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Promoter domain mediates guanosine tetraphosphate activation of the histidine operon

(stringent control/alarmones/coupled *in vitro* protein synthesis/*Salmonella typhimurium*/transcription)

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ABSTRACT **We have analyzed the effects of the "alarmone" guanosine 5′-diphosphate 3′-diphosphate (ppGpp) on regulation of the *Salmonella typhimurium* histidine operon *in vitro*. Expression of the wild-type promoter, measured in a DNA-dependent transcription–translation system, was strongly dependent on ppGpp; addition of ppGpp stimulated *his* expression 22-fold with plasmid DNA templates. Oligonucleotide-directed, site-specific mutations that increase the homology of the −10 hexamer to the consensus sequence of the $E\sigma^{70}$ promoters dramatically increased *his* expression in the absence of ppGpp and reduced the stimulation to less than a factor of 2. A deletion mutation that alters the sequence between the −10 hexamer and the start point of transcription, generated by BAL-31 nuclease, affected ppGpp regulation in a similar manner. We propose that the −10 hexamer sequence and the adjacent downstream region are both important in regulating transcription by ppGpp. Mechanisms to account for activation and repression of transcription by ppGpp are discussed.**

Prokaryotes have an extensive regulatory network to ensure an adequate, balanced supply of amino acids for protein synthesis. Although most amino acid biosynthetic genes are regulated specifically by the cognate amino acid through attenuation of transcription (1, 2) or a regulatory protein (3), a super-control mechanism adjusts the expression of these genes with respect to the total amino acid supply (4, 5). Amino acid starvation triggers the synthesis of the "alarmone" guanosine 5′-diphosphate 3′-diphosphate (ppGpp) by the RelA protein (6). The increased level of ppGpp stimulates expression of a number of amino acid biosynthetic genes and catabolic genes while repressing numerous stable RNA and ribosomal protein genes. This transcriptional regulation redirects energy and resources from the unproductive synthesis of additional translational components toward the synthesis of enzymes required to overcome the starvation. Levels of ppGpp vary inversely with growth rate during balanced growth by a *relA*-independent mechanism; growth-rate-dependent regulation of certain genes (e.g., stable RNA genes) may involve ppGpp (7).

In this study we identify sequences in the promoter of the *Salmonella typhimurium* histidine operon that are involved in regulation by ppGpp. Mutations in either the −10 hexamer sequence (Pribnow box) or the region between the −10 hexamer and the start point of transcription (the "discriminator" region) alter regulation of *his* expression by ppGpp. While the discriminator region has been implicated in repression of transcription by ppGpp (8, 9), our results indicate that activation of transcription by ppGpp involves the sequence of the −10 hexamer. Possible mechanisms of regulation by ppGpp are considered.

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MATERIALS AND METHODS

Bacterial Strains and Bacteriophages. *Escherichia coli* strain JM103, Δ(*lac-pro*) *supE thi strA sbcB15 endA hspR4* [F′ *traD36 proAB lacI*($LacI^q$) Δ*lacZM15*] (10), was used to propagate plasmids and M13mp phages. Phages containing the *his* mutations (Table 1) were derived from M13mp9::*his*1 and M13mp9::*his*4 (11).

Growth Media. JM103 was grown in 2× YT (12), and transformations were done as described by Hanahan (13). Plasmid templates for *in vitro* protein synthesis were isolated from cells grown in M9 medium with casamino acids (14) supplemented with 10% (vol/vol) LB (12), 0.17 μg of thiamine hydrochloride per ml, 164 μg of L-proline per ml, and 50 μg of ampicillin per ml.

***In Vitro* Mutagenesis.** The synthetic oligonucleotide used to generate the *hisGp3400* and *hisGp3401* mutations in M13mp9::*his*1 by standard methods (15, 16) was a gift of Systec Corporation. The Δ*hisGpe3420*, Δ*hisGpe3421*, and Δ*hisGpe3422* mutations were constructed in M13mp9::*his*4 RF DNA. This phage has a 1.6-kilobase pair deletion, Δ*hisGD* (11), that removes a *Mlu* I site. Replicative form (RF) DNA (200 μg/ml) made linear by digestion with *Mlu* I at a unique site was digested with BAL-31 nuclease (5 units/ml) in 50 μl at 37°C for 2 min and ligated. Strain JM103 was then transfected (13) with these deletion phages. RF DNAs from plaque-purified phages were digested with *Hae* III and electrophoresed on a polyacrylamide gel to determine the approximate deletion size (11). Nucleotide sequence analysis identified the deletion endpoints.

Nucleotide Sequence Analysis. The *his* promoter/regulatory region sequences were determined by the dideoxy method (10). Plasmid sequences were determined by the modifications of Wallace *et al.* (17). Synthetic primers complementary to the *his* leader-peptide sequence from +34 to +53 or to the 5′ end of *hisG* from +250 to +269 (relative to the start point of transcription) were a gift of Mickey Urdea of Chiron Corporation (18).

Plasmid Construction and Template Isolation. The *lacUV5* promoter template, pRS229 (19), and the translational fusion vector pJES35 were kindly provided by S. Kustu. pJES35 is derived from pMLB1034 (20) by modification of the polylinker reading frame (21). Plasmids and phage RF DNAs were isolated from JM103 by the cleared lysate method as modified by Messing (10). Plasmid templates (described in *Results*) for *in vitro* protein synthesis were amplified with chloramphenicol (14), isolated by the cleared lysate protocol and purified with two CsCl-gradient centrifugations.

***In Vitro* Protein Synthesis Conditions.** Reaction conditions and β-galactosidase assays were essentially as described (4, 5).

Abbreviations: ppGpp, guanosine 5′-diphosphate 3′-diphosphate; RF, replicative form.

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Table 1. *his* genotypes of corresponding phages and plasmids

Phage	Plasmid	*his* genotype
M13mp9::*his*1	PAZ2	*hisGp*$^+$, *hisGa*$^+$, *hisG*$^+$*D*$^+$
M13mp9::*his*4	pAZ13	*hisGp*$^+$, Δ*hisGal242*, Δ*hisGD*
M13mp9::*his*5	pAZ16	*hisGp3400*, *hisGa*$^+$, *hisG*$^+$ *D*$^+$
M13mp9::*his*6	pAZ17	*hisGp3401*, *hisGa*$^+$, *hisG*$^+$ *D*$^+$
M13mp9::*his*7	pAZ7	Δ*hisGpe3420*, Δ*hisGal242*, Δ*hisGD*
M13mp9::*his*8	pAZ5	Δ*hisGpe3421*, Δ*hisGal242*, Δ*hisGD*
M13mp9::*his*9	pAZ3	Δ*hisGpe3422*, Δ*hisGal242*, Δ*hisGD*

Construction and characterization of phages M13mp9::*his*1 and M13mp9::*his*4 have been described (11); all other phages and plasmids were constructed for this study.

RESULTS

ppGpp Efficiently Stimulates Expression from the *his* Promoter on a Supercoiled Template *in Vitro*. The stimulatory effect of exogenous ppGpp on *his* expression was analyzed with a DNA-dependent, coupled transcription–translation system containing an S-30 cell extract from a *relA* mutant strain of *S. typhimurium*. The *relA* mutation diminishes *de novo* synthesis of ppGpp in the S-30 system (5). The DNA templates directing *his* expression were translational fusion plasmids containing the promoter proximal portion of the *S. typhimurium his* operon: the promoter/regulatory region, *hisG*, *hisD*, and part of the *hisC* gene fused in-frame to *lacZ*, which encodes β-galactosidase (Fig. 1). Synthesis of the amino-terminal substituted β-galactosidase was dependent on transcription from the *his* promoter; negligible β-galactosidase activity was detected from the fusion vector template (pJES35).

Expression of the wild-type *his* promoter templates, pAZ2 and pAZ13, *in vitro* was stimulated more than 20-fold by the addition of physiological concentrations of ppGpp (Tables 2 and 3). Maximal *his* expression from both templates required about 50 μM ppGpp while 25 μM ppGpp yielded half-maximal stimulation (data not shown). These plasmid templates were significantly more responsive to ppGpp than the linear templates used in earlier studies in which half-maximal stimulation occurred at 100 μM ppGpp with 10-fold stimulation at a saturating concentration of 200 μM ppGpp (5). As observed (5), the presence or absence of the *his* attenuator did not significantly alter the regulation by ppGpp (Tables 2 and 3).

Mutations in the *his* Promoter −10 Hexamer Abolish Regulation by ppGpp. Mutations increasing the homology of the *his* promoter to the consensus −10 hexamer sequence were

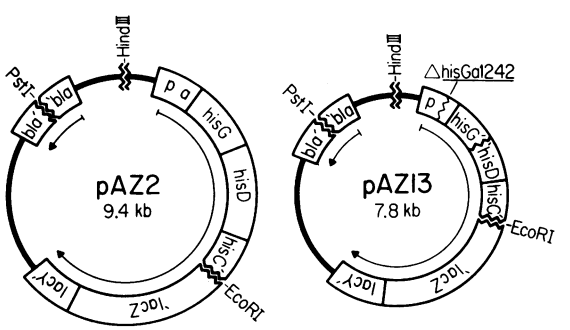

FIG. 1. Structure of the two *hisGp*$^+$ translational fusion plasmids pAZ2 and pAZ13. The plasmid pAZ2 contains the wild-type *his* promoter (p) and regulatory region, and pAZ13 contains the *his* attenuator (a) deletion Δ*hisGal242* and the Δ*hisGD* mutation (11). The ninth codon of the 5′-truncated *lacZ* gene is fused in-frame with the 42nd codon of *hisC*. The *Eco*RI–*Pst* I fragment was from pJES35 (21), the *Pst* I–*Hin*dIII fragment was from pBR322 (14), and the *Hin*dIII–*Eco*RI fragments were from M13mp9::*his* phages (11) as indicated in Table 1.

Table 2. Effects on ppGpp regulation of oligonucleotide-directed mutations in the −10 region of the *his* operon

Plasmid	Promoter	β-galactosidase activity, A_{420}/hr per 50 μl −ppGpp	β-galactosidase activity, A_{420}/hr per 50 μl +ppGpp	Ratio of activities, +ppGpp/−ppGpp
pAZ2	*hisGp*$^+$	0.34	7.5	22.0
pAZ16	*hisGp3400*	3.2	5.4	1.7
pAZ17	*hisGp3401*	3.0	3.3	1.1
pRS229	*lacUV5*	15.4	21.4	1.4

Reaction mixtures contained, where indicated, 0.2 mM ppGpp. The relative template activities and magnitudes of stimulation were reproducible in different experiments; absolute activities were variable. The template concentrations were as follows: pAZ2, 66 nM; pAZ16, 57 nM; pAZ17, 52 nM; pRS229, 55 nM.

constructed by oligonucleotide-directed mutagenesis of the *his* operon fragment in phage M13mp9::*his*1 (11). The synthetic oligonucleotide 5′ TACCTTTTATTATAAACCAC 3′ is complementary to the *his* promoter from positions +1 to −19 except for positions −9, −10, and −11 (with respect to the start point of transcription at +1) (Fig. 2). The two promoter mutations generated, *hisGp3400* and *hisGp3401*, have the respective −10 hexamer sequences TATAAT and TAGAAT (Fig. 2). Occurrence of the 2-base mutation may be due to partial mismatch repair of the heteroduplex generated during mutagenesis. Nucleotide sequence analysis of the entire *his* promoter/regulatory region of each mutant revealed no additional base changes.

The −10 hexamer mutations *hisGp3400* and *hisGp3401* altered regulation by ppGpp *in vitro*. Table 2 shows the stimulatory effect of saturating ppGpp concentrations on *his* expression from the two mutant promoters as measured by the activity of *de novo* synthesized β-galactosidase. In the absence of ppGpp, the level of *his* expression from the mutant promoters was almost 10-fold higher than that from the wild-type promoter. In addition to relieving the ppGpp requirement, the two mutations reduced the stimulation by ppGpp to less than a factor of 2 in contrast to a factor of 22 for the wild-type promoter (Table 2). Expression of the *lacUV5* promoter was relatively unaffected by ppGpp as observed (Table 2) (4). The similar effects of the *hisGp3400* and *hisGp3401* mutations show that the base pairs in the fourth and fifth positions of the *his* −10 hexamer are critical to the activation of transcription by ppGpp.

Mutations Downstream of the *his* Promoter −10 Hexamer Alter Regulation by ppGpp. The sequence between the −10 hexamer and the start point of transcription was changed *in vitro* by deleting sequences in a *his* fragment contained in the phage M13mp9::*his*4 (11). Phage RF DNA was digested with *Mlu* I at the only *Mlu* I site in the *his* regulatory region to linearize it (Fig. 2), then it was digested with BAL-31 nuclease and ligated. The three deletions generated,

Table 3. Effects on ppGpp regulation of BAL-31-generated deletions in the *his* promoter-leader region

Plasmid	Promoter	β-galactosidase activity, A_{420}/hr per 50 μl −ppGpp	β-galactosidase activity, A_{420}/hr per 50 μl +ppGpp	Ratio of activities, +ppGpp/−ppGpp
pAZ13	*hisGp*$^+$	0.05	1.1	22.0
pAZ7	Δ*hisGpe3420*	0.14	1.4	10.0
pAZ5	Δ*hisGpe3421*	0.51	0.94	1.8
pAZ3	Δ*hisGpe3422*	≤0.002	0.03	≥15.0

Experimental conditions were as indicated in Table 2. The template concentrations were as follows: pAZ13, 87 nM; pAZ7, 88 nM; pAZ5, 81 nM; pAZ3, 73 nM.

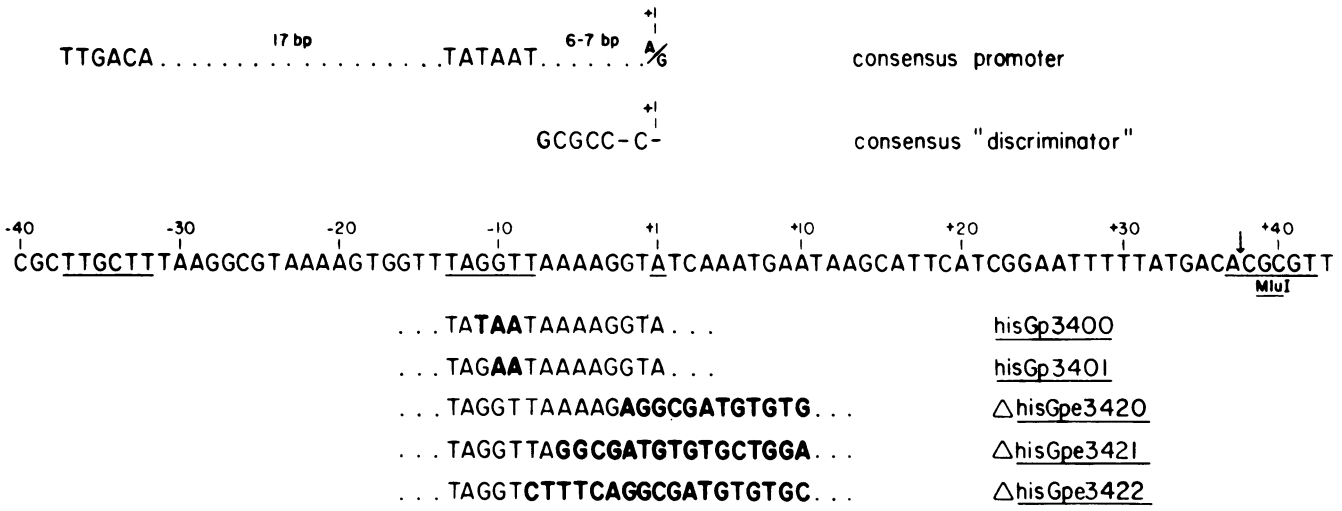

FIG. 2. Wild-type and mutant *his* promoter sequences, the consensus promoter sequence (22), and the consensus discriminator sequence for ppGpp repressible genes (23). The wild-type *his* promoter sequence (2) is shown with the −35 hexamer, −10 hexamer, and start point of transcription underlined. The oligonucleotide-directed mutations *hisGp3400* and *hisGp3401* are shown in boldface type. The arrow indicates the *Mlu* I site, which was the origin of the BAL-31 digestion used to construct the *hisGpe* deletions. The leftward extent of each deletion is indicated by the junction of normal and boldface lettering. The right end points of the deletions are: Δ*hisGpe3420*, +88; Δ*hisGpe3421*, +89; and Δ*hisGpe3422*, +83.

Δ*hisGpe3420*, Δ*hisGpe3421*, and Δ*hisGpe3422*, alter the promoter sequence as shown in Fig. 2 (bases in boldface). To alleviate any possible effects of the deletion mutations on attenuation, the mutations were constructed in M13-mp9::*his*4 phage, which lacks the attenuator as a result of the Δ*hisGa1242* mutation (11).

The three promoter deletions had differing effects on ppGpp regulation of *his* expression (Table 3). The Δ*hisGpe3421* mutation, which extends into the four consecutive adenosine residues immediately downstream of the −10 region, had the largest effect. In the absence of ppGpp, the level of expression of the Δ*hisGpe3421* promoter was about 10-fold higher than that of the wild-type promoter. In addition, this mutation reduced the stimulation by ppGpp to less than a factor of 2. The regulatory effects of the Δ*hisGpe3421* mutation were, therefore, very similar to those of the *hisGp3400* and *hisGp3401* −10 hexamer mutations demonstrating that the region between the −10 hexamer and the start point of transcription is critical for regulation of the *his* promoter by ppGpp. The Δ*hisGpe3420* mutation, which extends to position −2, had a smaller effect on *his* regulation: ppGpp-independent *his* expression was elevated about 3-fold and stimulation by ppGpp was 10-fold. The Δ*hisGpe3422* mutation extends furthest upstream and substitutes a cytidine residue for the highly conserved thymidine residue in the sixth position of the −10 hexamer sequence. Although this substitution drastically reduces promoter strength, the Δ*hisGpe3422* promoter was stimulated by ppGpp at least as much as the wild-type promoter.

DISCUSSION

In this paper we show that mutations in the *his* promoter increase *his* expression in the absence of ppGpp and dramatically reduce the stimulatory effect of ppGpp. The results provide strong evidence for the role of ppGpp as a positive regulator of the *his* operon (and, by analogy, other genes involved in amino acid production). The physiological significance of the *in vitro* results is supported by preliminary analyses of the regulatory effects of the *hisGp3400* and *hisGp3401* promoter mutations *in vivo* (data not shown).

The Target of ppGpp Activation Is a Step Associated with Initiation of Transcription. Studies in an uncoupled transcription–translation system showed that ppGpp acts on transcription of the *his* operon (5). The site-directed mutations in the nontranscribed region of the *his* promoter that alter the response to ppGpp now establish that this nucleotide acts primarily at initiation of transcription of the *his* operon. Apparently ppGpp does not significantly regulate *his* transcription during elongation either directly by influencing pausing of transcribing RNA polymerase at specific sites as has been observed for the *rrnB* operon (24) or in conjunction with the nusA protein, which affects transcription of a number of genes (24, 25). A "Box A-like" sequence in the *his* operon leader peptide region (25) that might be recognized by the nusA protein is deleted by our three BAL-31 generated mutations. Results presented here indicate that regulation of *his* transcription by ppGpp is at a step related to the function of the −10 hexamer sequence and of the region extending through the start point of transcription. This is the DNA region known to be melted in the open promoter complex prior to initiation.

Steps Associated with Initiation of Transcription Potentially Regulated by ppGpp. Initiation of transcription (the joining of the first two nucleotides in the growing RNA chain) is preceded by a series of RNA polymerase holoenzyme–DNA interactions including closed promoter complex formation followed by transition to the open promoter complex (26). During formation of the closed complex, RNA polymerase makes ionic bonds with the DNA backbone phosphates, which contribute much of the binding energy, and makes hydrogen bonds to determinants in the DNA helical grooves that define the specificity of binding. Open complex formation involves the melting of approximately 10–17 base pairs of DNA (27–29); the melted region encompasses the sequence from at least −9 to +2, or +3, for the phage T7 wild-type A3 promoter and the mutant *lacUV5* promoter, respectively (28). Subsequent to initiation, RNA polymerase may produce short, aborted transcripts before a stable, elongation complex is formed and the σ factor is released. Any of these steps (closed or open complex formation, abortive initiation, or σ release) could potentially be regulated by ppGpp.

Kinetic analyses indicate that the transition from the closed to the open complex is a minimum two-step process: an RNA polymerase conformational change that "nucleates" DNA melting followed by DNA strand separation (30, 31). The nucleation step is relatively insensitive to ionic strength and,

therefore, does not involve major changes in protein–DNA ionic interactions or in DNA conformation (31). This step could, however, involve a shift of the hydrogen bond contacts, making subsequent DNA melting more favorable. Nucleation provides an intermediate step in open complex formation at which ppGpp regulation could occur. This possibility is supported by analysis of the effects of *lac* promoter mutations.

The wild-type *lac* promoter, which is strongly stimulated by ppGpp (4, 32), differs from the consensus ("ideal") sequence for promoters recognized by the $E\sigma^{70}$ form of RNA polymerase in the fourth and fifth positions of the −10 hexamer sequence (consensus sequence-TATAAT; *lacZp*$^+$-TATGTT). The *lacUV5* mutation converts these two positions to the consensus base pairs and nearly abolishes ppGpp regulation (4, 32; Table 2). Whereas the *lacUV5* mutation has a negligible effect on closed complex formation, there is a 35-fold increase in the rate of transition to the open complex (33). Another *lac* promoter mutation, *lacP*^{s}, changes the fourth position of the −10 hexamer sequence to the consensus base pair (to give TATATT) and increases the transition rate much less than *lacUV5* (33, 34). The fact that the two mutant *lac* promoters have the same A+T content in the melted region makes it unlikely that the difference in transition rates results from differences in DNA duplex stability. The *lacUV5* mutation may increase the transition rate primarily by an effect on the nucleation step rather than by a direct effect on strand separation.

ppGpp appears to modulate the same kinetic step that is altered by the *lacUV5* mutation. We suggest, therefore, that it may be the nucleation step that is regulated by ppGpp in the activation of transcription. The *hisGp3400* and *hisGp3401* mutations, which like *lacUV5* introduce consensus base pairs into the fourth and fifth positions of the −10 hexamer sequence (Fig. 2) and relieve the requirement for ppGpp, support this proposal.

A Model for Regulation of Transcription by ppGpp. The working model we propose for activation of transcription by ppGpp is as follows. Certain promoters such as the wild-type *his* and *lac* promoters have sequences that present unfavorable contacts for nucleation in the absence of ppGpp. These sequences may have contacts that are inhibitory for nucleation; alternatively, optimal contacts may be missing. For these promoters, the nucleation step is, therefore, strongly rate-limiting in the pathway to open complex formation. The sequence positions that are important for nucleation include the fourth and fifth base pairs of the −10 hexamer sequence and possibly one or more base pairs between the −10 hexamer and the start point of transcription (consistent with the effects of the Δ*hisGpe3421* mutation). Mutations such as *hisGp3400*, *hisGp3401*, Δ*hisGpe3421*, and *lacUV5* may either eliminate inhibitory contacts or provide contacts favorable for nucleation, thereby circumventing the requirement for ppGpp to activate transcription. In the presence of ppGpp, RNA polymerase assumes a modified conformation that results in recognition of DNA contacts favorable for nucleation at promoters subject to activation by ppGpp, thus increasing the rate of open complex formation.

We suggest that ppGpp regulates a step after closed complex formation at repressible promoters as well. Kajitani and Ishihama (35) have reported that ppGpp inhibits transcription *in vitro* from preformed open complexes of several ppGpp-repressible promoters. This result implies that ppGpp is capable either of reversing the formation of open complexes or enhancing abortive initiation of transcription (26). The ppGpp repressible promoters have favorable contacts for nucleation in the −10 hexamer sequence (see below), but DNA melting or escape from abortive initiation may be rate-limiting because of the high G+C content of the discriminator sequence (23). Nucleation, DNA melting, or escape from abortive initiation could be inhibited by a ppGpp-induced conformational change in RNA polymerase.

The role we have proposed for ppGpp in regulating transcription after closed complex formation is quite different than that proposed by Lamond and Travers (36). They argued that ppGpp might inhibit transcription of repressible promoters primarily by further reducing the affinity of RNA polymerase for an already poor binding sequence. As they point out, their model fails to explain stimulation of transcription by ppGpp of activable promoters. Although the validity of our proposal requires additional evidence of a more direct nature, it is consistent with the available promoter mutant evidence and provides a possible mechanistic relationship between repression and activation of transcription by ppGpp.

Role of Promoter Sequence in ppGpp Regulation. Several studies (8, 9, 23, 37) have demonstrated the importance of a G+C-rich "discriminator" between the −10 hexamer sequence and the start point of transcription in promoters repressed by ppGpp. The discriminator regions of ppGpp activable promoters (e.g., those for amino acid biosynthetic genes) (5) are A+T-rich with a strong preference for adenosine or thymidine in the third and fourth positions downstream of the −10 hexamer sequence (23). The Δ*hisGpe3421* mutation, which introduces four consecutive guanosine or cytidine residues into the discriminator sequence (Fig. 2), establishes that this region of the *his* promoter is important in the regulation of transcription by ppGpp.

We have not found DNA sequences (e.g., dyad symmetries) that define a common interaction site for a hypothetical ppGpp binding protein (although this does not rule out possible participation of a DNA binding protein). ppGpp could modify RNA polymerase conformation either by binding directly to the enzyme, or in conjunction with an accessory protein. The further characterization of RNA polymerase mutants altered in ppGpp regulation (7, 38) should aid in distinguishing between these possibilities.

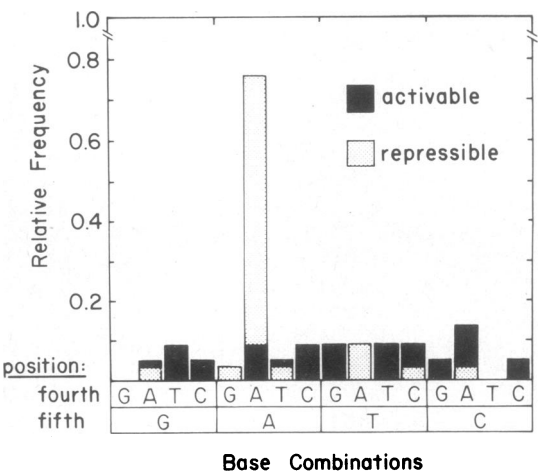

FIG. 3. Relative frequencies of base combinations in the fourth and fifth positions of the −10 hexamer sequences of ppGpp activable and repressible promoters (from *E. coli* unless noted). For ppGpp activable promoters, we have included all available promoter sequences for amino acid biosynthetic genes as follows: *argCp*, *argEp*, *argGp*, *argIp*, *aroHp*, *glyAp*, *hisGp*, *ilvBp*, *ilvGp*, *leuAp*, *trpEp*, *thrAp* (22); *aroFp*, *pheAp* (39); *asnAp* (40); *hisGp* (*S. typhimurium*) (2); *ilvIIp* (41); *lysAp* (42); *lysCp* (43); *metBp* (44); and *trpEp* (*S. typhimurium*) (45). The repressible promoter sequences are those of genes shown or predicted to be inhibited by ppGpp as follows: *argTp* (*S. typhimurium*), *leuVp*, *metTp*, *metYp*, *serTp*, *thrUp*, *tyrTp* (46); *pyrB2p* (47); *rplTp* (48); *rpsA1p* (49); *rpsOp* (50); *rpsU1p*, *rpsU2p* (51); *rrnAB1p*, *rrnD1p*, *rrnE1p*, *rrnG1p*, *rrnX1p*, *rplJp*, *rplKp*, *rpmH1p*, *rplNp* (spc), *rpsJp* (S10), *rpsLp* (str), *rpsMp* (*rpoA*), *rpsT1p*, *rpsT2p*, *tufBp* (22).

Activable promoters were compared with promoters known or predicted to be repressed by ppGpp (e.g., those for stable RNA and ribosomal protein genes) to identify sequence features in addition to the discriminator that might define the response to ppGpp. Comparison of the −35 hexamer sequences revealed no compelling correlation between sequence and the mode of regulation. However, we did find a striking similarity among the −10 hexamer sequences of repressible promoters that was absent from the −10 hexamer sequences of activable promoters. As shown in Fig. 3, most repressible promoters (76%) have A residues in the fourth and fifth positions of the −10 hexamer sequence, while activable promoters have a random distribution of bases in these two positions. Together with the analyses of the *hisGp3400, hisGp3401*, and *lacUV5* mutations, this observation supports the idea that the fourth and fifth positions of the −10 hexamer sequence are important in the activation of transcription by ppGpp. The presence of thymidine, guanosine, or cytidine in one or both of these two positions of most amino acid biosynthetic gene promoters (Fig. 3) would provide unfavorable contacts for the nucleation step of closed to open complex formation and the potential for a ppGpp activation mechanism. It will be interesting to test genetically the function of the −10 hexamer sequence in regulation of ppGpp repressible promoters and to determine those sequence changes that are required to interconvert repressible and activable promoters.

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