RC^+ GENE-DEPENDENT INHIBITION OF RNA SYNTHESIS WITHOUT ppGpp ACCUMULATION

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SUMMARY The lack of a required amino acid or a block in the synthesis of an aminoacyl-tRNA inhibits net RNA synthesis and results in the accumulation of the nucleotides ppGpp and MS_{II} in RC^+ but not in RC^- cells of E. coli. It would be conceivable that ppGpp is the mediator of the inhibition of net RNA synthesis by the RC^+ control system. However, the following results indicate that this is not (or at least not always) the case: Trimethoprim, an inhibitor of the formation of the peptide chain initiator formylmethionyl-$tRNA_f$, blocks net RNA synthesis in RC^+ but not in RC^- cells. We found that in RC^+ E. coli cells, trimethoprim (i) does not cause ppGpp or MS_{II} accumulation if no required amino acid is missing, and (ii) does not inhibit ppGpp accumulation elicited by the lack of a required amino acid.

RNA accumulation in E. coli is stated to be under "stringent" control (RC^+) if it is inhibited upon deprivation of a required amino acid, or by a block in the aminoacylation of a tRNA species. The control of RNA accumulation is stated to be "relaxed" in E. coli mutants (RC^-) in which this is not the case. The genetic site responsible for the control is the RC locus (1, 2, 3). In RC^+ (but not in RC^-) cells, two unusual nucleotides (MS_I and MS_{II}) are accumulated upon deprivation of a required amino acid (4). The structure of MS_I is ppGpp, that of MS_{II} (which is presumably also a guanine nucleoside polyphosphate) has not been established (5).

The fact that the increase in ppGpp level and the inhibition of RNA accumulation are elicited by the same treatment raised the hypothetical possibility that ppGpp (or MS_{II}) may be the inhibitor of RNA accumulation. In line with

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this it was reported that ppGpp inhibits: (i) the synthesis of the RNA precursors AMP and GMP (6), and (ii) the action of a factor (ψ) presumed to be involved in the synthesis of ribosomal RNA and possibly tRNA (7).

Trimethoprim (an inhibitor of dihydrofolate reductase (8)) which blocks the formylation of methionyl-$tRNA_f$ and thereby peptide chain initiation (9), also inhibits RNA accumulation in RC^+ E. coli cells (10). However, in RC^- cells, trimethoprim blocks peptide chain initiation but does not affect RNA accumulation (10). In this communication we describe experiments testing if the inhibition of RNA accumulation by trimethoprim is accompanied by ppGpp accumulation or not.

The curves in Figure 1(A) verify that depriving leucine-requiring RC^+ cells of leucine or supplementing their medium with trimethoprim inhibits RNA accumulation to a larger extent than either treatment alone. The curves in Figure 1(B) reveal that the concentration of ppGpp is the same in control cells and in cells to which trimethoprim has been added, whereas in cells deprived of leucine, it is greatly increased. The increase in ppGpp concentration resulting from leucine-deprivation is only slightly enhanced (if at all) if trimethoprim is also present. The curves in Figure 1(C) indicate that trimethoprim has no effect on MS_{II} accumulation either in the presence or in the absence of leucine. In the experiments shown in Figures 1(B) and 1(C) the cells had been equilibrated with ^{32}P-phosphate prior to the experiment; thus, the concentration of ppGpp in the cells was determined. In other studies (not shown), ^{32}P-phosphate was added simultaneously with leucine-deprivation and trimethoprim addition. These studies revealed that (i) the rate of accumulation of ^{32}P in ppGpp was much faster in leucine-deprived than in control cells, and (ii) trimethoprim did not affect the rate of ppGpp accumulation in either the presence or the absence of leucine. In further experiments the stringent response was elicited by adding valine, thereby blocking the synthesis of isoleucine and depriving the cells of this amino acid (11). The conclusions drawn from these experiments were the same as those outlined above.

All these results indicate that trimethoprim, which blocks RNA accumulation in RC^+ but not in RC^- cells, accomplishes this without altering detectably either the level or the rate of accumulation of ppGpp. Consequently, at least when the stringent response is elicited by trimethoprim, ppGpp is not the agent inhibiting RNA synthesis. In line with these conclusions, Zubay et

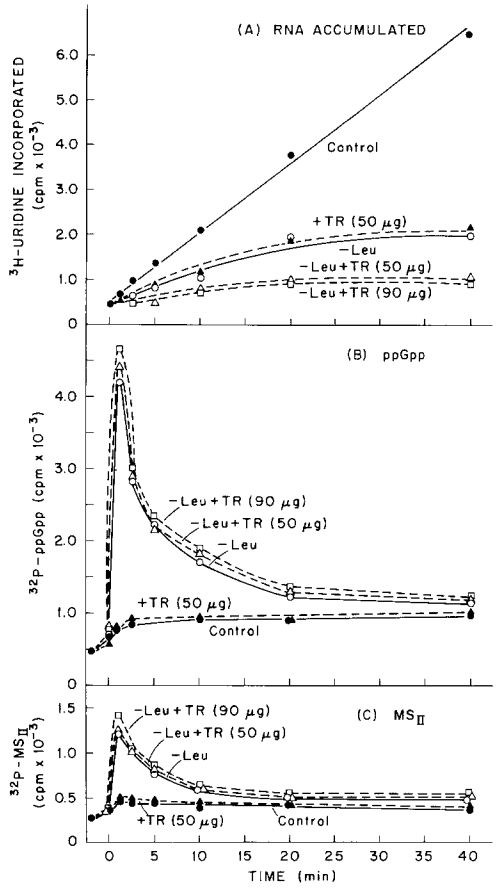

Figure 1 RNA accumulation and ppGpp and MS_{II} concentration in leucine-deficient RC^+ E. coli: Effects of trimethoprim addition and leucine deprivation.

CP78 RC^+ and CP79 RC^- E. coli K12 strains (19) which are isogenic except in the RC gene and require Arg, His, Leu, Thr, and vitamin B1 were grown at 37° on a rotary shaker in medium A (containing in 1 liter: Tris-HCl, 6.05g; KH_2PO_4, 3.48g; KCl, 1.33g; $(NH_4)_2SO_4$, 1.0g; glucose, 1.0g; sodium citrate 5 H_2O, 0.5g; $MgSO_4$ 7 H_2O, 0.1g; vitamin B1, 0.005g; as well as 0.05g of each of thymine, uracil, adenine, and guanine, and 0.1g of each of the twenty common amino acids, with the pH of the solution adjusted to 7.4 with HCl). The cells were grown overnight, sedimented next morning by centrifugation, resuspended at an A_{700} of approximately 0.18 in the same medium except containing less KH_2PO_4 (0.68g/l), and further incubated till the A_{700} reached about 0.65. At this time (-45 min) the culture was divided into two portions: portion I was supplemented with 50μc/ml of ^{32}P (0.12μg/ml), and both portions were incubated further. Aliquots (0.1ml) were taken from portion I at -45, -15, and -2 minutes to determine ppGpp and MS_{II}. 43 minutes later (at -2 minutes) five 2ml samples (1-5) from portion I and five 2ml samples (1-5) from portion II were filtered through nitrocellulose filters (Millipore HAWP-025-00) at room temperature. The filters on which the cells were retained were washed at room temperature with medium A lacking leucine. Subsequently the cells from individual filters were resuspended by vortexing in a different medium (2ml): in

medium A (control), 2) in medium A containing trimethoprim (50μg/ml), 3) in medium A without leucine, 4) in medium A without leucine, containing trimethoprim (50μg/ml), 5) in medium A without leucine, containing trimethoprim (90μg/ml). The trimethoprim (2, 4-diamino-5(3', 4', 5' trimethoxybenzyl) pyrimidine) (8), donated by Dr. G.H. Hitchings, Burroughs Wellcome Co.) has been dissolved in 95 per cent ethanol before being added to medium A. The same amount of ethanol (final concentration 1 per cent v/v) was added to all samples. Each of the media in which the cells in the samples (1-5) from portion II were resuspended contained ^{3}H uridine (25μg/ml, 23.7 C/mole), in addition to the components indicated for the corresponding samples of portion I. The filters were removed after the vortexing and each sample was further incubated at 37°C. (2-3 minutes elapsed between collecting the cells by filtration and starting their further incubation.)

a) Assay for RNA accumulation (Fig. 1(A)) in the samples of portion II.

Aliquots (0.1ml) were taken from samples 1-5 at the times indicated, pipetted into 10 per cent cold trichloroacetic acid (5ml), incubated in the cold for 3-4 hours, and filtered. The filters were washed (first with 5 per cent trichloroacetic acid containing uridine (25μg/ml) then with 95 per cent ethanol) dried and counted in 5ml of a toluene-based scintillator.

b) Assay for ppGpp (Fig. 1(B)) and MS_{II} (Fig. 1(C)) in the samples of portion I.

Aliquots (0.1ml) were taken from samples 1-5 at the times indicated, and pipetted into ice cold 4N formic acid (0.03ml). ppGpp and MS_{II} were isolated, identified and assayed in the aliquots according the the procedure of Cashel and Kalbacher (5). The results are expressed as cpm x 10^{-3} of ^{32}P in ppGpp or MS_{II} in 0.01ml of the culture. The experiments were repeated four times with similar results. One typical experiment with strain CP78 RC^{+} is presented in the Figure. The slight increase in the ppGpp concentration between -2 and +7 minutes is presumably a consequence of the manipulations in collecting and resuspending the samples. In each of the three experiments, 50μg/ml of trimethoprim inhibited RNA accumulation to the same extent as 90μg/ml (not shown). In CP79 RC^{-}, leucine deprivation or the addition of trimethoprim caused much less inhibition of RNA accumulation then in CP78 RC^{+} and trimethoprim addition did not affect the level of ppGpp. (In the figure, trimethoprim is abbreviated as TR.)

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al (12) failed to detect an inhibition of tRNA synthesis by ppGpp in extracts of RC^{+} E. coli cells though in vivo tRNA synthesis is under stringent control. A further point demonstrated by the results of the experiments with trimethoprim is that the RC^{+} gene-dependent inhibition of RNA accumulation does not necessarily result in the increased synthesis or accumulation of ppGpp or MS_{II}. (See also 13-18).

Finally, it should be noted that it is not inconceivable that stringent control may regulate RNA accumulation by a different mechanism in response to the deprivation of a required amino acid than in response to trimethoprim.

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