 F^+ with or without valine.

chloramphenicol, from Sigma Chemical Co.; amino acids, from Nutritional Biochemicals; [^{14}C]uracil (47 mCi/mmole) and [^{32}P]orthophosphoric acid, from International Chemical and Nuclear Corp.; [^{3}H]2-adenine (23 Ci/mmole), from Amersham–Searle Corp.; polyethyleneimine–cellulose thin-layer plates (MN-polygram cel 300 PEI), from Brinkmann Instruments.

Assay of ppGpp. Carrier-free [^{32}P]orthophosphate was added to the cultures to a final concentration of 50–200 μCi/ml at A_{500} = 0.3, which was at least 30 min before the start of the experiments. The concentration of phosphate ions in TMM was 10^{-3} M. At intervals indicated in the figures, samples were withdrawn and assayed for MS I by formic acid extraction and thin-layer chromatography as described previously (Cashel *et al.*, 1969).

Assay of Labeled RNA. For cumulative labeling of RNA, aliquots of the cultures were mixed with one-tenth the volume of a [^{14}C]uracil labeling mixture, which contained per ml: 5 μCi of [^{14}C]uracil, 50 μg of unlabeled uracil, and 200 μg of cytosine, or with a [^{3}H]adenine labeling mixture which contained per ml: 5 μCi of [^{3}H]adenine and 10 μg of unlabeled adenine. For the [^{14}C]uracil labeling, 0.1-ml portions of the culture were removed at intervals and assayed for labeled RNA (cold trichloroacetic acid insoluble radioactivity) by the filter paper disk method (Bollum, 1968). When the [^{3}H]-adenine mixture was used, an additional 0.1-ml sample was removed and treated with 0.5 N NaOH to estimate the amount of incorporation into DNA (Roodyn and Mandel, 1960). This amount was subtracted from the amount of incorporation into cold trichloroacetic acid insoluble radioactivity to give incorporation of label into RNA.

Relative Rates of RNA Synthesis. Relative rates of RNA synthesis were determined by the method of Lazzarini and Dahlberg (1971), using [^{3}H]2-adenine as described by Gallant *et al.* (1970). To determine the relative specific activity of the ATP pool, a culture was prelabeled for at least 30 min with 100 μCi/ml of [^{32}P]phosphate, and aliquots were pulse labeled with [^{3}H]2-adenine (4 nmoles/ml, 23 Ci/mmole) for 1 min. Nucleotides were then extracted with 1 N formic acid and chromatographed one dimensionally in 0.75 M KH_2PO_4 (Cashel *et al.*, 1969). The ATP spots were cut out and counted in a Packard Tri-Carb scintillation counter using channel settings which minimize the cross-contamination of ^{3}H and ^{32}P. The rate of incorporation of [^{3}H]adenine into RNA was determined by pulse labeling an aliquot of unlabeled culture with [^{3}H]adenine for 1 min and measuring total trichloroacetic acid precipitable counts and the counts incorporated into DNA (Roodyn and Mandel, 1960). The counts incorporated into DNA were subtracted from total trichloroacetic acid precipitable counts to give incorporation into RNA. This value was then divided by the relative specific activity (^{3}H : ^{32}P ratio) of the ATP pool at the same interval to give the relative rate of RNA synthesis.

Results

Formation of ppGpp in Infected Cells. The addition of the antibiotic rifampicin to the infected culture allows only RNA phage-specific macromolecular biosynthesis (Fromageot and Zinder, 1968) due to the specific inhibition of host RNA synthesis (Wehrli *et al.*, 1968). The addition of rifampicin (500 μg/ml) at 15 min postinfection does not substantially affect replication of R17 phage RNA for at least 15 min (J. D. Rothwell, personal communication), while nearly complete inhibition of host-specific RNA synthesis is achieved within 5 min. Thus, this experimental condition was used in the following experiments. Figure 1 shows the effect of isoleucine starvation on the rifampicin-treated R17 infected cells. Isoleucine starvation was caused by the addition of valine, which acts as a feedback inhibitor of the isoleucine–valine biosynthetic pathway (Leavitt and Umbarger, 1962). It has previously been shown that the addition of valine causes a similar reduction in the rate of accumulation of phage proteins in both stringent and relaxed hosts (Khan and Yamazaki, 1970). In the stringent host, CP78 F^+, the level of ppGpp rose gradually to a peak at 15–20 min after the addition of valine and subsequently declined. The autoradiogram indicated a simultaneous accumulation of smaller amounts of MS II (data not shown). When chloramphenicol (100 μg/ml) was added along with the valine, no measurable amounts of MS compounds were formed (Figure 1). In the relaxed host, CP79 F^+, no measurable amounts of MS compounds were formed with or without isoleucine starvation.

The same experiments, performed using another pair of stringent and relaxed hosts, F74 and F8, yielded similar results.

To allow a quantitative evaluation of the present data, the effect of isoleucine starvation on the uninfected stringent host without rifampicin was studied. Figure 2 shows that upon addition of valine the level of ppGpp rose sharply, then remained constant for the duration of the experiment. It is noted that this level was significantly less than the maximal level of ppGpp produced in the infected stringent host (Figure 1). Also, the kinetics of ppGpp production were different from those of the infected cell, in which ppGpp accumulated more slowly to the maximum level and then declined.

The second control experiment was performed to examine the effect of rifampicin on ppGpp accumulation in the uninfected stringent host. The experimental conditions were the same as described in Figure 1 except that the uninfected host was used. Rifampicin was added to the [^{32}P]phosphate prelabeled CP78 F^+ culture 5 min prior to the addition of valine. As has been previously reported by Wong and Nazar

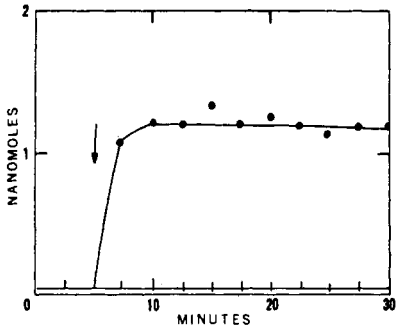

FIGURE 2: Accumulation of ppGpp in an uninfected stringent strain during isoleucine starvation. A CP78 F$^+$ (*rel*$^+$) culture prelabeled with [^{32}P]phosphate was divided into two portions at A_{500} = 0.4 (zero time). To one portion, rifampicin was added to a final concentration of 500 μg/ml. Valine (500 μg/ml) was added 5 min later to cause isoleucine starvation. Samples taken before and after the addition of valine were assayed for ppGpp as described in Materials and Methods. The amounts of phosphate incorporated into ppGpp were calculated as nanomoles of phosphate per milliliter of culture at A_{500} = 1.0. (●) Untreated culture with valine; (−) levels of ppGpp produced in the untreated culture without valine, and the rifampicin-treated cultures with and without valine.

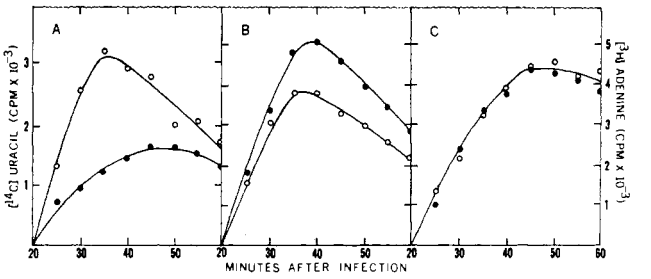

FIGURE 3: Effects of isoleucine starvation on the incorporation of [^{14}C]uracil and [^{3}H]adenine into phage RNA. CP78 F$^+$ (*rel*$^+$) and CP79 F$^+$ (*rel*) cultures were infected with R17 phage and treated with rifampicin as described in Figure 1. At 20-min postinfection valine was added to a final concentration of 500 μg/ml to cause isoleucine starvation and the culture exposed to either a [^{14}C]uracil- or a [^{3}H]adenine-labeling mixture. Samples were taken at 5-min intervals thereafter and assayed for labeled RNA as described in Materials and Methods. Part A shows [^{14}C]uracil incorporation in infected CP78 F$^+$ cells, parts B and C show [^{3}H]adenine incorporation into infected CP78 F$^+$ and CP79 F$^+$ cells, respectively. (●) Plus valine; (○) minus valine.

(1970), no detectable amounts of ppGpp and MS II were formed under these conditions. These results have been incorporated into Figure 2.

The accumulation of ppGpp occurred in the R17-infected stringent cells during isoleucine starvation under conditions where host RNA and protein biosynthesis were severely inhibited by the addition of rifampicin. Since ppGpp production by the host alone does not occur in the presence of rifampicin, the accumulation of ppGpp in the infected host must be phage specific.

Phage RNA Synthesis. Friesen (1969) and Khan and Yamazaki (1970) have used the incorporation of radioactive uracil into phage RNA as a measure of RNA accumulation during amino acid starvation of stringent and relaxed infected cells. However, Lazzarini and Dahlberg (1971) have shown that the rate of entry of radioactive uracil into the UTP pool is drastically reduced during amino acid starvation of stringent strains, and such a phenomenon may also occur in an infected stringent cell. Since the rate of entry of radioactive adenine into the ATP pool is less susceptible to this effect of amino acid starvation (Lazzarini and Dahlberg, 1971), the incorporation of this precursor into phage RNA in infected stringent and relaxed cells was compared with that of [^{14}C]uracil (Figure 3A,B). In the stringent host, [^{14}C]uracil incorporation into phage RNA was substantially reduced by isoleucine starvation, whereas [^{3}H]adenine incorporation was actually increased. No effect of isoleucine starvation was observed in the relaxed strain (Figure 3C). It is obvious that the choice of label would greatly influence any interpretation of the effect of amino acid starvation on phage RNA accumulation.

To circumvent any specific activity changes occurring during amino acid starvation we have measured relative rates of RNA synthesis by the method of Lazzarini and Dahlberg (1971). Rates of RNA synthesis were measured by pulse-labeling RNA with [^{3}H]adenine and correcting for the specific activity of the ATP pool.

It has been reported (Khan and Yamazaki, 1970) that degradation of preformed phage RNA begins at about 35 min postinfection under our conditions (Figure 3B). The mechanism of this degradation will be reported elsewhere. For this reason we have determined the rates of RNA synthesis for only 16 min after rifampicin addition, during which time the major part of phage RNA synthesis occurs.

Figure 4A shows the effect of isoleucine starvation on the relative specific activity of the ATP pool in the infected stringent and relaxed cells. A slow decline in specific activity occurred using the relaxed cells with and without isoleucine starvation, and a similar decline occurred in the unstarved stringent cells. When the stringent cells were starved for isoleucine

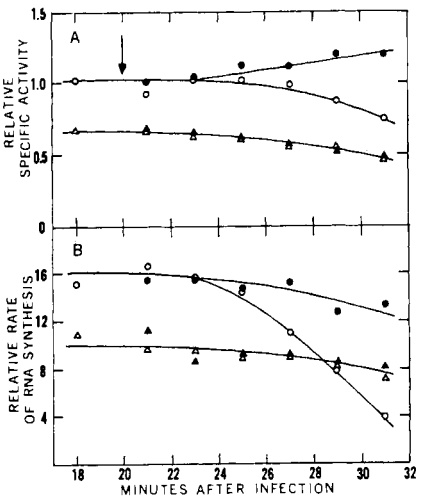

FIGURE 4: Effects of isoleucine starvation on the relative specific activities of the ATP pools, and relative rates of phage RNA synthesis. Unlabeled and [^{32}P]phosphate-prelabeled CP78 F$^+$ (*rel*$^+$) and CP79 F$^+$ (*rel*) cultures were infected with R17 phage and treated with rifampicin with or without valine as described in Figure 1. In part A, aliquots were removed from the [^{32}P]phosphate-prelabeled culture at the indicated intervals and mixed with [^{3}H]adenine. After 1 min an equal volume of 2 M formic acid was added, and the relative specific activity of the ATP pool determined as described in Materials and Methods. In part B, aliquots were removed from the unlabeled culture at the indicated intervals and mixed with [^{3}H]-adenine. After 1 min, samples were removed for assay of [^{3}H]-adenine incorporation into RNA as described in Materials and Methods. The incorporation into RNA at each interval was divided by the corresponding relative specific activity from part A to obtain the relative rate of RNA synthesis. (○) CP78 F$^+$ without valine; (●) CP78 F$^+$ with valine; (△) CP79 F$^+$ without valine; (▲) CP79 F$^+$ with valine.

a slow increase in specific activity resulted. These slow specific activity changes are in contrast with the marked decrease which occurs in the starved host (Lazzarini and Dahlberg, 1971). There was also a significant difference between the overall specific activity of the ATP pools of the stringent and the relaxed cells during infection.

The corrected relative rates of RNA synthesis are shown in Figure 4B. Without isoleucine starvation the rate of RNA synthesis declined sharply in the infected stringent host starting at about 24-min postinfection. When isoleucine starvation was imposed the rate of RNA synthesis was actually stimulated over that of the control. Obviously, a so-called stringent response did not occur in the infected host. Therefore, the [^{3}H]adenine incorporation data (Figure 3B) is a better representation of RNA accumulation than that of [^{14}C]uracil (Figure 3A). Isoleucine starvation had no effect on the rate of phage RNA synthesis in the infected relaxed host. Initially, there was a significant difference between the rates of phage RNA synthesis in stringent and relaxed hosts.

The reason for the sharper decrease in the rate of RNA synthesis in the infected unstarved stringent strain is not known. However, this decrease was paralleled by a decrease in the size of the ATP pool (unpublished data).

Discussion

MS compounds are accumulated in infected stringent cells in response to valine addition. However addition of valine does not cause a decrease in rate of phage RNA synthesis in either a stringent or a relaxed cell. We conclude that there is no correlation between the rate of phage RNA synthesis and MS compound accumulation.

Wong and Nazar (1970) have shown that pretreatment with rifampicin prevents the formation of MS nucleotides by stringent cells, thus suggesting that nascent RNA synthesis is necessary for MS compound formation. This interpretation has been disputed by Erlich *et al.* (1971), who demonstrated that MS compounds can be accumulated in the presence of rifampicin if aminoacylation of tRNA is restricted. Similarly, we have shown that starvation may produce MS compounds in the presence of rifampicin if the stringent cell is infected with R17. In the infected cell, the continuing presence of phage mRNA maintains the valine-imposed isoleucine starvation, whereas in the uninfected host rifampicin results in a depletion of the host mRNA required for continued protein synthesis, and a reestablishment of the isoleucine pool results.

Travers *et al.* (1970b) have reported that ψ factor stimulated rRNA synthesis may be inhibited by ppGpp *in vitro*. It has been postulated that the phage RNA synthetase, which contains the host ψ factor as a component, may be similarly inhibited by this nucleotide. However, since high levels of ppGpp are accumulated in the phage-infected stringent cell (Figure 1) without a corresponding inhibition of the rate of phage RNA synthesis (Figure 4B), we conclude that ppGpp does not inhibit the phage RNA synthesizing system.

It is interesting to note that without isoleucine starvation the rate of RNA synthesis in the stringent host declines sharply at about 24-min postinfection, and that isoleucine starvation prevents such a decline (Figure 4B). However, the relaxed cell exhibits only a slow decrease in rate of synthesis with and without isoleucine starvation. How the *rel* gene affects phage RNA synthesis in this unconventional manner is not clear.

In summary, phage RNA synthesis is not influenced by the *rel* gene of the host in the same manner as is host RNA itself, nor is the accumulation of ppGpp dependent upon a decrease in RNA synthesis.

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