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Correlation between Guanosine Tetraphosphate Accumulation and Degree of Amino Acid Control of Ribonucleic Acid Accumulation During Nutritionally Slowed Growth in *Escherichia coli*†

Sabih R. Khan‡ and Hiroshi Yamazaki*

ABSTRACT: Amino acid starvation causes a severe curtailment of RNA accumulation and elicits accumulation of guanosine 3′,5′-bis(diphosphate) in stringent strains of *Escherichia coli* grown in glucose minimal media. However, it is shown here that amino acid starvation only partially inhibits RNA accumulation when the bacterial growth rate is reduced by utilizing poor carbon sources or by limiting the intracellular supply of thiamine. Even under these conditions, the observed rate of RNA accumulation is inversely correlatable to the level of guanosine 3′,5′-bis(diphosphate) accumulated.

When stringent (*rel*$^+$) bacteria are starved for an essential amino acid or fail to aminoacylate a tRNA species, not only does protein accumulation cease but also RNA accumulation is substantially reduced (Sands and Roberts, 1952; Pardee and Prestidge, 1956; Neidhardt, 1966; Gallant and Margason, 1972). This response, called the stringent response, depends on the function of the *rel* gene. Mutations at this locus give rise to relaxed mutants (*rel*$^-$) which continue to accumulate RNA for at least one-third doubling time after amino acid starvation (Borek *et al.*, 1956; Stent and Brenner, 1961; Fiil and Friesen, 1968). Amino acid starvation elicits a second response in *rel*$^+$ cells: rapid accumulation of guanosine 3′,5′-bis(diphosphate) (ppGpp) which does not occur in *rel*$^-$ cells (Cashel and Gallant, 1969; Cashel, 1969; Swanton and Edlin, 1972). The correlation between the kinetics of ppGpp accumulation and the cessation of RNA accumulation suggests that this nucleotide plays a role in the regulation of synthesis of rRNA (Cashel, 1969). However, this presumption has not been directly demonstrated (Haseltine, 1972; Lazzarini and Johnson, 1973; Murooka and Lazzarini, 1973).

The correlation between the high intracellular levels of ppGpp and the cessation of RNA accumulation has been previously established under relatively rich growth conditions such as glucose minimal medium. In the present work, the generality of this relationship is examined. At slow growth rates, created by poor carbon sources or limitation in the intracellular supply of thiamine, stringent (*rel*$^+$) strains of *Escherichia coli* adopt a partially relaxed character: amino acid starvation does not completely halt RNA accumulation. The observed rate of RNA accumulation under these conditions depends on the previous growth rate. Even under these conditions, the inverse correlation between the ppGpp levels and RNA accumulation is maintained.

Experimental Section

Bacteria and Culture Conditions. Three *E. coli* K-12 stringent (*rel*$^+$) strains were used: HY 1, CP 78A, and CP 78B. A prototrophic strain which we have named HY 1 was obtained from Dr. Ira Pastan, National Cancer Institute, Bethesda, Md. CP 78A (leaky for thiamine requirement) and CP 78B (thiamine revertant) were isolated from a single clone of CP 78 (*thi, arg, his, thr, leu, rel*$^+$) (obtained from Dr. Barbara Bachmann, Yale University, New Haven, Conn.) after prolonged cultivation in the absence of thiamine. These strains grow in

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‡ Present address: Laboratory of Molecular Biology, NINDS/NIH, Bethesda, Md. 20014.

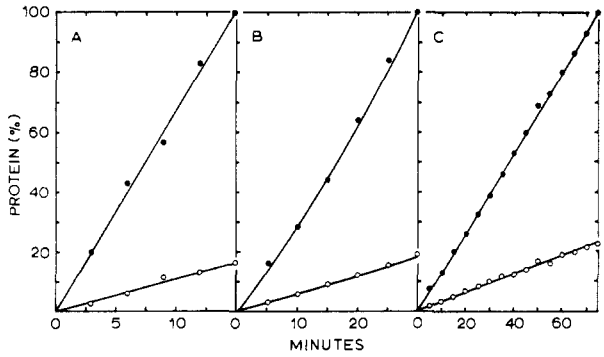

FIGURE 1: Effect of valine-imposed isoleucine starvation on protein accumulation in HY 1 grown on three different carbon sources. Strain HY 1 (*rel*$^+$) was cultured at 37° in glucose (panel A), acetate (panel B), or aspartate (panel C). When the cells had reached logarithmic growth, each culture was divided into two equal portions. At zero time, the first portion was mixed with one-tenth the volume of a protein-labeling mixture and one-tenth the volume of L-valine (5 mg/ml). The second portion was similarly labeled in the absence of valine, thus serving as an unstarved control. Fifty-microliter portions of the labeled cultures were periodically withdrawn from the starved (○) and unstarved (●) cultures and assayed for labeled protein as previously described (Bollum, 1969). The data are expressed as a percentage of the radioactivity in the unstarved control culture at one-quarter doubling time after the addition of valine.

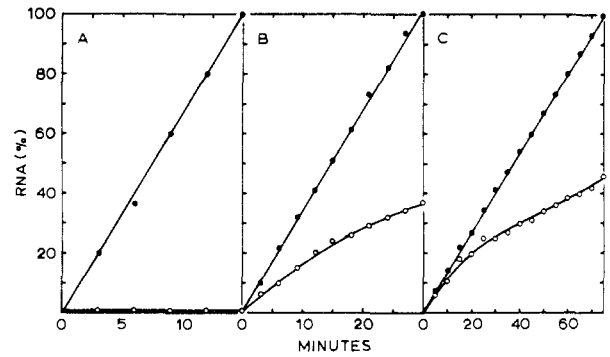

FIGURE 2: Effect of valine-imposed isoleucine starvation on RNA accumulation in HY 1 grown on three different carbon sources. Strain HY 1 (*rel*$^+$) was cultured at 37° in glucose (panel A), acetate (panel B), or aspartate (panel C). Carrier-free [^{32}P]orthophosphate was added to the log-phase cultures to a final specific activity of 5 Ci/mol (5 μCi/ml of culture). After at least one doubling, the labeled culture was divided into two equal portions. At zero time, L-valine was added to the first portion to a final concentration of 500 μg/ml. The second portion served as an unstarved control. Two 50-μl portions were removed at intervals from both the starved (○) and unstarved (●) cultures and assayed for labeled RNA as previously described (Watson and Yamazaki, 1973). The data are presented as a percentage of the RNA radioactivity in the control culture at one-quarter doubling time after the addition of valine.

the thiamine-free glucose medium with doubling times of approximately 330 min for CP 78A and 70 min for CP 78B. In the presence of thiamine, both grow at the same rate as CP 78 with a doubling time of 60 min. They retained all other characteristics of CP 78 except for the thiamine requirement.

Tris-maleate minimal medium (TMM) (Khan and Yamazaki, 1972) containing 1 mM phosphate was used throughout. Three carbon sources were used to create different growth rates: 0.4% glucose; 1.0% sodium acetate trihydrate; 0.5% aspartic acid. Whenever necessary, the media were further supplemented with thiamine-HCl (10 μg/ml) and amino acids (100 μg/ml). For CP 78A and CP 78B, isoleucine (100 μg/ml) was supplemented. Growth was followed by measuring absorbance at 500 nm (A_{500}) with a Bausch and Lomb Spectronic 20 spectrophotometer using a round cuvet (11-mm i.d.).

Assay of RNA Accumulation. Carrier-free [^{32}P]orthophosphate (Atomic Energy of Canada, Ltd.) was added to a log-phase culture at a final concentration of approximately 5 μCi/ml and a final specific activity of approximately 5 Ci/mol. The culture was equilibrated with the label for at least one doubling prior to sampling. Two 50-μl portions of the labeled culture were periodically withdrawn and were assayed for the acid-insoluble radioactivity (total nucleic acids) and the alkali-insoluble radioactivity (DNA) according to the procedure of Watson and Yamazaki (1973). The radioactivity in RNA was determined by subtracting the alkali-insoluble radioactivity from the acid-insoluble radioactivity.

Assay of Protein Accumulation. Protein accumulation was monitored by following L-[U-^{14}C]arginine incorporation. When arginine was present as a supplement in the medium, one-tenth the volume of [^{14}C]arginine (10 μCi/ml; 300 Ci/mol) was added to one volume of culture. In all other cases, one volume of culture was mixed with one-tenth the volume of a protein-labeling mixture which consisted of [^{14}C]arginine (5 μCi/ml) and L-arginine (100 μg/ml). Portions (50-μl) of the labeled culture were withdrawn periodically, and were assayed for the hot trichloroacetic acid insoluble radioactivity by the filter paper disk method (Bollum, 1968).

Assay of ppGpp. Carrier-free [^{32}P]orthophosphoric acid was added to log-phase cultures to a final specific activity of 150–300 Ci/mol at least one doubling prior to withdrawal of the first sample. The samples were mixed with an equal volume of 2 M formic acid. The acid-extracted ppGpp was assayed as described previously (Cashel, 1969).

Results

The prototrophic *E. coli* stringent strain, HY 1, was used, because of the possibility that if an amino acid auxotroph were used, a required amino acid could be employed as a carbon source rather than the added poor carbon source. The doubling times of HY 1 were approximately 60 min in glucose, 120 min in acetate, and 300 min in aspartate medium. Amino acid (isoleucine) starvation was effected by the addition of valine, a potent feedback inhibitor of an early enzyme of the isoleucine synthetic pathway (Leavitt and Umbarger, 1962; Nierlich, 1968; Fiil, 1969). Figure 1 shows the effects of valine-imposed isoleucine starvation on the accumulation of labeled protein in HY 1 at these three growth rates. In order to permit comparison in the three cultures, the amounts of the labeled proteins are expressed as a percentage of the amounts in the unstarved control of each culture at one-quarter doubling time after the addition of the label. The addition of valine caused similar inhibition of accumulation of labeled protein in the three cultures indicating that the degree of isoleucine starvation was approximately the same. In contrast, the effect of isoleucine starvation on RNA accumulation is profoundly different as shown in Figure 2. As expected, RNA accumulation in this stringent strain grown in the glucose medium (panel A) was severely inhibited during isoleucine starvation, whereas the same strain growing at slower rates in acetate (panel B) and aspartate (panel C) medium continued to accumulate RNA up to one-quarter doubling times (30 and 75 min, respectively) after the onset of amino acid starvation. Beyond those times, the rate of RNA accumulation rapidly declined and ceased completely after one-half doubling times. The determination of the relaxed character is valid only during an early period of amino acid starvation since even highly relaxed mutants (Fiil and Friesen, 1968) accumulate RNA at a normal rate only up to one-third doubling time after the onset of amino acid starvation.

The second response to amino acid starvation that is affected

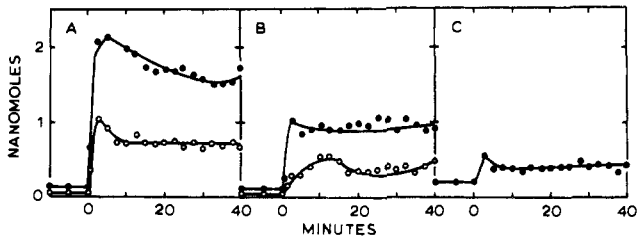

FIGURE 3: Effect of valine-imposed isoleucine starvation on accumulation of ppGpp by HY 1 grown on three different carbon sources. Strain HY 1 (*rel*$^+$) was grown at 37° in glucose (panel A), acetate (panel B), or aspartate (panel C) as a carbon source. [^{32}P]Orthophosphate was added to the log-phase cultures at a final specific activity of 10–300 Ci/mol. After at least one doubling, L-valine was added to a final concentration of 00 μg/ml (zero time). Fifty-microliter portions of the culture were removed periodically and were assayed for ppGpp (●) and pppGpp (○) as previously described (Cashel, 1969). The data are presented as the amount of phosphate incorporated into these nucleotides in 1 ml of the culture of A_{500} = 1.0.

by the *rel* gene is the accumulation of ppGpp. Figure 3 shows the effect of carbon sources on the cellular capacity to accumulate ppGpp upon isoleucine starvation. In all three cultures, isoleucine starvation immediately elicited the accumulation of ppGpp. However, the amounts of ppGpp that the cells accumulated above the basal levels were considerably different: the largest in the glucose culture, intermediate in acetate, the least in aspartate. Therefore, by this criterion as well, the cells grown in aspartate exhibited the most relaxed character; the acetate culture, the intermediate; the glucose culture, the most stringent character. The amounts of accumulation of guanosine 3′-triphosphate 5′-diphosphate (pppGpp) (Cashel and Gallant, 1969; Cashel, 1969; Sy and Lipmann, 1973) were also different: greater in the glucose culture than that in the acetate culture. No detectable amounts of pppGpp accumulated in the aspartate culture.

To further delineate the primary cause of the observed relaxation of RNA control, we examined stringent cells whose growth rates were slowed by other means—reduced culture temperature and vitamin deprivation. HY 1 growing in glucose medium at 20° (doubling time = 160 min) did not exhibit the relaxed control of RNA accumulation. Consequently, it appears that the chemical environment of the cells is primarily responsible for the relaxed character. This tentative conclusion is further strengthened by experiments performed with the strain, CP 78A (leaky for thiamine requirement) whose growth was restricted by thiamine limitation. Figure 4 illustrates the effects of thiamine limitation on the degree of the stringent response. β-Thienylalanine was used to create phenylalanine starvation (Ezekiel, 1965; Sköld, 1970; Khan and Yamazaki, 1970). In the thiamine-supplemented medium, the leaky mutant, CP 78A, exhibited a typical stringent response in terms of accumulation of RNA and ppGpp (panels C and E) whereas in the thiamine-free medium it exhibited a highly relaxed response (panels D and F). In the thiamine-free culture, an unusually high basal level of ppGpp was observed (panel F). The thiamine revertant, CP 78B, exhibited the typical stringent response whether the cells were grown in the absence or presence of thiamine.

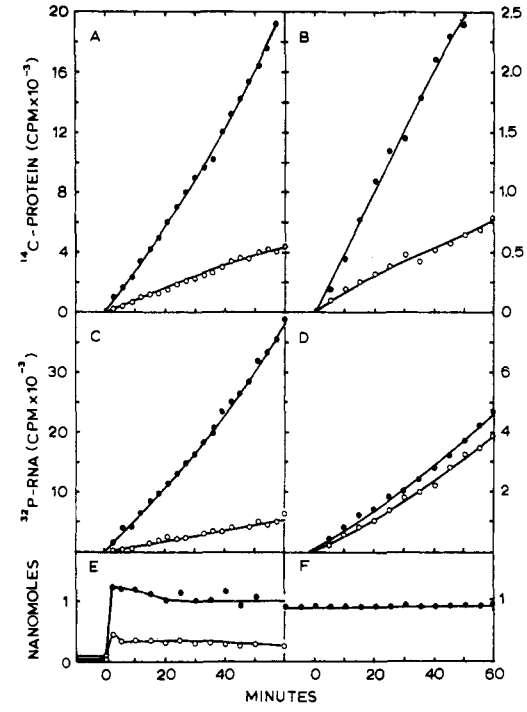

FIGURE 4: Effect of thienylalanine-imposed phenylalanine starvation on accumulation of protein, RNA, and ppGpp in CP 78A. A log-phase culture of CP 78A grown on glucose in the presence of thiamine was divided into two portions. The effect of β-thienylalanine-imposed phenylalanine starvation was studied on the first portion in the continued presence of thiamine: panel A indicates protein accumulation; panel C, RNA accumulation; panel E, ppGpp accumulation. The second portion was transferred to a thiamine-free medium. The cells were cultured for several generations until the final thiamine-limited exponential growth (doubling time of 330 min) was obtained. The effect of β-thienylalanine-imposed phenylalanine starvation was then studied on this thiamine-limited culture: panel B indicates protein accumulation; panel D, RNA accumulation; panel F, ppGpp accumulation. β-Thienylalanine was added at zero time to a final concentration of 500 μg/ml. Otherwise, the analytical procedures were the same as described in the preceding figures. The data in panels A, B, C, and D represent the amounts of radioactivity in 50 μl of the labeled cultures. The open circles represent the starved samples; the closed circles, the unstarved samples. The data in panels E and F represent the amount of phosphate incorporated into ppGpp (●) and pppGpp (○) in 1 ml of the culture of A_{500} = 1.0. No detectable amount of pppGpp was accumulated in the culture grown in the absence of thiamine (panel F).

Discussion

Stringent *E. coli* cells which were growing slowly on poor carbon sources exhibited a partially relaxed response in terms of the two conventional criteria, *i.e.*, accumulation of RNA and ppGpp. An even greater relaxed response was observed when the growth rate was greatly reduced due to the limited supply of thiamine. This phenomenon must be caused by the chemical environment of the cell rather than the physical environment since it was not observed when the growth rate of a stringent strain was reduced by lowering the incubation temperature.

There is an apparent inverse correlation between the extent of ppGpp accumulation and the degree of relaxed response. The *in vitro* synthesis of ppGpp has been shown to require the interaction between an uncharged tRNA and ribosomes carrying the codon specific to that tRNA (Haseltine and Block, 1973; Pedersen *et al.*, 1973). These and the *in vivo* studies (Lund and Kjeldgaard, 1972a,b; Fiil *et al.*, 1972) raise the possibility that ppGpp is normally required by some step in protein synthesis. If this is correct, the intracellular requirement for this nucleotide is expected to be smaller in a slowly growing cell. Different turnover rates of this nucleotide might result in different levels of ppGpp accumulation upon the onset of amino acid starvation, which may, in turn, cause different degrees of inhibition of RNA accumulation. Various other possibilities can be speculated. For example, amino acid starvation is known to cause an increase in degradation of proteins (Sussman and Gilvarg, 1969; Brunschede and Bremer, 1971). A nu-

tritionally poor condition may additionally enhance protein degradation during amino acid starvation, which would then reduce the effect of amino acid starvation by recharging of tRNA. A small increase in the level of charging of tRNA is shown to significantly reduce the rate of ppGpp accumulation *in vitro* (Pedersen *et al.*, 1973).

The purpose of this paper is to report this new phenomenon of a nutritionally imposed relaxed response and to demonstrate the generality of the inverse correlation between ppGpp accumulation and RNA accumulation.

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