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Correlation between Histidine Operon Expression and Guanosine 5′-Diphosphate-3′-Diphosphate Levels during Amino Acid Downshift in Stringent and Relaxed Strains of *Salmonella typhimurium*

RICHARD F. SHAND,† PAUL H. BLUM,‡ REINHOLD D. MUELLER,§ DANIEL L. RIGGS, ‖ AND STANLEY W. ARTZ*

Department of Microbiology, University of California, Davis, California 95616

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We have analyzed the correlation of attenuator-independent expression of the *Salmonella typhimurium* histidine operon in vivo with levels of the "alarmone" guanosine 5′-diphosphate 3′-diphosphate. Amino acid downshift caused by serine hydroxamate addition increased *his* expression in a *relA*$^+$ strain and decreased *his* expression in a *relA* mutant, whereas levels of guanosine 5′-diphosphate-3′-diphosphate varied in parallel with the changes in *his* expression in the two strains. In several experiments, overall variations in *his* expression ranged from 20- to 60-fold after downshift. The mild downshift allowed growth of the cultures to continue at near-preshift rates. Serine hydroxamate addition was also used to analyze the effect of amino acid downshift on induced expression of wild-type and mutant *lac* promoters. There was a 12-fold difference in *lac* expression when a *relA*$^+$-*relA1* pair was subjected to mild starvation but only a 3-fold difference when the strains carried the *lacZpL8UV5* promoter mutation. These results suggest that guanosine 5′-diphosphate-3′-diphosphate stimulates gene expression in vivo at the level of transcription initiation.

The histidine operons of *Salmonella typhimurium* and *Escherichia coli* are under control of two distinct regulatory mechanisms: (i) a well-characterized mechanism with translational control of transcription termination (attenuation mechanism; reviewed in references 1 and 28); and (ii) a less well-defined mechanism involving the "alarmone" guanosine 5′-diphosphate 3′-diphosphate (ppGpp), which mediates general amino acid control (reviewed in references 1 and 5). Attenuation regulates *his* expression in response to the levels of charged histidyl-tRNA specifically, whereas ppGpp attunes *his* expression to the availability of amino acids in general (24) and is the focus of this study.

Expression of the *his* operon has been correlated with ppGpp levels both in vivo (22, 24, 29, 30) and in vitro (21, 24). Correlations in vivo have usually made use of changes in steady-state growth conditions or mutants (*spoT*) defective in ppGpp degradation, which alter ppGpp levels at most two- to fourfold. Correspondingly, the expression of the *his* operon varies only two- to fourfold under these conditions. In addition, expression of the *his* operon is stimulated to nearly maximum levels by the basal ppGpp concentration present in cultures grown in minimal medium (24). Therefore, amino acid downshift of a stringent (*relA*$^+$) strain grown in minimal medium, which causes a large accumulation of ppGpp, does not result in much increase in *his* expression. In contrast to these results in vivo, attenuator-independent *his* expression in vitro has been shown to vary over a 10- to 20-fold range in correlation with ppGpp levels (20, 24). These results were obtained with a DNA-dependent in vitro protein-synthesizing system with an S-30 (30,000 × *g* supernatant) cell extract devoid of ppGpp so that the full extent of stimulation by added ppGpp could be assessed.

Determination of the full range of effect of ppGpp levels on *his* expression in vivo requires that ppGpp concentrations be decreased below basal levels. Amino acid starvation of a relaxed (*relA*) mutant provides an experimental condition to produce this effect (10, 12, 23) and has been used to study ppGpp regulation of stable RNA synthesis (10, 11, 13) (see Discussion).

We have devised a mild starvation method by using the serine analog serine hydroxamate, which increases ppGpp levels in a *relA*$^+$ strain and decreases ppGpp levels in a *relA* mutant. The mild starvation allows growth to continue at near-normal rates so that differential rates of enzyme synthesis can be measured accurately. The method works well in both complex and minimal media, and we have used it to show that the full range of regulation of attenuator-independent *his* expression in vivo is 20- to 60-fold. The relative insensitivity of expression of a known ppGpp-insensitive promoter (*lacZpL8UV5*) (16, 17) to serine hydroxamate addition in *relA*$^+$ and *relA1* strains suggests that a large component of the regulatory effect occurs at the level of transcription initiation.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The *S. typhimurium* LT2 derivatives used in this study are listed in Table 1. Unless otherwise indicated, complex medium (supplemented nutrient broth [SNB medium]) contained 0.8% (wt/vol) nutrient broth (Difco Laboratories) supplemented with 0.5% (wt/vol) NaCl, 1× Vogel and Bonner Salts (27), 0.4% (wt/vol) glucose, and 0.75 mM serine (since nutrient broth is deficient in this amino acid; see Results). Phosphate limited (0.2 mM) minimal MOPS (morpholinepropanesulfonic acid) medium was essentially as described previ-

* Corresponding author.
† Present address: Department of Biochemistry/Biophysics, University of California, San Francisco, CA 94143.
‡ Present address: Department of Medical Microbiology, Stanford University, Stanford, CA 94305.
§ Present address: Department of Biology, Marquette University, Milwaukee, WI 53233.
‖ Present address: Department of Biology, University of California, San Diego, CA 92093.

TABLE 1. Bacterial strains

Strain	Genotype	Source
TA2437	*zga-607*::Tn*10 relA1 dhuA1*	B. Ames
AZ1413[a]	*pyrC1502 ΔhisGa1242 Φ(hisHAF-lac)*	This work
AZ1460[a]	*ΔhisGa1242 Φ(hisGD-lac)*	This work
AZ1471	Isogenic to AZ1460 but *zga-607*::Tn*10 relA*$^+$	P22(TA2437) × (AZ1460)[b]
AZ1472	Isogenic to AZ1460 but *zga-607*::Tn*10 relA1*	P22(TA2437) × (AZ1460)
AZ1477	*F' lacZpL8UV5 proAB*$^+$ (*pro*::Tn*10 dhuA1*)	This work
AZ1478	Isogenic to AZ1477 but *relA1*	This work
AZ1480	*F' lacZp*$^+$ *proAB*$^+$ (*pro*::Tn*10 dhuA1*)	This work
AZ1481	Isogenic to AZ1480 but *relA1*	This work
AZ2199	Isogenic to AZ1413 but *zga-607*::Tn*10 relA*$^+$	P22(TA2437) × (AZ1413)
AZ2200	Isogenic to AZ1413 but *zga-607*::Tn*10 relA1*	P22(TA2437) × (AZ1413)

[a] Mu dl *cts* (Ap^r *lac*) fusions were isolated and immobilized as described previously (3). The point of insertion of the Mu dl phage in strain AZ1413 was localized to the *hisH*, *hisA*, or *hisF* gene and in strain AZ1460 to the *hisG* or *hisD* gene. The immobilization procedure renders the strain heat resistant, so that growth experiments can be done at 37°C.

[b] Phage P22 transductional crosses are described as follows: P22(donor) × (recipient).

ously (4) and supplemented with 50 μg of histidine hydrochloride per ml and 40 μg of uracil per ml. All cultures were grown at 37°C with shaking. Severe amino acid starvation in minimal MOPS medium was induced by the addition of DL-serine hydroxamate (Sigma Chemical Co.) to a final concentration of 10 mM (concentrations are for DL-serine hydroxamate; note that only the L form is active [25]). Mild amino acid starvation in SNB medium and in minimal MOPS medium supplemented with 1.0 mM serine was induced by adding serine hydroxamate to a final concentration of 2 mM. Stock solutions of serine hydroxamate (0.5 M) were prepared fresh and kept on ice before use. Cultures were in exponential growth for three to four generations before the addition of the analog. Serine hydroxamate addition was done at an optical density at 650 nm (OD_{650}) between 0.1 and 0.25 in different experiments. One OD_{650} was determined to be 1.3×10^9 cells per ml in minimal MOPS medium with a Gilford 250 spectrophotometer.

Mild amino acid starvation of strains carrying *lac* promoters was done by the addition of serine hydroxamate (2 mM final concentration) to cultures growing in SNB medium. Ten minutes later, *lac* expression was induced by adding isopropyl-β-D-thiogalactoside (1 mM final concentration).

Sample preparation and β-galactosidase assays. Growth and protein synthesis were stopped by adding 1-ml culture samples to 100 μl of ice cold ethanol containing 11 mg of chloramphenicol per ml. Cells were lysed by adding 15 μl of freshly prepared lysis solution (18) and vortexing for 15 s. The toluene was evaporated by incubation at 37°C for 30 min. Samples (10 to 100 μl) were assayed in duplicate at 28°C in a reaction mixture containing 100 μl of orthonitrophenyl-β-D-galactopyranoside (4 mg/ml) in 0.1 M phosphate buffer (pH 7.0) and sufficient Z buffer (14) to bring the total reaction mixture to 0.5 ml. Reactions were terminated by adding 250 μl of 1 M Na_2CO_3 (stop solution) and chilling on ice for 10 min. Cell debris was then removed by centrifugation for 5 min in a Fisher model 235B microfuge, and the A_{420} was determined.

Specific activities were determined as differential rates of enzyme synthesis from differential rate plots with at least five samples taken at about 10-min intervals during growth. β-Galactosidase units were calculated by using the formula: β-galactosidase units = $[A_{420}/(t)(v)(2)(0.666)] \times 1{,}000$, where t is the time of reaction in minutes, and v is the volume of culture used in the assay in milliliters. The A_{420} is divided by 2 because the assays were performed in 0.5 ml instead of 1.0 ml and divided by 0.666 to take into account the dilution of the assay mixture by the stop solution. Specific activities were calculated by dividing enzyme units by the OD_{650} of the culture at the time of sampling.

Labeling nucleotide pools with $^{32}P_i$. Cultures were labeled as described previously (4) with the following modifications. Overnight cultures (10 ml) were grown in supplemented minimal MOPS medium with limiting glucose (0.04% wt/vol) at 37°C with shaking. The cells were harvested by centrifugation at room temperature and suspended in 0.1 volume of 0.85% (wt/vol) NaCl. Then 10 ml of supplemented minimal MOPS medium (0.4% [wt/vol] glucose) containing 63 to 126 μCi of $^{32}P_i$ (carrier-free, in acid-free aqueous solution from Amersham Corp.) per ml was inoculated with 30 μl of suspended cells (initial OD_{650} of ~0.01). The labeled cultures were incubated at 37°C, and the increase in OD_{650} was monitored by using a 250-ml Erlenmeyer flask modified with a sidearm cuvette. The addition of the radioisotope increased the generation times of strains AZ2199 and AZ2200 from 47 to 61 min. Culture samples (200 μl) were extracted with formic acid (20 μl) on ice, and unincorporated P_i was precipitated by the tungstate-tetraethylammoniumprocaine method (4).

Quantitation of ppGpp by thin-layer chromatography. Basal ppGpp levels in the *relA*$^+$ and *relA1* strains and *relA1* postshift ppGpp levels were determined by two-dimensional thin-layer chromatography. All procedures were performed at 4°C since this was found to maximize recovery of ppGpp. Polyethyleneimine-cellulose thin-layer chromatography plates (Brinkmann Instruments, Inc.) were prerun with 10% (wt/vol) NaCl in double-distilled water (19). Extracts (20 μl) were spotted in two 10-μl applications and blown dry. The first-dimension solvent system (solvent system 2 of Gallant et al. [9]: 2 M HCOOH, 1.5 M LiCl [pH 3.4]) was titrated by adding solid NaOH to just less than pH 3.4 and then making the final adjustment with 10 M NaOH. The plates were developed in the first dimension to the top (~4 to 6 h), washed in cold methanol for 20 min, and blown dry (~2 h). The second dimension (1.5 M KH_2PO_4 [pH 3.4]) was run until the front reached the top of the plate (~6 h). The plate was removed from the chamber and blown dry. Autoradiography was performed by sandwiching a sheet of Kodak XAR-5 film between two Lightning Plus intensifying screens (screens face each other) and placing them on top of the thin-layer plate(s). Exposure was for 3 days at −80°C. The areas on the thin-layer chromatograms containing ppGpp, and equivalent blank areas for background counts were scraped off with a scalpel and placed in 10 ml of liquid

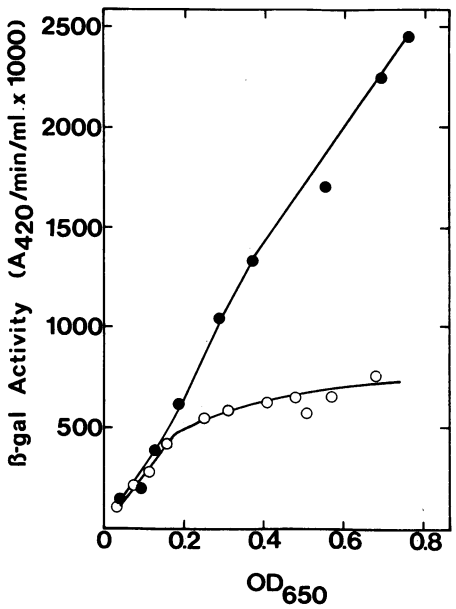

FIG. 1. Differential rates of *his-lac* expression in strain AZ1471 (*relA*$^+$; ●) and strain AZ1472 (*relA1*; ○) during unrestricted growth in SNB medium lacking serine.

scintillation cocktail [631.2 ml of toluene, 368.7 ml of Triton X-100, 2.68 g of 2,5-diphenyloxazole, 0.18 g of 1,4-bis(5-phenyloxazolyl)benzene]. Samples were counted for 10 min on a Beckman LS3145P liquid scintillation counter with the variable discriminator module set at a lower limit of 397 and an upper limit of 940. Efficiencies of counting [(counts per minute)/(disintegrations per minute) × 100] of ^{32}P samples of known radioactivity (disintegrations per minute) were between 95 and 100%. Quenching by polyethyleneimine-cellulose was negligible.

Postshift ppGpp levels in the *relA*$^+$ strain were determined by spotting 10-μl extracts onto a polyethyleneimine-cellulose plate prerun as above followed by one-dimensional chromatography in 1.5 M KH_2PO_4 (pH 3.4 at 4°C). The polyethyleneimine-cellulose plate was dried and treated as described above, except film exposure was reduced to 4 h.

RESULTS

Altered histidine operon expression in Rel$^+$ and Rel$^-$ strains during unrestricted growth in complex medium. We initially made the surprising observation that the differential rate of expression of the *his* operon changed in a *relA*$^+$ strain and a *relA* mutant during unrestricted, exponential growth in a nutrient broth-based (Difco) complex medium (SNB medium lacking serine) (Fig. 1). In this experiment, as in other experiments in this paper, *his* expression was determined by measurements of β-galactosidase activity encoded by the *lacZ* gene, which had been transcriptionally fused to the *his* operon promoter (Table 1). In addition, the strains carry the Δ*hisGal1242* mutation, which renders *his* operon expression constitutively derepressed and independent of the attenuation mechanism.

During early exponential growth in SNB medium lacking serine, both the *relA*$^+$ strain (AZ1471) and the *relA1* mutant (AZ1472) maintained a differential rate of about 2,500 units per OD_{650} unit. At an OD_{650} of about 0.2 the differential rate of *his* expression in both strains underwent an apparent shift, with the *relA*$^+$ strain transiently increasing the rate slightly and the *relA1* mutant decreasing the rate about 10-fold. Throughout this period of shift in differential rates there was no measurable alteration in the 25-min generation time of either strain.

The abrupt shift in differential rates of *his* expression during unrestricted growth in complex medium implied some change in nutrient supply. Because we had previously observed that nutrient broth fails to support full growth of a serine auxotroph, we tested whether serine supplementation would alter the shift in differential rates and found this to be the case (data not shown). In particular, the marked decrease in *his* expression in the *relA1* mutant at an OD_{650} of 0.2 (Fig. 1) was eliminated by the addition of serine to the medium, implying that serine depletion was the cause of the shift.

The above results suggested that attenuator-independent regulation of the *his* operon was responsive to increased and decreased levels of ppGpp caused by mild starvation in the *relA*$^+$ strain and *relA* mutant, respectively. Since serine might vary in amount in different lots of nutrient broth, we standardized the mild amino acid downshift condition by using the serine analog serine hydroxamate in serine-supplemented media (either complex SNB medium or minimal MOPS medium; see Materials and Methods).

Effect of serine hydroxamate on growth of an isogenic pair of *relA*$^+$/*relA1* strains. The serine analog serine hydroxamate is a competitive inhibitor of seryl-tRNA synthetase (26). We added serine hydroxamate at different concentrations to exponentially growing bacterial cultures to vary starvation conditions from severe, where cell growth was rapidly arrested, to mild, where cell growth was only moderately decreased (Fig. 2). During severe starvation (10 mM serine hydroxamate), cultures of the *relA*$^+$ strain AZ2199 and the *relA1* mutant AZ2200 continued to increase in optical density slightly but with apparent generation times greater than 6 h (Fig. 2A). During mild starvation (2 mM serine hydroxamate), cultures of the *relA*$^+$ strain and the *relA1* mutant continued at the preshift growth rate for about 15 min and then established slower growth rates for about 30 to 40 min before growth ceased.

Although growth of the cultures after starvation is not required for ppGpp synthesis, severe starvation makes it difficult to analyze the differential rate of enzyme synthesis since the increase in culture mass is so slow. The mild starvation conditions we employed allow growth and protein synthesis to continue at relatively rapid rates while causing substantial perturbation of the ppGpp pools (see below).

It should be noted that, although the results shown in Fig. 2 were obtained with cultures grown in minimal MOPS medium, very similar results were obtained in complex SNB medium (data not shown). SNB medium included the addition of sufficient serine (0.75 mM) to overcome the serine depletion effect shown in Fig. 1 while still allowing 2 mM serine hydroxamate to elicit mild starvation. (A comparable mild starvation effect of 2 mM serine hydroxamate on cells grown in minimal MOPS medium occurred when serine was added to the medium at 1.0 mM.)

Response of *his* operon expression to mild amino acid starvation caused by serine hydroxamate. Figure 3 shows the effect of mild starvation by serine hydroxamate on *his* expression in immobilized Mu dl *his-lac* fusion derivatives of a *relA*$^+$ strain (AZ1471) and an isogenic *relA* mutant (AZ1472); both strains lack the *his* attenuator (Δ*hisGal1242*). Before the addition of serine hydroxamate, the steady-state differential rate of *his* expression in the *relA* mutant (1,900 U/OD_{650}) was approximately the same as that in the *relA*$^+$

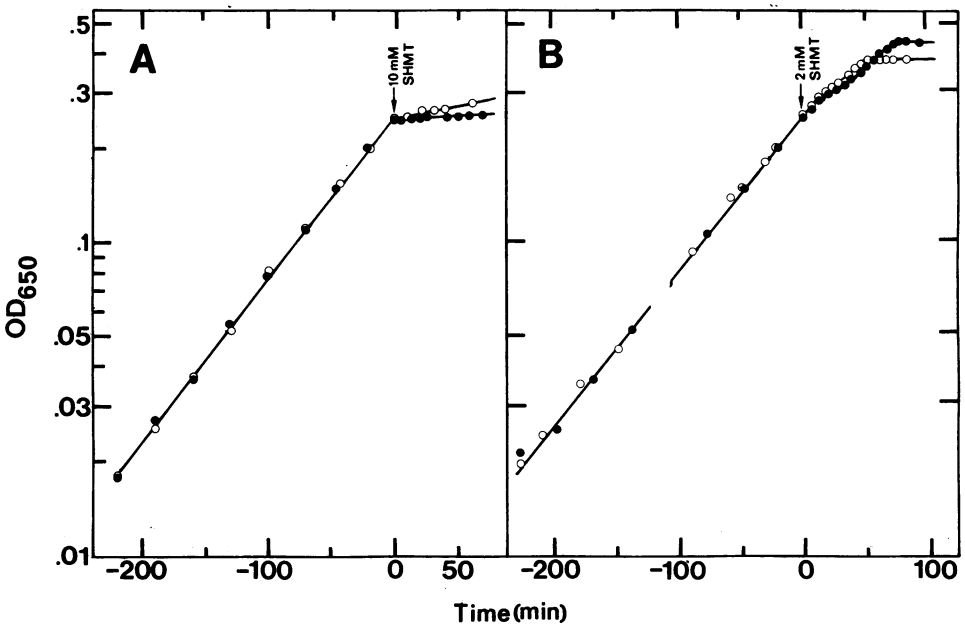

FIG. 2. Effect of severe (A) and mild (B) starvation by serine hydroxamate (SHMT) on growth rate of strain AZ2199 (*relA*$^+$; ●) and strain AZ2200 (*relA1*; ○). Strains were grown in minimal MOPS medium containing 63 μCi of $^{32}P_i$ per ml (A) or 126 μCi of $^{32}P_i$ per ml (B). The preshift doubling time for both strains was 61 min. Serine hydroxamate was added to cultures (arrows) at an OD_{650} of ~0.25.

strain (1,960 U/OD_{650}). In the *relA*$^+$ strain, the differential rate of *his* expression increased to 4,600 U/OD_{650} after serine hydroxamate addition. This 2.4-fold increase in differential rate occurred after a delay of about 7 min, as determined by extrapolation from the corresponding growth curve (data not shown, but similar to those in Fig. 2B). The postshift differential rate of 4,600 U/OD_{650} lasted for 20 min before an intermediate differential rate of about 3,300 U/OD_{650} was established. In the *relA* mutant, *his* expression decreased to a differential rate of 80 U/OD_{650} 10 min after the addition of the analog.

The 2.4-fold increase in differential rate in the *relA*$^+$ strain and the 24-fold decrease in differential rate in the *relA* mutant represent about a 60-fold range of *his* expression in response to mild amino acid starvation. In different experiments, the overall range of effect varied from 20- to 60-fold. The variability in the overall effect depended largely on the error in measuring the differential rate of *his* expression in the *relA* mutant after serine hydroxamate addition. The differential rate under these conditions is quite small.

Effect of severe and mild starvation by serine hydroxamate on ppGpp levels and the correlation with *his* expression. Severe amino acid starvation (10 mM serine hydroxamate) in a *relA*$^+$ strain (Fig. 4A) elicited an increase in ppGpp from a basal level of 28.7 pmol/OD_{650} to a maximum level of 1,042 pmol/OD_{650}. This 36-fold increase in the ppGpp level started immediately upon serine hydroxamate addition and was completed within 7 min. A new steady-state level of ppGpp was established after approximately 15 min. In contrast, ppGpp levels in the *relA1* strain decreased from a basal level of 21.8 pmol/OD_{650} to about 2 pmol/OD_{650}. This 10-fold decrease in the ppGpp level started immediately upon serine hydroxamate addition, and the new, lowered steady-state level was established in about 3 min. Therefore, there was about a 500-fold difference in ppGpp levels between the *relA*$^+$ strain and the *relA1* mutant after severe starvation.

After mild amino acid starvation (2 mM serine hydroxamate) (Fig. 4B), the magnitude of the effect on ppGpp levels

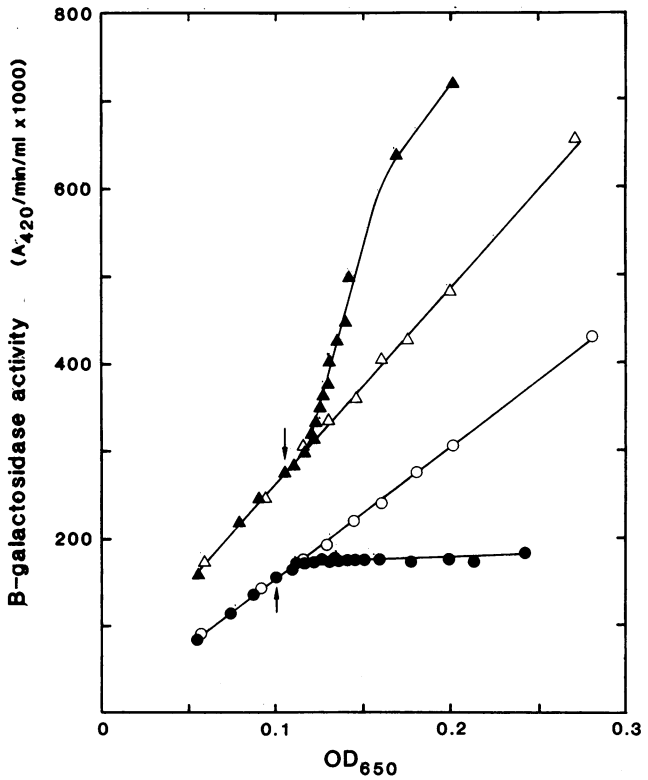

FIG. 3. Effect of mild starvation on the differential rate of *his-lac* expression in strain AZ1471 (*relA*$^+$; ▲, △) and strain AZ1472 (*relA1*; ●, ○). Strains were grown in SNB medium and shifted with 2 mM serine hydroxamate (▲, ●) at arrows or not shifted (△, ○). The effect of the mild starvation on growth was similar to that shown in Fig. 2B.

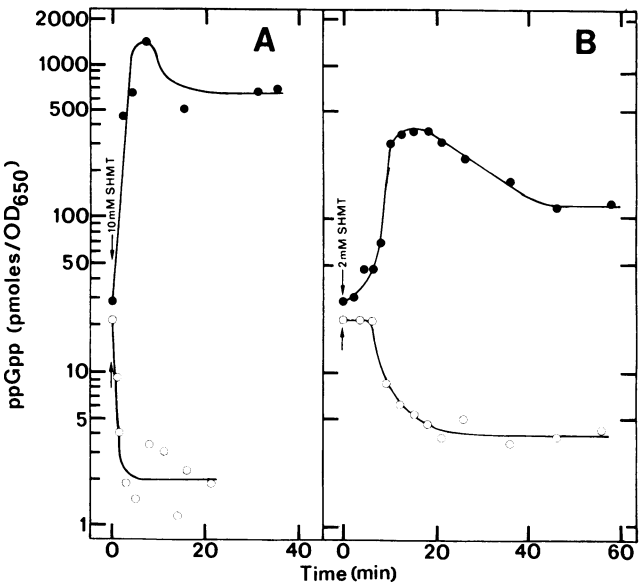

FIG. 4. Effect of severe (A) and mild (B) starvation by serine hydroxamate (SHMT) on ppGpp levels in strain AZ2199 (*relA*$^+$; ●) and strain AZ2200 (*relA1*; ○). Growth conditions were as described in the legend to Fig. 2. Basal ppGpp levels are averages of 5 samples: for AZ2199, 28.7 pmol of ppGpp per OD_{650} (22.2 μM intracellular concentration); for AZ2200, 21.8 pmol of ppGpp per OD_{650} (16.7 μM intracellular concentration). The variation in basal ppGpp levels in five experiments was ±10%. The parameters used to estimate intracellular concentrations were 1.3×10^9 cells per ml per OD_{650} and an assumed average volume of 10^{-15} liter per cell.

was smaller and the kinetics of the response was delayed as compared with those under severe starvation conditions. In the *relA*$^+$ strain, ppGpp increased slowly over the first 6 to 8 min after serine hydroxamate addition, reached a maximum (400 pmol/OD_{650}, or about 13 times higher than the basal level) by 15 min, and then declined to a new steady-state level of 120 pmol/OD_{650}. After mild starvation of the *relA1* mutant the ppGpp level was unchanged for 6 to 8 min and then decreased 5.5-fold to 4 pmol/OD_{650} over the next 14 min (Fig. 4B). The overall difference in ppGpp levels between the mildly starved *relA*$^+$ strain and *relA1* mutant was about 100-fold.

A comparison of Fig. 3 and 4B shows the correlation between the changes in differential rate of *his* expression and the changes in ppGpp levels after mild amino acid starvation. In the *relA*$^+$ strain, there was a similar lag before both the increase in *his* expression and the increase in ppGpp level. Both parameters reached maximal levels at about the same time and then declined to new steady-state levels with a similar time course. In the *relA* mutant, there was a similar lag before both the decline in *his* differential rate of expression and the decline in ppGpp level, after which both parameters remained constant over the duration of the experiment. Although it is not possible to establish a definitive cause-and-effect relationship between ppGpp levels and changes in differential rates of *his* expression from kinetic experiments, the overall correlation is striking.

Effect of mild starvation by serine hydroxamate on expression of wild-type (*lacZp*$^+$) and mutant (*lacZpL8UV5*) *lac* promoters. The response of the wild-type *lac* promoter to mild amino acid starvation by serine hydroxamate in *S. typhimurium* was dependent upon the *relA* allele (Table 2), consistent with previous experiments that used a strong

TABLE 2. Effect of the *relA* allele on expression of wild-type *lac* and *lacZpL8UV5* promoters upon amino acid downshift[a]

Strain	Relevant genotype	β-Galactosidase sp act[b]	*relA*$^+$/*relA1* ratio
AZ1480	*relA*$^+$ *lacZp*$^+$	2,750	12.2
AZ1481	*relA1 lacZp*$^+$	225	
AZ1477	*relA*$^+$ *lacZpL8UV5*	10,000	3.1
AZ1478	*relA1 lacZpL8UV5*	3,220	

[a] Cultures were grown in SNB medium. Serine hydroxamate was added at an OD_{650} of 0.15, and isopropyl-β-D-thiogalactoside was added 10 min later. Preshift generation times were ~35 min, and postshift generation times varied from 55 to 60 min. Each of the cultures was tested at the end of the experiment for retention of the F′ *lac*; greater than 95% of the cells retained the plasmid.

[b] Postshift specific activities were determined from differential rate plots as described in Materials and Methods with the units $[(A_{420}/\text{min})/OD_{650}] \times 1{,}000$.

starvation in *E. coli* (16). The differential rate of β-galactosidase synthesis of the induced wild-type *lac* promoter was 12.2-fold lower in the *relA1* mutant (AZ1481) than in the *relA*$^+$ strain (AZ1480) after the addition of serine hydroxamate. In contrast, expression of the *lacZpL8UV5* promoter was only 3.1-fold lower in the *relA1* mutant (AZ1478) than in the *relA*$^+$ strain (AZ1477). The interpretation of these experiments is that the rate of transcription initiation of the wild-type *lac* promoter correlates with changes in ppGpp levels (16, 17). By analogy, we conclude that changes in *his* expression in vivo most likely occur largely at the level of transcription initiation.

DISCUSSION

Results in this paper show that attenuator-independent expression of the *his* operon in vivo undergoes marked changes in response to mild amino acid downshift caused by serine hydroxamate addition to cultures of *S. typhimurium* strains. The changes in *his* expression correlate well with variations in ppGpp levels, implying that ppGpp may be an effector of *his* expression in vivo. This implication is supported by experiments that revealed a correlation in vivo between *his* expression and ppGpp levels under steady-state growth conditions (22, 24, 29, 30) and in vitro in a cell-free protein-synthesizing system (21, 24). A strong, transient decrease in *his* expression during mild amino acid downshift of a *relA* mutant has also been observed (24). In this earlier experiment, however, it was difficult to distinguish between the independent effects of ppGpp and the *his* operon-specific attenuation mechanism since the downshift was caused by a mild histidine starvation and the strains had intact attenuation regulation. In addition, it was not known that *relA* mutants respond to amino acid starvation by decreasing their ppGpp levels, as was shown subsequently by Lagosky and Chang (10).

The regulatory mechanism(s) responsible for the 20- to 60-fold changes in *his* expression observed during mild amino acid downshift cannot be determined with certainty from the experiments reported here. Although the changes clearly are independent of the *his* attenuation mechanism, synthesis or degradation of *his-lac* mRNA, translational effects, or a combination of these could be involved in the regulatory response.

Several lines of evidence, however, support the conclusion that a significant component of the regulatory effect on *his* expression occurs at the level of transcription initiation of the *his* promoter. First, expression of the *his* operon in vitro is stimulated 10- to 20-fold by physiological concentrations (50 to 100 μM) of ppGpp in a cell-free protein-

synthesizing system (21, 24), and experiments in which transcription was uncoupled from translation indicate that the stimulation by ppGpp occurs entirely at the level of transcription (24). Second, some mutations that alter the sequence of the *his* promoter either in the −10 hexamer sequence or between the −10 hexamer sequence and the start point of transcription at +1 alleviate the stimulation by ppGpp and render expression of the *his* operon nearly independent of ppGpp in vitro (21). Other *his* promoter mutations cause *his* expression to become hyperstimulated by ppGpp in the cell-free system (D. Riggs and S. Artz, manuscript in preparation). Third, expression of the wild-type *lac* promoter is stimulated by ppGpp in a cell-free protein-synthesizing system (17, 31), and the differential rate of β-galactosidase synthesis under control of the wild-type *lac* promoter correlates with ppGpp levels during amino acid downshift in vivo (Table 2, Fig. 4) (16). In contrast, expression of the mutant *lacZpL8UV5* promoter is relatively insensitive to changes in ppGpp concentration both in vitro (17, 21) and in vivo (Table 2) (16).

Experiments with wild-type and mutant *lac* operon promoters are consistent with a general role for ppGpp in stimulating transcription initiation of certain promoters. Results with the *lac* operon, however, do not provide an ideal experimental control for fused *his-lac* expression. For example, the *lacZ* gene in the *his-lac* transcriptional fusion construct is translated from a *trpA* ribosome-binding site (15), which could behave differently than the *lacZ* ribosome-binding site during amino acid downshift. Definitive evidence concerning the extent to which changes in transcription initiation frequencies are involved in determining the *his* regulatory response to amino acid downshift awaits the analysis in vivo of *his* promoter mutants that alter the response of *his* expression to ppGpp in vitro (see above). These experiments are in progress.

Little is known about the synthetic pathway(s) and regulatory mechanism(s) responsible for maintaining basal ppGpp levels that occur in unstarved cultures during balanced growth (5). There is substantial evidence, however, that the ribosomal, *relA*-dependent pathway is only partially responsible for basal ppGpp levels in *E. coli* or *S. typhimurium*. For example, the *relA1* mutant of *S. typhimurium* used in this work maintained a ppGpp level during balanced growth in minimal glucose medium at about 75% of that of its isogenic *relA*$^+$ counterpart (i.e., 22 as compared with 29 pmol/OD_{650}; Fig. 4). (The *S. typhimurium relA1* mutation behaves in all respects similarly to tight, loss-of-function, *E. coli relA* mutations including deletion and insertion mutations [2, 7].)

A substantial amount of the variation in *his* expression in correlation with ppGpp levels occurred during amino acid downshift in the *relA* mutant (Fig. 1 and 3) and presumably, therefore, reflects changes in the *relA*-independent rate of accumulation of ppGpp. Although we used serine hydroxamate addition to *relA* mutant cultures as a convenient but artificial means to examine the full range of ppGpp-dependent regulation of *his* expression, we believe that the results likely represent a physiologically relevant response.

Friesen et al. (8) observed in *E. coli* that an upshift from minimal acetate medium to minimal glucose medium containing an excess of the 20 amino acids resulted in a rapid decline (20- to 40-fold) in basal ppGpp levels that persisted for 30 to 40 min. Concomitant with the decline in basal ppGpp levels there was an increase in the rate of accumulation of stable RNA. Since the decline in ppGpp level occurred similarly in a *relA*$^+$ strain and in a tight *relA* mutant, it may be concluded that the *relA*-independent pathway(s) is primarily responsible for adjusting ppGpp levels to conditions of amino acid excess. In addition, the extent of the decline in basal ppGpp level was similar in a *spoT*$^+$ strain and a *spoT1* mutant (6), indicating that it is the synthesis rather than the degradation of ppGpp that is regulated during upshift.

Thus, the *relA*-independent pathway(s) responds to both amino acid starvation (10) and excess (6, 8) by reducing ppGpp synthesis. A physiological rationale for this response is as follows. During amino acid starvation of a wild-type strain, the *relA*-dependent mechanism predominates and is responsible for the accumulation of large amounts of ppGpp. (In this situation, the *relA*-independent pathway is superfluous and is shut off.) This prepares the cell for slower growth during amino acid starvation conditions (e.g., by increasing amino acid biosynthesis and decreasing rRNA synthesis). In contrast, during amino acid excess, ppGpp levels decline because both the *relA*-dependent and *relA*-independent pathways are inhibited. This prepares the cell for faster growth during amino acid excess conditions (e.g., by decreasing amino acid biosynthesis and increasing rRNA synthesis). Starvation of a *relA* mutant (as by serine hydroxamate addition) mimicks the physiological situation of amino acid excess in a wild-type strain.

The wide range in levels of ppGpp that result from variations in the activities of the *relA*-dependent and *relA*-independent pathways provokes a complex array of both positive and negative regulatory responses that allow the cell to adapt efficiently to conditions of amino acid starvation or excess (24).

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