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Magic Spot Metabolism in an *Escherichia coli* Mutant Temperature Sensitive in Elongation Factor Ts

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A temperature-sensitive mutant of *Escherichia coli* HAK88 which has been shown to have a lesion in elongation factor Ts (EFTs) was studied with repsect to its metabolism of guanosine 5'-diphosphate, 2'(3')-diphosphate (ppGpp) and the associated failure of ribosomal ribonucleic acid (rRNA) accumulation at the nonpermissive temperature. Results reported here show that (i) when EFTs is nonfunctional, a full complement of charged transfer RNA (tRNA) cannot prevent accumulation of ppGpp (magic spot) and the stringent failure of rRNA accumulation; (ii) chloramphenicol prevents magic spot (MS) formation and the stringent response not by increasing the percentage of charged tRNA, but possibly by somehow interfering directly with the synthesis of MS; and (iii) tetracycline can lead to MS disappearance without resumption of RNA synthesis. Thus, the absence of MS and the presence of a functional RNA polymerase and charged tRNA are not sufficient to support rRNA accumulation in vivo. An additional element in the regulatory system is suggested.

From increasingly strong circumstantial evidence, the nucleotide ppGpp, guanosine 5'-diphosphate, 2'(3')-diphosphate (magic spot [MS]), has been implicated in the regulation of ribosomal ribonucleic acid (rRNA) accumulation during amino acid starvation of stringent cells (2). Accumulation of magic spot precedes the shut off of rRNA accumulation, and on the addition of the deprived amino acid, a reduction in the MS concentration precedes the resumption of rRNA accumulation (2). Also, chloramphenicol (CHL), which severely inhibits protein synthesis, prevents MS synthesis and restores rRNA accumulation in starved cells. Relaxed mutants do not accumulate MS during amino acid starvation. However, they do accumulate MS and preferentially restrict rRNA accumulation during shift-down transitions as do stringent cells (11).

Studies with whole cells have also shown that the presence of a pool of one or more uncharged transfer RNA (tRNA) species is somehow responsible for both MS accumulation and subsequent differential control of rRNA accumulation (1, 3). The trigger for the accumulation of MS has been shown in vitro to be the presence of uncharged tRNA in the ribosomal acceptor site (7). The ribosome-catalyzed reaction does not require functional elongation factor T (EFTsTu), but is in fact inhibited by functional T factor (7, 17).

This paper reports the metabolism of MS associated with failure of rRNA accumulation in an *Escherichia coli* mutant with a temperature-sensitive elongation factor Ts (EFTs). The strain, HAK88, is a stringent strain known to show a stringent response at the nonpermissive temperature (42 C) (9, 10). The data reported here support the idea that there must be not only a full complement of charged tRNA, but also a functional T factor (EFTu + EFTs) to prevent accumulation of MS and the stringent failure of rRNA accumulation (7). In addition, our data show that CHL prevents MS formation and the stringent response not by increasing the percentage of charged tRNA, as had been suggested (8), but possibly by somehow interfering directly with the synthesis of MS. The evidence presented also indicates that the absence of MS and the presence of a functional RNA polymerase are not sufficient to support rRNA accumulation in vivo.

MATERIALS AND METHODS

Strains of *E. coli* used in this study were: HAK88 (9, 10), which has the following characteristics: *trp*$^-$, *rel*$^+$, RNase I$^-$, temperature-sensitive EFTs, RNA^{perm} (tryptophan auxotroph, stringent control of rRNA accumulation, ribonuclease [RNase] I deficient, temperature-sensitive EFTs, permeable to RNA); NP29, formerly 1-9 (3), which is *rel*$^+$, *thi*$^-$, *valS* (stringent control of rRNA accumulation, thia-

mine auxotroph, temperature-sensitive valine synthetase); and D10 (5) which is *met*$^-$, *RNase* I$^-$ (methionine auxotroph, RNase I deficient). Strain NP29 was used in all of the following experiments to provide controls in addition to those for strain HAK88 at 30 C.

Media. Enriched medium I is composed of (per liter): Na_2HPO_4, 7 g; KH_2PO_4, 3 g; NH_4Cl, 1 g; $NaSO_4$, 0.8 g; $FeCl_3$, 1.75 mg; $MgCl_2$, 48.8 mg; $CaCl_2$, 2.75 mg, plus 0.4% glucose, 0.2% technical grade Casamino Acids, 0.2% yeast extract, and 0.005% tryptophan. Enriched medium II is composed of enriched medium I without yeast extract.

Low phosphate growth medium is composed of (per liter): tris(hydroxymethyl)aminomethane base, 6.05 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $(NH_4)_2SO_4$, 1.00 g; trisodium citrate$\cdot 3H_2O$, 0.50 g; KCl, 1.86 g, $FeCl_3$, 1.6 mg, and KH_2PO_4 at a final concentration of 2×10^{-3} M plus 0.4% glucose, 0.2% PO_4-free Casamino Acids, and 0.005% tryptophan. The pH was adjusted to 7.4. Low phosphate labeling medium is the same as low phosphate growth medium except for the decrease in two components, KCl to 0.15 g and KH_2PO_4 to 2×10^{-4} M.

Measurement of tRNA charging levels. The levels of charging in strains HAK88 and NP29 at 30, 42, and 42 C plus CHL were measured according to the procedure of Folk and Berg (4) as modified by Lewis and Ames (13), with the exception that a nucleic acid free S-100 (6) from strain D10 was used as a source of aminoacyl-tRNA synthetase. The cells were grown in enriched medium I and harvested during midlog phase. Four ^{14}C amino acids were used in the in vitro charging of tRNA. They were: phenylalanine, 460 mCi/mmol; leucine, 300 mCi/mmol; valine, 270 mCi/mmol; and methionine, 260 mCi/mmol.

Measurement of MS, guanosine 5′-triphosphate, and adenosine 5′-triphosphate levels. The levels of MS, guanosine 5′-triphosphate (GTP), and adenosine 5′-triphosphate (ATP) were measured as described by Cashel (2) and modified by Kaplan, Atherly, and Barrett (8), with minor variations. HAK88 and NP29 cells were grown in low phosphate growth medium to an absorbancy at 420 nm (A_{420}) of approximately 0.5. The cells were then collected by centrifugation, washed with low phosphate labeling medium, and resuspended in an equal volume of low phosphate labeling medium. Samples (1 ml) of the culture in the presence of 50 μCi of carrier-free $^{32}PO_4$ were incubated at 30, 42, and 42 C plus tetracycline (TET) or CHL added at zero time or at 30 min after the shift to 42 C. Samples (100 μliters) were taken at various time intervals into an equal volume of 2 N formic acid. After 15 min on ice, the cells were sedimented, and the supernatant fluids were applied directly on polyethyleneimine cellulose chromatograms. The one-dimensional chromatograms were run in 1.5 M KH_2PO_4 (pH 3.4) to 17 cm above the origin (the top of the plate) in closed chambers at room temperature. The remaining procedure is as previously described (2), except that the entire chromatogram was cut into 0.5 by 2 cm strips, and the dry samples were counted by utilizing Cerenkov radiation. Incorporation of $^{32}PO_4$ was corrected for decay and adjusted to incorporation per 5×10^6 cells.

Labeling of RNA. The cells were grown in enriched medium II to an A_{420} of approximately 0.5. The nucleotide pool was pre-expanded with cold uracil (final concentration 5 μg/ml). [^{3}H]uracil (18.5 Ci/mmol, final concentration 5 μC/ml) was added at −45 minutes. A portion of the culture was transferred to 42 C at 0 min with CHL and TET added as indicated. Samples (0.2 ml) were transferred to 1 ml of cold 10% trichloroacetic acid, and duplicate samples were processed for RNA and DNA. Radioactivity incorporated into DNA was subtracted from radioactivity incorporated into RNA.

Materials. Uniformly labeled ^{14}C amino acids and [5-^{3}H]uracil were obtained from Schwartz Bioresearch, Inc.; ^{32}P sodium phosphate, carrier-free, was obtained from Mallinckrodt Nuclear. CHL was a gift of Parke, Davis, and Co. TET was obtained from Lederle, as the solid (30% TET, 70% ascorbic acid). Polyethyleneimine cellulose thin-layer chromatograms were purchased from Brinkmann Instruments, Inc.

RESULTS

Levels of tRNA charging. Previous in vivo and in vitro data have indicated that uncharged tRNA is necessary for MS accumulation. The lesion in strain HAK88 is known to be in EFTs, so that uncharged tRNA would not be expected to accumulate. In fact, levels of charging of four representative tRNA's were checked (Table 1), and the percentages of charged tRNA were normal in strain HAK88 at 42 C. In contrast, with a standard mutant temperature sensitive in valine tRNA synthetase (NP29), the level of uncharged valyl-tRNA is quite low even at 30 C. Studies after the addition of CHL further demonstrate that the percentages of charged tRNA in strain HAK88 at 42 C, in which the temperature-sensitive lesion is slightly leaky, were very near their maxima. In contrast, the level of charged valyl-tRNA in strain NP29 at 42 C was increased 400% by the addition of CHL.

Magic spot, GTP, and ATP metabolism. Strain HAK88 has a full complement of charged tRNA at 42 C, and one might therefore expect that, although stringent, it would not produce MS at 42 C. Even if MS should form, CHL, which is thought to prevent MS synthesis by permitting a build up of charged tRNA (1), would not be expected to inhibit MS synthesis, because as shown above CHL does not significantly increase the percentages of charged tRNA in strain HAK88 at 42 C.

By following the procedure of Cashel (2) the metabolism of MS was studied in strain HAK88 at 30, 42, and at 42 C in the presence of CHL. Contrary to expectations based on known levels of charged tRNA, high levels of MS were formed

TABLE 1. *Percentage of tRNA charged in strains HAK88 and NP29*[a]

Strain	Conditions	Leu tRNA	Phe tRNA	Meth tRNA	Val tRNA
HAK 88	30 C	48	71	97	94
	42 C	55	92	100	87
	42 C + CHL	65 Δ + 18[b]	76 Δ − 17	87 Δ − 13	92 Δ + 5
NP 29	30 C	48			19
	42 C	70			10
	42 C + CHL	78 Δ + 11			50 Δ + 400

[a] Percentage of charging was determined as (no. of counts bound in oxidized fraction per no. of counts bound in unoxidized fraction) × 100.

[b] The percentage of charge was determined as Δ (% charged at 42 C − % charged 42 C + CHL) ÷ % charged at 42 C.

at 42 C but not at 30 C. When CHL was added at zero time, no MS was formed; whereas when CHL was added after MS had reached a steady state level further synthesis of MS was prevented. The MS then present was degraded with a half-life of approximately 1 min (Fig. 1).

The relative levels of GTP and ATP were also measured by thin-layer chromatography and were found to be lower at 42 C than at 30 C. The levels of these two nucleotides at 42 C, however, increased rapidly after the addition of CHL (Fig. 1).

Recently TET has been shown to prevent the synthesis of MS by presumably binding to the ribosomal acceptor site in a fashion analogous to charged tRNA and thereby preventing the synthesis of MS (8). We predicted, therefore, that TET would prevent the synthesis of MS seen in strain HAK88 at 42 C. The results shown in Fig. 2 demonstrate that this is, in fact, the case. In addition, the levels of GTP and ATP increase after the addition of TET as they do after the addition of CHL.

From these results, strain HAK88 appears to show a normal, stringent response with regard to MS, GTP, and ATP metabolism at 42 C in the presence or absence of CHL or TET.

Accumulation of rRNA. Strain HAK88 has been shown by DNA-RNA hybridization (11) to stop accumulation of rRNA at 42 C. This can be easily assayed by the rapid halt of RNA labeling, because Nierlich (16) has shown that when the accumulation of stable RNA (rRNA, tRNA) is inhibited, RNA labeling stops, even though unstable RNA is still formed. The accumulation of stable RNA, including rRNA, stops very rapidly in strain HAK88 at 42 C (Fig. 3) and also shows that CHL partially restores RNA accumulation.

As Fig. 2 indicated, TET, like CHL, prevented synthesis of MS. However, unlike CHL, TET fails to restore RNA accumulation (Fig. 4).

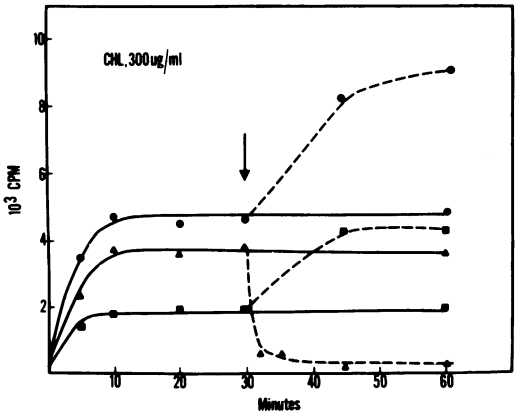

FIG. 1. *Metabolism of MS, GTP, and ATP in the presence and absence of CHL. HAK88 cells were grown to midlog phase, shifted to 42 C, and labeled with* ^{32}P *at zero time. Symbols: (—●—) ATP in the absence of CHL, (---●---) ATP in the presence of CHL, (—▲—) MS in the absence of CHL, (---▲---) MS in the presence of CHL, (—■—) GTP in the absence of CHL, (---■---) GTP in the presence of CHL.*

DISCUSSION

The analysis of the physiology of strain HAK88 at 42 C suggests that functional EFTs is not required for either MS or rRNA formation and defines some features of the relationship between ribosome function, MS formation, and rRNA synthesis.

MS synthesis. In vitro synthesis of MS has been shown to be dependent on codon-specific mRNA, the homologous uncharged tRNA, the stringency factor (which seems to be a pyrophosphoryl transferase), GDP, GTP, ATP, and ribosomes (7, 19). In the experiments with purified components, including charged tRNA, the synthesis of MS is prevented if EFTs and EFTu are present, suggesting a competition

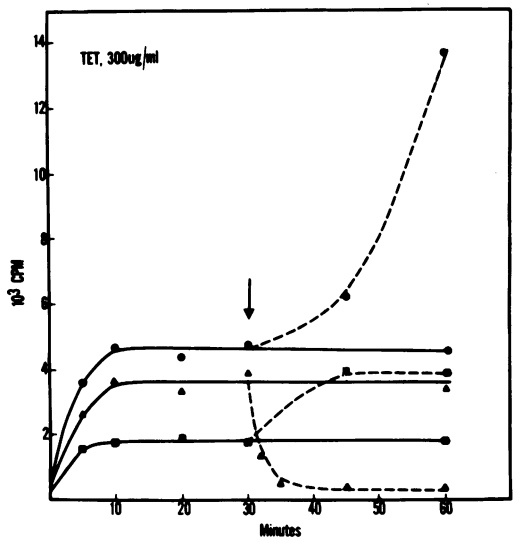

FIG. 2. *Metabolism of MS, GTP, and ATP in the presence and absence of TET. HAK88 cells were grown to midlog phase, shifted to 42 C, and labeled with ^{32}P at zero time. Symbols: (—●—) ATP in the absence of TET, (---●---) ATP in the presence of TET, (—▲—) MS in the absence of TET, (---▲---) MS in the presence of TET, (—■—) GTP in the absence of TET, (---■---) GTP in the presence of TET.*

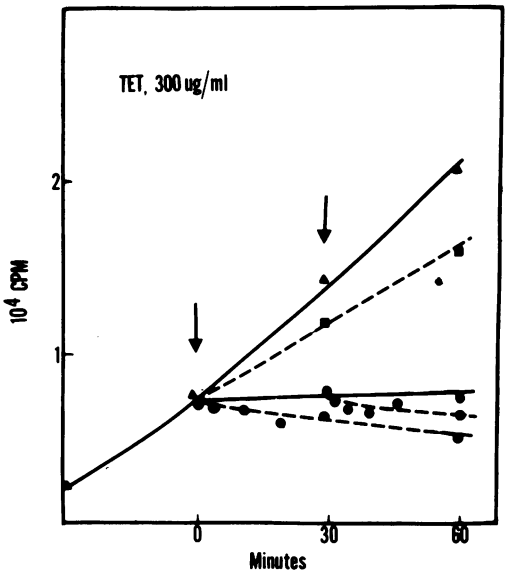

FIG. 4. *Accumulation of stable RNA in the presence and absence of TET. HAK88 cells were prelabeled with [5-^3H]uracil, and portions were transferred to 42 C at zero time. TET was added as indicated. Symbols: (—▲—) 30 C in the absence of TET, (—●—) 42 C in the absence of TET, (---■---) 30 C in the presence of TET, (---●---) 42 C in the presence of TET.*

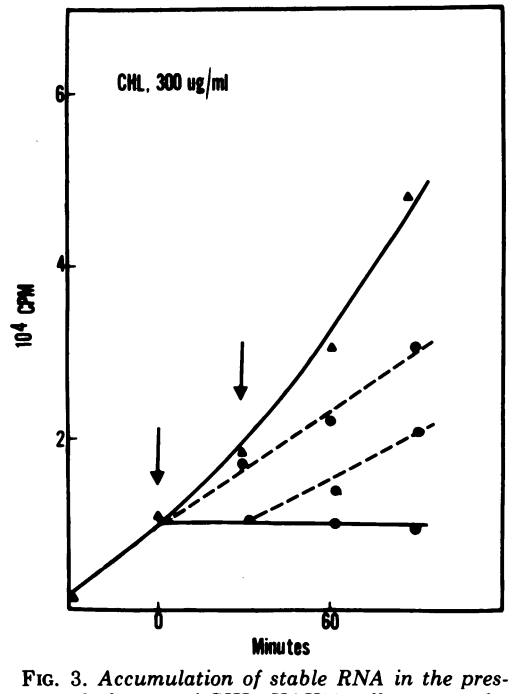

FIG. 3. *Accumulation of stable RNA in the presence and absence of CHL. HAK88 cells were prelabeled with [5-^3H]uracil, and portions were transferred to 42 C at zero time. CHL was added as indicated. Symbols: (—▲—) 30 C in the absence of CHL, (—●—) 42 C in the absence of CHL, (---●---) 42 C in the presence of CHL.*

between uncharged tRNA and aminoacyl-tRNA for the ribosomal acceptor site (7). Our results with strain HAK88 have shown that MS is formed even in the presence of a full complement of charged tRNA when EFTs is nonfunctional, in agreement with the results in vitro.

The signal for MS formation could still be the presence of uncharged tRNA in the acceptor site of the ribosome, as suggested by Haseltine and Block (7). This could occur as follows: according to standard formulations for protein synthesis (12), the addition of an amino acid to a growing peptide chain is followed by transfer of the peptidyl tRNA from acceptor to donor site. In presence of active EFTu, the next amino acyl tRNA then enters the acceptor site. However, if EFTs is inactive, as in strain HAK88 at 42 C, then EFTu is blocked as inactive EFTu-GDP. Uncharged tRNA has a high codon-specific affinity for the acceptor site (14). Therefore, even the low level of uncharged tRNA under these conditions would bind to the ribosome and could be sufficient to start MS synthesis. Furthermore, as soon as MS has begun to accumulate, it will bind very tightly to EFTu (15). This

would antagonize the formation of the aminoacyl-tRNA-EFTu-GTP complex even more severely, intensifying the block of amino acyl-tRNA binding to ribosomes.

Thus, the ultimate effect of the lesion in EFTs in strain HAK88 can be to increase the level of uncharged tRNA on the ribosome even in the presence of a full complement of charged tRNA in the cytoplasm. The formation of MS would then occur as if uncharged tRNA were present in the cell in large amounts (8).

Control of rRNA synthesis. Previous in vivo work by Atherly (1), with a temperature-sensitive synthetase mutant and a temperature-sensitive G factor mutant, has shown that the point at which arrest of protein synthesis elicits stringent control of rRNA accumulation lies between the activation of tRNA and the G factor-dependent hydrolysis of GTP. Our results in vivo further localize this point in protein synthesis by showing that it is apparently at or just after the binding of tRNA to the ribosomal acceptor site. The inference is based on the fact that although strain HAK88 has a known lesion in EFTs, and has a full complement of charged tRNA, it is nevertheless stringent at the nonpermissive temperature.

The in vivo results with strain HAK88 also show that CHL acts to relieve the stringent response, but apparently not, as has been previously suggested (8), by increasing the percentage of tRNA charged with amino acids. At least in this case there is a possible direct interaction of CHL with machinery involved in MS synthesis—in other words, at the ribosome. Perhaps, in line with Pestka's formulation of CHL actions (18), this might somehow decrease the fraction of uncharged tRNA at the acceptor site. The results with CHL might, however, be interpreted differently, consistent with earlier suggestions (8). For example, the growth of strain HAK88 is known to be partially recoverable at 42 C by added tRNA (9), and it might be possible that CHL addition leads to a small but important increase in the level of charging of tRNA.

The correlation of the presence of MS and the failure of rRNA accumulation has held in all of a large number of previous cases. The correlation, however, seems to break down in one case presented here. TET prevents the synthesis of MS in HAK88 cells at 42 C, but fails to restore rRNA accumulation. This suggests that the absence of MS and the presence of functional RNA polymerase are not sufficient to guarantee rRNA accumulation. The effect of TET may be independent of the lesion in EFTs, for when RNA accumulation was limited by tryptophan starvation at 30 C (the stringent response), TET again failed to restore RNA synthesis in strain HAK88. Presumably, MS must interact with an additional molecule, possibly a "psi factor" (20), receptor protein, or a negative control element, to produce the stringent response. Among the possible candidates for such a factor are ribosomal proteins, or as Travers envisioned, EFTuTs (21). However, any such factor apparently does not include functional EFTs, because in the presence of CHL at 42 C, HAK88 cells accumulate RNA (Fig. 3). An independent genetic argument for the existence of additional elements comes from the isolation of mutants specifically temperature-sensitive in rRNA accumulation (S. Chaney and D. Schlessinger, manuscript in preparation).

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