Mutations That Render the Promoter of the Histidine Operon of Salmonella typhimurium Insensitive to Nutrient-Rich Medium Repression and Amino Acid Downshift

XAVIER J. DA COSTA* AND STANLEY W. ARTZ

Section of Microbiology, University of California, Davis, California 95616

Received 3 May 1996/Accepted 30 May 1997

The effects of mutations in the promoter of the histidine operon of Salmonella typhimurium were examined in vivo. The wild-type chromosomal copy of the his promoter was replaced with mutations in the −10 hexamer sequence and in the region between the −10 hexamer and the transcriptional start point—termed the discriminator sequence. The substitutions were performed with a phage M13 allele replacement system. Expression of the his operon is known to correlate with levels of guanosine 5′,3′-bispyrophosphate (ppGpp) in vivo. Strains containing either the wild-type his promoter or his promoter mutations were grown in both nutrient-rich and minimal media under steady-state conditions known to alter intracellular levels of ppGpp in a predictable way. The effect of the presence or absence of the his attenuator was assessed under these conditions as well. Expression of the his operon was studied by measuring the differential rate of β-galactosidase synthesis with a his-lac transcriptional fusion. Regulation of the his operon in the promoter mutants was also studied under conditions of a transient amino acid downshift induced by the addition of serine hydroxamate to cultures growing in nutrient-rich medium. These growth conditions cause elevated levels of ppGpp.

The results provide physiological confirmation of previous evidence obtained with a coupled transcription-translation system in vitro which indicated that ppGpp regulates interaction of RNA polymerase at the his promoter. More specifically, the in vivo evidence shows that the region of the his promoter that includes the −10 hexamer and discriminator sequences is the target at which ppGpp stimulates transcription.

Expression of the histidine operon in the enteric bacteria Salmonella typhimurium and Escherichia coli is subject to two different control mechanisms; (i) an operon-specific attenuation mechanism and (ii) a global regulatory mechanism dependent on overall amino acid availability (1, 26). The attenuation mechanism is well characterized and is governed primarily by the levels of His-tRNAHis in the cell. When His-tRNAHis levels decline, his operon expression rises and vice versa. The global regulatory mechanism is less well understood, but the available evidence indicates that it is mediated by levels of the general signal molecule, or alarmone, guanosine 5′,3′-bispyrophosphate (also known as guanosine tetraphosphate or ppGpp). When levels of ppGpp rise, his operon expression is stimulated and vice versa.

The intracellular level of ppGpp depends on the activities of two enzymes that are the products of the relA and spoT genes (6, 29). The ribosome-associated RelA enzyme synthesizes ppGpp when uncharged tRNA occupies the ribosome’s acceptor site (e.g., during amino acid downshift). The mechanism of regulation of the cytoplasmic SpoT enzyme is somewhat obscure (9, 19), but ppGpp accumulation as a function of SpoT activity appears to correlate inversely with the growth rate in response to the nutrient richness of the growth medium (5). In addition, SpoT synthesizing activity is inhibited during amino acid downshift concomitant with the activation of the RelA enzyme (13, 21).

Historically, the role of ppGpp as a regulatory molecule has been most closely associated with control of stable RNA (rRNA and tRNA) synthesis. Enteric bacteria use at least two regulatory systems, stringent control and growth rate-depen-
–10 hexamer, termed the discriminator sequence. The discriminator regions of stable RNA promoters have been implicated in stringent control and growth rate-dependent control and have a well-defined consensus sequence (GGC GCC GG) (11, 23, 24). The wild-type his operator discriminator is AT rich, as are the discriminators of some other putative ppGpp-stimulated promoters, but no obvious consensus sequence has been defined (17, 24).

The experiments described in this report were designed to address two important issues concerning the role of ppGpp in stimulating expression of the his operon. The first issue is whether promoter mutations in the –10 hexamer and discriminator regions found to alter ppGpp regulation in the coupled system in vitro have a corresponding physiological effect when tested in vivo. To address this issue, we recombined promoter mutations into the chromosomal his operon and tested their regulatory responses to medium richness during steady-state growth and to amino acid downshift conditions. The second issue has to do with the type of promoter mutation. As mentioned above, the two ppGpp-insensitive his promoter mutations analyzed in the coupled system both had an up promoter effect. It could be argued, therefore, that these mutations set the target of ppGpp stimulation could be something other than transcription initiation. To address this issue, we constructed and analyzed a new discriminator mutation that has a down promoter effect.

Our results support the following major conclusions: (i) stimulation of his expression by ppGpp occurs at or near the his promoter (ii) the his promoter region containing both the –10 hexamer and discriminator sequences is crucial for stimulation by ppGpp, and (iii) results obtained with the coupled system in vitro are physiologically relevant. Furthermore, the results support an independent finding (30) that the presence of a GC-rich consensus discriminator sequence is not sufficient for negative control by ppGpp. The his promoter with a GC-rich consensus discriminator was insensitive to stimulation by ppGpp but was not inhibited by it.

**MATERIALS AND METHODS**

**Bacterial strains, media, and growth conditions.** The *S. typhimurium* strains used in this work were all derived from wild-type strain LT2 and are shown in Table 1. The nutrient-rich, complex medium used was LB medium (14) supplemented with 0.4% (wt/vol) glucose and 1% Vogel and Bonner salts (25). The minimal medium used was MM (14) supplemented with 0.2% (wt/vol) glucose and 40 μg each of histidine hydrochloride and tryptophan per ml. All cultures were grown at 37°C with shaking. The media used for allele replacement (3) were 2% Y1 (14) containing 20 μg of chloramphenicol per ml to select integrants and nutrient agar (Difco) containing sodium deoxycholate (0.3%, wt/vol) to select segregants. MacConkey lactose agar was from Difco.

**Construction of his promoter mutations.** The his promoter mutations hisgp3423, hisgp3424, and hisgp3425 were constructed in three successive steps by utilizing the dut method of oligonucleotide-directed mutagenesis (12). A kit for this purpose was obtained from Bio-Rad Laboratories. The procedure started with phage M13mp9:his5 (17), which carries a 3.1-kb fragment containing the beginning of the *S. typhimurium* his operon with a promoter mutation that converted the wild-type –10 hexamer to the consensus E 

\[
\begin{align*}
\text{his}\text{D-lac} & \text{ promoter mutations for measurement of his expression. The donor P22 phage lysates}
\end{align*}
\]

were made on strains AZ316 and AZ750 (2). The *hisD-lac* transcriptional fusion carried by some of the strains resulted from an immobilized Mu d1 cts(Apr lac) insertion that has already been described (2, 20, 21). The immobilization procedure (2) allowed the fusion to be moved by phage P22 generalized transduction for strain constructions and rendered the strains heat resistant so that growth experiments could be done at 37°C. The fusion previously was designated *hisD-Glac* (20, 21) because, based on a histidinol-negative growth phenotype, the location of the insertion was imprecisely known. Direct sequencing of *his* DNA PCR amplified from strain AZ316 has since determined that the Mu d1 cts(Apr lac) insertion within hisD with the left endpoint of the insertion 151 bp downstream of the hisD translation initiation codon (15).

The *hisD-lac* transcriptional fusion carried by some of the strains resulted from an immobilized Mu d1 cts(Apr lac) insertion that has already been described (2, 20, 21). The immobilization procedure (2) allowed the fusion to be moved by phage P22 generalized transduction for strain constructions and rendered the strains heat resistant so that growth experiments could be done at 37°C. The fusion previously was designated *hisD-Glac* (20, 21) because, based on a histidinol-negative growth phenotype, the location of the insertion was imprecisely known. Direct sequencing of *his* DNA PCR amplified from strain AZ316 has since determined that the Mu d1 cts(Apr lac) insertion within hisD with the left endpoint of the insertion 151 bp downstream of the hisD translation initiation codon (15).

**Allele replacement.** To efficiently recombine the promoter mutations into the *S. typhimurium* chromosome, the 3.1-kb his DNA fragments containing the different mutations were first subcloned into the M13mp11cat plasmid (3). The M13mp11cat and M13mp11lac phage vectors allow any gene that has been cloned into the phage multiple cloning site to be integrated into the homologous region of the bacterial chromosome by selecting for resistance to chloramphenicol. Segregants were selected by resistance to the bile salt sodium deoxycholate. Details of the events associated with the allele replacement have already been described (3).

The recipient used in the allele replacement, strain AZ302, contained a deletion in the his leader region extending from and including positions +20 to +89 with respect to the transcriptional start point (designated +1). This deletion, *hisGe3419*, causes a tight His− phenotype and allowed easy screening of His+ segregants in which the deletion had been replaced. Strain AZ302 was grown in LB broth for 5 h at 37°C to mid-log phase. Samples (0.1 ml) of the culture were dispensed into small, sterile glass test tubes, and 0.1-ml volumes of the recombinant M13mp11cat plasmid phage were added to the tubes to give a multiplicity of infection in the range of 10 to 100. The tubes were incubated for 1 h at 37°C, after which 0.1 ml of each mixture was spread over 2% YT-chloramphenicol (20 μg/ml) plates. The plates were incubated overnight at 37°C, by which time integrant colonies appeared. The donors and recipient were spread separately on the same medium, and no colonies appeared on these control plates. Several chloramphenicol-resistant colonies were picked at random from each cross and streaked directly onto nutrient agar plates containing 0.3% sodium deoxycholate to select segregants that had lost the M13mp11cat plasmid. The plates were incubated overnight at 37°C, and isolated colonies were picked and streaked onto LB plates for single-colony purification. The segregants were restested for loss of the M13mp11cat recombination plasmid on the chloramphenicol-containing medium, as well as for the ability to grow on minimal medium lacking histidine. The ability of the segregants to grow on minimal medium was a good indication of successful allele replacement, since the recipient strain, strain AZ302, is a his− strain. Based on the results of experiments that measured relative expression of the different mutant his promoters in vitro (7), segregants that had retained either the *hisGp3400* or the *hisGp3424* mutant promoter were expected to grow well on minimal medium lacking histidine. An exception was the *hisGp3425* promoter mutant, which had low expression in vitro and, in fact, grew poorly without histidine. To verify the presence of the promoter mutations, the his promoter regions in representative segregants were PCR amplified and the DNA was sequenced as described below. The strains carrying the three mutant promoters that resulted from the allele replacement procedure were designated AZ7505, AZ7506, and AZ7507 (Table 1).

**Fusion of Mu d1 cts(Apr lac) to his promoter mutations for measurement of his-lac expression.** Donor P22 phage lysates were made on strains AZ316 and AZ460. Both donor strains contained the *ΔhisD-g422* mutation and an immobilized Mu d1 cts(Apr lac) insertion in the *hisD* gene (*hisD-lac*; Table 1).

**TABLE 1. Bacterial strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZ302</td>
<td>ΔhisGp3419 trp::Tn5/F pl32 F12[3] p501 trp+</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>AZ314</td>
<td>ΔhisD-lac ΔhisGα1242 trp::Tn5</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>AZ315</td>
<td>Same as AZ314 but ΔhisGα+</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>AZ316</td>
<td>ΔhisGα1367 ΔhisGα1242 ΔhisD-lac trp::Tn5</td>
<td>Laboratory collection</td>
</tr>
</tbody>
</table>

* The ΔhisD-lac transcriptional fusion carried by some of the strains resulted from an immobilized Mu d1 cts(Apr lac) insertion that has already been described (2, 20, 21). The immobilization procedure (2) allowed the fusion to be moved by phage P22 generalized transduction for strain constructions and rendered the strains heat resistant so that growth experiments could be done at 37°C. The fusion previously was designated *hisD-Glac* (20, 21) because, based on a histidinol-negative growth phenotype, the location of the insertion was imprecisely known. Direct sequencing of his DNA PCR amplified from strain AZ316 has since determined that the Mu d1 cts(Apr lac) insertion within hisD with the left endpoint of the insertion 151 bp downstream of the hisD translation initiation codon (15).
Whereas strain AZ1460 is wild type for the his promoter, strain AZ316 forms white colonies when plated on MacConkey lactose agar while strain AZ1460 forms dark red colonies. The rationale for using these two donor lysates was based on the results of in vitro experiments which indicated that the hisGp3425, hisGp3424, and hisGp3424 by oligonucleotide-directed mutagenesis. The top strand in each step represents the uracil-incorporated template strand, and the bottom strand represents the oligonucleotide primer, containing the desired base changes (in boldface). Each mutant, in turn, served as the template for the subsequent mutant (progressing from step 1 to step 3). The PCR-amplified DNAs were sequenced to verify the mutations present. All six recombinant classes were obtained, and 11 of the 14 templates sequenced were of the predicted genotype; the remaining three recombinants apparently resulted from quadruple crossover events. Strains AZ7516 through AZ7521 (Table 1) are the representatives of the six recombinant classes that were used to study his expression as a function of promoter repression. Donor P22 phage lysates (D) were made on strains AZ1460 and AZ316 and were either wild type for the his promoter or carried the hisGp3425 down promoter mutation, respectively. Both strains AZ1460 and AZ316 contained the hisGa1242 attenuator deletion and an Mu d1 ts(Ap') lac' insertion in the hisD gene. Recipient strains (R) AZ7505, AZ7506, and AZ7507 each contained one of the three promoter mutations (hisGp3400, hisGp3424, or hisGp3425) described in Materials and Methods. In every case, the rightmost crossover was selected but there were three possible locations for the second crossover event (shown as circled numbers 1, 2, and 3). The recombinants resulting from the different crossover events are shown at the bottom. Crossovers 2 and 3 produced the desired recombinants. In these cases, the recipient promoters (hisGp3424') were retained and the hisGa1242 deletion was inherited together with the Mu d1 ts(Ap' lac') insertion in crossover 2 but not in crossover 3, which resulted in inheritance of the wild-type attenuator site.

However, depending on the location of the second crossover, his promoter mutations may segregate with or without the hisGa1242 mutation. In the AZ7505 and AZ7506 (recipient) X P22/AZ316 (donor) transduction, crossovers 2 and 3 resulted in dark red and light red recombinant colonies, respectively. For both of these crossovers, the mutant promoter of the recipient was retained and the attenuator deletion was inherited from the donor in crossover 2 but not in crossover 3; both recombinant classes were distinguishable from the donor class (crossover 1), which gave dark red colonies. Whereas white recombinants retained the wild-type attenuator. Both classes were distinguishable from the donor class (crossover 1), which gave dark red colonies. Fourteen colonies representing each of the six recombinant classes of interest (hisGp3400, hisGp3424, and hisGp3425, each combined with either the wild-type attenuator or the hisGa1242 mutation) were chosen, and genomic DNA was isolated and PCR amplified (see below). The PCR-amplified DNAs were sequenced to verify the mutations present. All six recombinant classes were obtained, and 11 of the 14 templates sequenced were of the predicted genotype; the remaining three recombinants apparently resulted from quadruple crossover events. Strains AZ7516 through AZ7521 (Table 1) are the representatives of the six recombinant classes that were used to study his expression as a function of...
β-galactosidase synthesis in the lac fusion derivatives. F’123 was segregated from these strains following growth in media nonselective for retention of the episome.

β-Galactosidase assays with samples obtained during steady-state growth and following serine hydroxamate addition. Strains were grown in 2 ml of nutrient-rich medium or minimal medium with required supplements at 37°C overnight and then subcultured into 25 ml of the same medium to give an initial optical density at 650 nm (OD_{650}) of about 0.01. β-Galactosidase specific activities were determined from the slopes of differential rate plots of five samples taken in approximately equivalent incremental steps between OD_{650} of about 0.1 and 0.5 for cultures in steady-state growth. A mild downshift condition was induced by addition of tri-serine hydroxamate (Sigma) to a concentration of 2 mM (21). Serine hydroxamate was prepared as a 0.5 M stock solution immediately prior to use and kept on ice. The analog was added to cultures in exponential growth in nutrient-rich medium at an OD_{650} of about 0.15. Samples were taken every 2 min for 0.5 h and every 10 min thereafter for the next 0.5 h following addition of serine hydroxamate. β-Galactosidase assays were performed and specific activities were calculated as described previously (21).

Preparation of DNA templates, PCR amplifications, and DNA sequencing. Genomic DNA was isolated by the following method (15). Cultures were grown in 2 ml of LB broth supplemented with 1.2% (wt/vol) glucose and 0.7% Vogel and Bonner salts and incubated overnight at 37°C. A sample (1.5 ml) of the culture was then centrifuged for 5 min in a microcentrifuge, and the supernatant was discarded. The cell pellet was resuspended in 567 µl of TE buffer (14), 30 µl of 10% sodium dodecyl sulfate plus 3 µl of 20 mg/ml protease K (Sigma) was added, and the tubes were incubated for 1 h at 37°C. Proteinase K was denatured by incubating the tubes for 5 min at 80°C, and the lysate was treated with 5 µl of 10-mg/ml DNase-free RNase A (Sigma) for a further 15 min at 37°C. The DNA was purified of protein by phenol-chloroform extraction (1:1, vol/vol) and precipitated with isopropanol. DNA pellets were washed with 70% ethanol, dried, and resuspended in 0.5 ml of TE buffer.

Two primers were designed to amplify the his region by PCR. The primers were spaced about 1,500 bp apart. Primer XAV1, a 20-mer, has the sequence 5’-TCTGATCGCGTCACTAGATG-3’ and was designed to anneal to the template strand approximately 400 bp upstream of the his transcriptional start point. The sequence contains a restriction site for the enzyme PstI (5’-CTGCAG-3’). Primer XAV2, a 26-mer, has the sequence 5’-GGTCCTAGAGCAGTCGAGCT-3’ and was designed to anneal to the nontemplate strand approximately 1,100 bp downstream of the transcriptional start site. A restriction site for the enzyme XbaI (5’-TCTAGA-3’) was designed into the primer, near its 5’ end. PCR amplifications were performed under standard conditions by using components of the Perkin-Elmer GeneAmp DNA Amplification Reagent Kit. Samples of the amplified reactions were electrophoresed to verify the product size. The PCR-amplified DNAs were purified by using Amicon 100 Microconcentrators (W. R. Grace & Co.) and sequenced by using Sequenase Version 2.0 enzyme (United States Biochemical). The template DNAs were sequenced by using a primer-template molar ratio of about 20:1. The primers used to sequence the his promoters have already been reported (17). A region of DNA encompassing approximately 200 nucleotides downstream and 100 nucleotides upstream was sequenced for each mutant construct to ensure that genetic and biochemical manipulations did not introduce extraneous mutations that might affect his expression. In particular, the presence of the wild-type attenuator site or the ΔhisGm1242 mutation was determined for each strain.

RESULTS

Expression of wild-type and mutant his promoters under steady-state conditions in minimal and nutrient-rich media. Figure 3 and Table 2 show the effects on his expression of growing strains containing either the wild-type his promoter or his promoter mutations in a minimal medium versus a nutrient-rich medium. The data in Table 2 are summarized as specific activities which were taken from the slopes of differential rate plots of β-galactosidase accumulation obtained during balanced, steady-state growth (21). Representative examples of the differential rate plots for cultures grown in minimal and nutrient-rich media are shown in Fig. 3, which gives results for strain AZ314 carrying the wild-type his promoter and strain AZ7521 carrying one of the promoter mutations (hisGp3425). Linearity of the data and extrapolation of the data from points at the origin of the graph indicate that the cultures, in fact, approached balanced growth and that β-galactosidase accumulated as a constant proportion of cell mass during the experiment. All of the strains grew with very similar generation times in the nutrient-rich medium (28 min) and in the minimal medium (55 min).

To simplify interpretation of the data, experiments reported in Fig. 3 and Table 2 were done with strains completely missing the operon-specific attenuation mechanism owing to deletion of the attenuator site by the gusA1242 mutation. Thus, these experiments measured only attenuator-independent regulatory events. Results obtained with the attenuator present will be addressed subsequently.

Expression of the his operon by strain AZ314 (hisGp-) was about 2.5-fold lower in nutrient-rich medium than in minimal medium (Fig. 3 and Table 2). In replicate experiments, the repression ratio varied from about 2.5-fold to 3.5-fold. This moderate fluctuation was observed to result primarily from error in the measurement of the differential rate of β-galactosidase synthesis in rich medium, apparently because of the constant depletion of nutrients in this medium, especially at higher ODs. The magnitude of the regulatory effect correlates well with the change in steady-state ppGpp levels found in strains grown under similar conditions (5). In contrast, regulation of his expression in strains AZ7517, AZ7521, and AZ7519 was relatively unaffected by the nutrient richness of the growth medium. Strain AZ7517 carries a mutation (hisGp3400) that converts the wild-type his promoter – 10 hexamer to the consensus sequence for interaction with E. coli RNA polymerase (i.e., 5’-TAGGTT → 5’-TATAAT). This mutation was previously shown to render the his operon relatively insensitive to ppGpp stimulation in the DNA-dependent, coupled transcription-translation (S-30) system in vitro (17). In vivo, the hisGp3400 mutant gave somewhat elevated his expression of minimal medium compared to the hisGp- strain and was highly resistant to repression in nutrient-rich medium (decrease of about 23%; Table 2). Strain AZ7521 carries a mutation (hisGp3425) that converts the wild-type, AT-rich his discriminator sequence to a GC-rich sequence (i.e., 5’-AAAAAGGT → 5’-GCGCCCC). This mutation resulted in a
strong down promoter effect. The hisGp3425 mutant was also highly resistant to repression in nutrient-rich medium, showing about 23% lower his operon expression in the latter medium than in minimal medium (Fig. 3 and Table 2). The hisGp3425 mutation has recently been shown to render his expression relatively insensitive to ppGpp stimulation in the coupled system in vitro (7). Strain AZ7519 carries the hisGp3424 mutation, which combines the sequence changes of the hisGp3400 and hisGp3425 mutations. The hisGp3424 mutant promoter was resistant to nutrient-rich medium repression, similar to the single mutants, and his expression was at about the hisGp3400 level found in strain AZ7517 (Table 2).

Table 3 shows the regulatory effects on his expression of growth in minimal medium versus nutrient-rich medium with strains isogenic to those in Table 2, except for the presence of the wild-type attenuator site. Expression of the his operon in Table 3, therefore, is much lower than that observed in Table 2 because of repression caused by the attenuation mechanism. Repression by attenuation in minimal medium was about 10-fold for strains with the wild-type promoter (compare strain AZ7516 [6,895 U/OD$_{650}$; Table 2] to strain AZ7515 [676 U/U of OD$_{650}$; Table 3]).

Nutrient-rich medium repression of his expression in strain AZ7515 (Table 3) is a combined effect of attenuation and promoter regulation. Expression of the his operon by strain AZ7515 (wild-type promoter, wild-type attenuator) was about 13-fold lower in nutrient-rich medium than in minimal medium. Strains AZ7516, AZ7520, and AZ7518, which carry promoter/regulatory mutations in the −10 and/or discriminator regions, showed reduced nutrient-rich medium regulation (3- to 4.5-fold) because only the attenuation mechanism was working in these strains. The results of Tables 2 and 3, taken together, are consistent with the interpretation that the steady-state, 13-fold nutrient-rich medium repression in strain AZ7515 results from additivity of two regulatory phenomena: ppGpp-dependent promoter regulation (about 2.5- to 3.5-fold) and attenuation (about 3- to 4.5-fold).

We assume that repression via the attenuation mechanism in nutrient-rich medium is due primarily to increased tRNA charging and growth rate-dependent changes in the relative rates of transcription and translation (5) with consequential effects on formation of the alternative RNA secondary structures that govern the mechanism (10). It is unlikely that changes in ppGpp levels directly influence his attenuation in vivo, since we have shown that the presence or absence of the attenuator does not significantly alter regulation by ppGpp in the DNA-dependent transcription-translation system in vitro (17).

**Effects of promoter mutations on differential rates of his expression following addition of serine hydroxamate to elicit the stringent response.** Serine hydroxamate is a competitive inhibitor of seryl-tRNA synthetase and elicits the stringent response by mimicking an amino acid downshift and provoking relA-dependent synthesis of ppGpp (6, 21). We previously described in detail the close correlation between attenuator-independent his expression and ppGpp levels during downshift caused by serine hydroxamate addition to cultures of S. typhimurium strains containing the wild-type his operon promoter (21). In those experiments, as well as the ones reported here, a moderate concentration of serine hydroxamate was used to allow growth to continue so that differential rates of enzyme synthesis could be monitored accurately during the downshift (21).

Table 4 shows the effects on his operon expression of adding serine hydroxamate to strains containing wild-type or mutant his operon promoters. All strains contained the ΔhisGa1242 attenuator deletion mutation. At the time of serine hydroxamate addition, the cultures were growing exponentially in nutrient-rich, complex medium. Samples for β-galactosidase assays were taken periodically before and after serine hydroxamate addition to establish preshift and postshift differential rates of his expression. The preshift differential rates in Table 4 are close to those shown in Table 2 for the same strains grown under steady-state conditions in nutrient-rich medium.

Following serine hydroxamate addition, the differential rate in strain AZ714 (wild-type promoter) increased fourfold, from a preshift level of 2,434 U/OD$_{650}$ to a postshift level of 10,285 U/OD$_{650}$ (Table 4). This increase in his operon expression

---

**TABLE 2.** Effects of promoter mutations on differential rates of his expression in attenuator-deleted strains in minimal medium and nutrient-rich medium

| Strain | Relevant DNA sequence* | β-Galactosidase sp act*
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−10 Hexamer</td>
<td>Discriminator</td>
</tr>
<tr>
<td>AZ314</td>
<td>TAGGTT (wt)</td>
<td>AAAAGGT (wt)</td>
</tr>
<tr>
<td>AZ7517</td>
<td>TATAAT</td>
<td>AAAAGGT (wt)</td>
</tr>
<tr>
<td>AZ7521</td>
<td>TAGGTT (wt)</td>
<td>GCGCCCC</td>
</tr>
<tr>
<td>AZ7519</td>
<td>TATAAT</td>
<td>GCGCCCC</td>
</tr>
</tbody>
</table>

* DNA sequences of the wild-type (wt) promoter elements are indicated.

**TABLE 3.** Effects of promoter mutations on differential rates of his expression with attenuation in minimal medium and nutrient-rich medium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant DNA sequence</th>
<th>β-Galactosidase sp act</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−10 Hexamer</td>
<td>Discriminator</td>
</tr>
<tr>
<td>AZ315</td>
<td>TAGGTT (wt)</td>
<td>AAAAGGT (wt)</td>
</tr>
<tr>
<td>AZ7516</td>
<td>TATAAT</td>
<td>AAAAGGT (wt)</td>
</tr>
<tr>
<td>AZ7520</td>
<td>TAGGTT (wt)</td>
<td>GCGCCCC</td>
</tr>
<tr>
<td>AZ7518</td>
<td>TATAAT</td>
<td>GCGCCCC</td>
</tr>
</tbody>
</table>

* See the footnotes to Table 2.
TABLE 4. Effects of promoter mutations on differential rates of his expression in attenuator-deleted strains following addition of serine hydroxamate

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant DNA sequence</th>
<th>β-Galactosidase sp act</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-10 Hexamer</td>
<td>Discriminator</td>
</tr>
<tr>
<td>AZ314</td>
<td>TAGGTT (wt)</td>
<td>AAAAGGT (wt)</td>
</tr>
<tr>
<td>AZ7517</td>
<td>TATAAT</td>
<td>AAAAGGT (wt)</td>
</tr>
<tr>
<td>AZ7521</td>
<td>TAGGTT (wt)</td>
<td>GCGCCCC</td>
</tr>
<tr>
<td>AZ7519</td>
<td>TATAAT</td>
<td>GCGCCCC</td>
</tr>
</tbody>
</table>

|       | Preshift | Postshift |
| AZ314 | 2,434    | 10,285    |
| AZ7517| 6,569    | 10,188    |
| AZ7521| 447      | 506       |
| AZ7519| 6,538    | 9,363     |

* Strains were grown in nutrient-rich medium at 37°C, and 2 mM DL-serine hydroxamate was added at an OD650 of about 0.15. Sampling for β-galactosidase activity occurred as described in Materials and Methods. For other information, see the footnotes to Table 2.

DISCUSSION

Previous evidence established a direct correlation between the level of his operon expression and the concentration of ppGpp both in vivo (18, 21, 22, 27, 28) and in vitro (17, 22). The correlation persisted in the absence of the cis-acting attenuator site, indicating that some regulatory mechanism other than the attenuation mechanism is involved. In addition, evidence obtained in vitro suggested the his promoter as the site at which the regulatory response to ppGpp occurs (17). The latter evidence was based on the finding that certain mutations that altered a domain of the his promoter, including the −10 hexamer and the adjacent discriminator sequence, greatly diminished the stimulation of his expression by ppGpp in a DNA-dependent, coupled transcription-translation (S-30) system (17). Whereas expression of the his operon containing the wild-type promoter was stimulated 10- to 20-fold by optimal ppGpp concentrations in the coupled system (17, 22), that of the promoter mutants were stimulated less than 2-fold (17).

Results reported here demonstrate for the first time that well-defined his promoter mutations disrupt the correlation between the level of his operon expression and the concentration of ppGpp in vivo. The results, therefore, provide important physiological evidence to supplement the evidence of the effects of his promoter mutations on ppGpp regulation in vitro. The results also lend added support to the conclusion that ppGpp regulates RNA polymerase interaction with the his promoter and point specifically to the region of the promoter containing the −10 hexamer and discriminator sequences as the target of ppGpp stimulation (17).

Analysis of the properties of the hisGp3425 mutation alleviates a reasonable objection to the interpretation that ppGpp regulates his expression at the level of RNA polymerase interaction with the promoter (6). The objection was based on the observation that the mutant promoters found to be ppGpp-insensitive in vitro resulted from up promoter mutations (17). It could be argued, therefore, that the coupled system expressed the mutant promoters at the maximum capacity of the system, thus masking any further stimulation of his expression by ppGpp. If this were the case, then stimulation by ppGpp could occur at some stage of protein synthesis other than RNA polymerase interaction with the promoter. The hisGp3425 mutation resulted in a strong down promoter effect and rendered attenuator-independent his expression insensitive to growth conditions that change ppGpp levels (Fig. 3 and Tables 2 and 4). In agreement with these in vivo results, the hisGp3425 mutant promoter has been found to be ppGpp insensitive in the coupled system in vitro despite being expressed at a low level compared to the wild-type his promoter (7). The behavior of the hisGp3425 mutant promoter, therefore, can most readily be explained by a defect in ppGpp regulation at or near the promoter.

Despite the validity of the conclusion described above, the data in Tables 2 to 4 are, interestingly, consistent with the interpretation that the combined effects of the ΔhisGp1242 attenuator mutation and the hisGp3400 up promoter mutation cause the his operon to be expressed in vivo at a level approaching a maximum. This maximum apparently corresponds to a level of β-galactosidase specific activity in the range of about 8,000 to 10,000 U/OD650 under the conditions analyzed. The up promoter effect of the hisGp3400 mutation, by itself, was about threefold in minimal medium (compare strain AZ315 [676 U/OD650] to strain AZ7516 [2105 U/OD650] in Table 3). When the effect of the hisGp3400 mutation was measured in the attenuator deletion background, however, the up promoter effect was reduced to less than 20% (compare strain AZ314 [6,895 U/OD650] to strain AZ7517 [8,052 U/OD650] in Table 3). This lack of additivity suggests that some step in β-galactosidase synthesis, other than initiations by RNA polymerase, became limiting when the hisGp3400 mutation was combined with the attenuator deletion. The limiting step could be, for example, transcription elongation or a step in translation. In addition, the specific activity of β-galactosidase achieved during nutritional downshift was about 10,000 U/OD650 (Table 4), which is consistent with the interpretation that this level is near the maximum level possible. (For comparison, the corresponding minimum level of his expression in the absence of ppGpp [measured in a double-null relA and spoT mutant] is about 1,000 U/OD650 [31]. The overall regulatory range of the ppGpp stimulatory effect at the his promoter in vivo is therefore about 10-fold.)

There is an interesting relationship between the hisGp3400 and hisGp3425 mutations. The data in Tables 2 to 4 and Fig. 3 all indicate that hisGp3400 (in strains AZ7516 and AZ7517) is an up promoter mutation, while hisGp3425 (in strains AZ7520 and AZ7521) is a strong down promoter mutation. However, when the two mutations were combined to make a new mutation, hisGp3424 (in strains AZ7518 and AZ7519), the effect on his expression mimicked that of the hisGp3400 mutation. That is, hisGp3400 is epistatic to hisGp3425. Assuming that the his transcription start site remains unaltered by the promoter mutations introduced, this epistatic relationship is consistent with the classical bacterial genetic interpretation (4) that hisGp3400 and hisGp3425 alter different steps in a common pathway and that the step altered by hisGp3400 precedes that altered by hisGp3425. In biochemical terms, the epistatic relationship between the two mutations may be explained by the idea that melting of his promoter DNA by RNA polymerase proceeds in at least two steps, with the first step being initiated within the −10 hexamer and the second step progressing into the discriminator region. It will be interesting to analyze this relationship in more detail with in vitro techniques.

Finally, it is interesting that the hisGp3425 mutation converted the wild-type his discriminator sequence (5′-AAAAAG GT) to the consensus sequence (5′-GGCGCCNC) normally as-
associated with promoters that are ppGpp inhibitable (11, 23, 24, 32). Although the hisGp3425 mutation caused a down promoter effect and rendered his expression insensitive to ppGpp stimulation (similar to a previously studied up promoter, his discriminator mutation [17]), the mutation did not introduce negative control. Thus, our results are consistent with other evidence indicating that the GC-rich consensus discriminator is necessary but not sufficient for negative control by ppGpp (30).

How the discriminator sequences of promoters that are susceptible to stimulation or inhibition by ppGpp contribute to this important regulation remains to be determined.

ACKNOWLEDGMENTS

We thank Jon Ness and Wei Zhang for helpful discussions and for comments on the manuscript.

This work was supported by National Institutes of Health grant GM27307.

REFERENCES


