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NOTES

Histidine Operon Deattenuation in *dnaA* Mutants of *Salmonella typhimurium* Correlates with a Decrease in the Gene Dosage Ratio between $tRNA^{His}$ and Histidine Biosynthetic Loci

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Expression of the histidine operon of *Salmonella typhimurium* is increased in *dnaA*(Ts) mutants at 37°C. This effect requires an intact *his* attenuator and can be suppressed by increasing the gene copy number of the *hisR* locus, which encodes the $tRNA^{His}$. We present data which suggest that the *his* deattenuation defect in *dnaA*(Ts) mutants results from the loss of a gene dosage gradient between the *hisR* locus, close to *oriC*, and the *his* operon, far from *oriC*. Some of the conclusions drawn here may apply to other operons as well.

In prokaryotes, the DnaA protein plays a central role in initiation of chromosomal replication. The protein binds to a specific 9-bp sequence, the DnaA box, which is repeated four times at the replication origin, *oriC* (13 [reviewed in reference 20]). Besides this primary function, DnaA acts as a transcription factor that can regulate the initiation or termination of transcription upon binding sequences related to DnaA boxes found in various genes (21). In this study, we describe an additional locus whose expression is influenced by the DnaA protein: the histidine biosynthetic operon of *Salmonella typhimurium*. The *his* operon is transcribed from the primary promoter (P1), but the main regulation results from a translation-dependent transcription attenuation mechanism whereby transcriptional levels are inversely correlated with the levels of histidyl-$tRNA^{His}$ in the cell (16 [reviewed in reference 32]). This system is very finely tuned, since as little as a 50% reduction in histidyl-$tRNA^{His}$ causes severalfold *his* deattenuation (18). Several *his* regulatory mutants affected in $tRNA^{His}$ biosynthesis have been described, including two classes resulting from changes in the closely linked genes for RNase P (*rnpA* [6]) and DNA gyrase (*gyrB* [24]). The possibility that additional mutations mapping in the same region might be *dnaA* alleles has been suggested (24).

Deattenuation of a chromosomal *his-lac* fusion in *dnaA*(Ts) strains at semipermissive temperature. Two thermosensitive *dnaA* alleles that prevent growth at 42°C, *dnaA727* and *dnaA747* (kindly provided by Russ Maurer, Case Western Reserve University, Cleveland, Ohio), were used in this study (Table 1). Introduction of either of these mutations into a strain carrying a *his-lac* chromosomal operon fusion (*hisC9968*::MudJ) results in an increase in β-galactosidase activity that is moderate (twofold) at 28°C and becomes substantial (eight- to ninefold) at 37°C, a temperature still permissive for growth (Table 2). In principle, such an increase in *his* operon expression might be ascribed either to a negative effect of the DnaA protein on the *his* promoter or to a positive role on the attenuation mechanism. To distinguish between these possibilities, the effect of the *dnaA747* allele was analyzed in a strain carrying a *his* attenuator deletion (*hisO1242* [16]). Results in Table 3 show that the *dnaA*-dependent increase in *his-lac* expression is abolished when the attenuator is absent. Similar results were obtained upon replacing the *hisC9968*::MudJ operon fusion with a protein fusion (15) (*hisD2504*::MudK) that generates approximately 100-fold-less β-galactosidase activity (data not shown), indicating that failure to observe a β-galactosidase increase is not attributable to the enzyme levels exceeding the measurable range. These findings rule out the *his* operon P1 promoter as the target of the DnaA protein and suggest that increased operon expression results from transcription deattenuation. A direct role of the protein on attenuator function is unlikely, due to the absence of sequences resembling a DnaA box at or near this site. The results presented below suggest that the DnaA protein affects the attenuation mechanism indirectly, by influencing the level of expression of the *hisR* locus, the single-copy gene encoding $tRNA^{His}$ (5).

Suppression of *his* deattenuation in a *dnaA* mutant by increasing the *hisR* gene dosage. Isogenic strains carrying the *dnaA747* mutation or its wild-type allele and a *his-lac* fusion, were transformed by $ptRNA^{His}$ CCA, a recombinant plasmid carrying the entire *hisR* locus (23), or by parental vector pACYC184. The resulting strains (MA4870, MA4871, MA4872, and MA4873) were grown in nutrient broth medium supplemented with tetracycline (5 μg/ml), and β-galactosidase enzyme activity was assayed as described in Table 2, footnote *a*. The β-galactosidase activities of the $dnaA^+$ and *dnaA747* strains were 120 and 861 Miller units, respectively, with plasmid pACYC184 and 30 and 24 Miller units, respectively, with plasmid $ptRNA^{His}$CCA. Thus, increasing the *hisR* gene dosage has two noticeable effects: (i) it causes a reduction in the basal level of *his* operon expression regardless of the *dnaA* allele, and (ii) it completely suppresses *dnaA*-dependent deattenuation. The former effect is consistent with the notion that the *his* operon is incompletely repressed in rich medium (32) and suggests that this reflects limiting $tRNA^{His}$ levels. The latter effect strongly suggests that *his* deattenuation in *dnaA* mutants results from a shortage of $tRNA^{His}$.

A potential DnaA box within the *hisR* promoter sequence is not involved in *his* deattenuation. Examination of the nucleotide se-

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TABLE 1. *S. typhimurium* strains and plasmids used in this study

Strain[a] or plasmid	Genotype[b]	Source or reference[c]
Strains		
RM374	*dnaA727 zid-1257*::Tn*10*dTc	19
RM595	*dnaA747 zid-2025*::Tn*10*dKm	R. Maurer
RM135	*dnaC141 zji-1255*::Tn*10 thyA2 deo*	19
RM2937	*dnaC602 zji-8183*::Tn*10 thyA2 deo*	19
DEH38	*zdi-6798*::Tn*10*dTet *proU2881*::MudJ	D. El-Hanafi
MA785	*hisC9968*::MudA(Ap) *zid-1257*::Tn*10*dTc *dnaA747*	
MA786	*hisC9968*::MudA(Ap) *zid-1257*::Tn*10*dTc	
MA922	*hisC9968*::MudA(Ap) *zid-2025*::Tn*10*dKm *dnaA747 zdi-6798*::Tn*10*dTc	
MA923	*hisC9968*::MudA(Ap) *zid-2025*::Tn*10*dKm *zdi-6798*::Tn*10*dTc	
MA2847	*zgb-6784*::Tn*10*dTc	4
MA4522	*hisC9968*::MudJ(Km) *zid-1257*::Tn*10*dTc *dnaA727*	
MA4526	*hisC9968*::MudJ(Km) *zid-1257*::Tn*10*dTc *dnaA747*	
MA4527	*hisC9968*::MudJ(Km) *zid-1257*::Tn*10*dTc	
MA4528	*hisC9968*::MudJ(Km) *hisR10107 zid-1257*::Tn*10*dTc *dnaA747*	
MA4529	*hisC9968*::MudJ(Km) *hisR10107 zid-1257*::Tn*10*dTc	
MA4530	*hisC9968*::MudJ(Km) *zji-1255*::Tn*10 dnaC141*	
MA4534	*hisC9968*::MudJ(Km) *zji-8183*::Tn*10 dnaC602*	
MA4535	*hisC9968*::MudJ(Km) *zji-8183*::Tn*10*	
MA4764	*hisO1242 hisC9968*::MudJ(Km) *zid-1257*::Tn*10*dTc *dnaA747*	
MA4765	*hisO1242 hisC9968*::MudJ(Km) *zid-1257*::Tn*10*dTc	
MA4870	*hisC9968*::MudA(Ap) *zid-2025*::Tn*10*dKm *dnaA747*/pACYC184	
MA4871	*hisC9968*::MudA(Ap) *zid-2025*::Tn*10*dKm *dnaA747*/ptRNA^{His}CCA	
MA4872	*hisC9968*::MudA(Ap) *zid-2025*::Tn*10*dKm/pACYC184	
MA4873	*hisC9968*::MudA(Ap) *zid-2025*::Tn*10*dKm/ptRNA^{His}CCA	
Plasmids		
pACYC184	Tc^r Cm^r	9
ptRNA^{His}CCA	Tc^r; pACYC184 derivative that carries the entire *hisR* locus	23
pTS1	Ap^r Tc^r; pUC18 derivative that carries a 5.3-kb *Bam*HI chromosomal DNA fragment from strain MA2847 including the Tn*10*dTc element and adjacent material	

[a] All bacterial strains are derived from *S. typhimurium* LT2. Bacteria were grown in nutrient broth (NB) medium throughout this study. Strain construction was performed as previously described (11).

[b] When appropriate, the "*z*" designation of transposon insertions was revised according to the latest edition of the *Salmonella* genetic map (26). MudA (Ap^r) refers to a conditionally transposition-defective derivative of the phage Mud1 constructed by Hughes and Roth (14). MudJ (Kn^r) refers to phage Mud1–1734 constructed by Castilho et al. (7). The *hisC*-Mud fusions used in this work were previously described (11).

[c] Where not specified, the source of the strain is this work.

quence of the *hisR* promoter region reveals that the segment between positions −12 and −4 (TTATCCACC in the nontemplate strand) matches exactly the consensus sequence for a DnaA box as defined by Schaefer and Messer (27). Thus, a tentative explanation for the *his*-deattenuated phenotype of *dnaA* mutants is that binding of DnaA protein to the *hisR* promoter is required for its optimal activity. The availability of a promoter mutation affecting the potential DnaA box allowed this hypothesis to be tested. Mutation *hisR10107* causes a C/G to T/A base pair change at the 6th position of the DnaA box (TTATCTACC [11]). This is a highly conserved position, and its alteration in different DnaA boxes was shown to reduce binding by the DnaA protein (13, 27). Thus, one might expect that the *hisR10107* change should either impair *hisR* promoter activity—resulting in *his* deattenuation even in a *dnaA*$^+$ background—or render the promoter independent of DnaA protein activity, thereby suppressing the deattenuation defect of *dnaA* mutants. The results in Table 3 show that the *hisR10107* mutation does neither of the above. The mutant promoter behaves like the wild-type promoter in its response to the

TABLE 2. Effect of *dnaA*(Ts) mutations on expression of a *his-lac* operon fusion at 28 and 37°C[a]

Strain	*dnaA* allele	β-Galactosidase activity in Miller units (deattenuation ratio)	
		28°C	37°C
MA4527	Wild type	93 (1.0)	110 (1.0)
MA4526	*dnaA747*	206 (2.2)	994 (9.0)
MA4522	*dnaA727*	220 (2.4)	863 (7.8)

[a] Bacterial cultures were grown overnight at 28°C in nutrient broth (NB), diluted 1 to 200, and incubated under aerobic conditions at 28 or 37°C in NB. Cell growth was monitored spectrophotometrically. At an optical density at 600 nm of 0.3 to 0.4, cell cultures were chilled on ice, and β-galactosidase activity was measured in toluene-permeabilized cells as described previously (22); the reported values represent the averages of at least three independent determinations. The standard error was below 10% in all cases.

TABLE 3. Effect of *hisO1242* and *hisR10107* mutations on *his-lac* expression at 37°C in the presence or absence of the *dnaA747* allele[a]

Relevant genotype	β-Galactosidase activity (Miller units)	
	dnaA$^+$	*dnaA747*
hisO$^+$ *hisR*$^+$	110	994
hisO1242 hisR$^+$	4,670	3,898
hisO$^+$ *hisR10107*	84	1,011

[a] All strains used (MA4527, MA4526, MA4765, MA4764, MA4529, and MA4528) carry the *hisC9968*::MudJ fusion (see Table 1). Cells were grown and processed for the determination of β-galactosidase activity as described in the footnote to Table 2.

TABLE 4. Effect of *dnaC*(Ts) mutations on *his-lac* expression at 28 and 43°C[a]

Strain	*dnaC* allele	β-Galactosidase activity in Miller units (deattenuation ratio)	
		28°C	43°C
MA4535	Wild type	93 (1.0)	119 (1.0)
MA4530	*dnaC141*	103 (1.1)	395 (3.3)
MA4534	*dnaC602*	90 (1.0)	396 (3.3)

[a] Bacterial cultures were grown overnight at 28°C in nutrient broth (NB), diluted 1 to 200, and incubated at 28 or 43°C in NB. β-Galactosidase activity was determined as described in the footnote to Table 2.

dnaA alteration. This contrasts with the effect of the *hisR10107* mutation on the promoter sensitivity to negative DNA supercoiling: in facilitating the promoter-opening step, the C/G-to-T/A base pair change renders the promoter insensitive to defects in DNA gyrase (11, 12). Overall, these results tend to indicate that the link between the DnaA protein and *his* regulation may not involve a direct interaction between the protein and the *hisR* promoter.

***dnaC* mutations cause *his* deattenuation.** The DnaC protein is required for initiation of DNA replication at a later stage than the DnaA protein (20). Mutations *dnaC141* and *dnaC602* result in thermosensitive alleles (19) that are not completely lethal, allowing some residual DNA synthesis and cell growth to take place at 43°C (13a). Both alleles were introduced in the *his*::MudJ genetic background, and β-galactosidase levels were measured as a function of temperature. The results showed a threefold increase in *his* expression when cells are grown at 43°C relative to cells grown at 28°C (Table 4). Although this increase is less dramatic than that observed with the *dnaA* mutants, the trend is clearly the same, and the smaller magnitude of the effects is ascribable to the "leaky" character of the *dnaC* alleles. These data strongly suggest that the *his* deattenuation defect is consequent to a defect in initiation of DNA replication and is independent of the nature of the initiation function affected.

***his* deattenuation in a *dnaA* mutant correlates with the loss of the gene dosage gradient between *hisR* and *his* loci.** In fast-growing bacteria, the time interval separating consecutive rounds of initiation of DNA replication is shorter than the time needed for each elongation cycle to go to completion. As a result, fast-growing bacteria have multiple replication forks, and genes that are near the origin of replication are normally present at a higher copy number than genes located in the terminus region (8, 10). The existence of such a gene dosage gradient has been inferred from measuring the expression of reporter genes introduced at various chromosomal positions (28, 30) and demonstrated by quantitative Southern hybridization analyses (2). In *S. typhimurium*, the *oriC* site is located at 85 map units on the genetic map (26). The *terC* site has never been exactly mapped, but it is likely to be around 34 map units, at the equivalent position as in *Escherichia coli*. From the positions of *hisR* and *his* biosynthetic loci at 85 and 45 map units, respectively, one predicts that, in rich medium, the *hisR* gene dosage should be in excess relative to the *his* operon. Conceivably, this imbalance might be essential to attain the $tRNA^{His}$ levels needed for full attenuation of *his* operon transcription. In order to test this possibility, we studied the effects of a *dnaA* mutation on the relative dosage of the same DNA sequence inserted near *oriC* or near *terC*. Chromosomal DNA from strains carrying a Tn*10*dTc element (Tn*10*Δ*16*Δ*17* Tc^r [31]) inserted either at 84 cs or at 38 cs was extracted from exponentially growing cells as described previously (3), digested with *Hpa*I restriction endonuclease, and subjected to Southern analysis by using ^{32}P-labelled DNA from a plasmid carrying the Tn*10*dTc element and sequences from the 62-cs region as a hybridization probe (Fig. 1A). For each lane in Fig. 1A, the Tn*10*dTc-specific 2-kb hybridization signal was quantified and normalized to the signal from the 62-cs sequence. In agreement with previous data (2), our results showed that, in the wild-type strain, the Tn*10*dTc element is amplified approximately twofold when located near *oriC*, compared to that when present in the terminus region (Fig. 1B). In contrast, this amplification is lost in the *dnaA747* mutant strain in which *oriC*- and *terC*-proximal sequences occur in equimolar amounts (Fig. 1B). Clearly, these data support the idea that the *his* deattenuation defect of DNA replication initiation mutants reflects a decrease in the *hisR*/*his* operon dosage ratio consequent to the decrease in the frequency of initiation events (Fig. 1C).

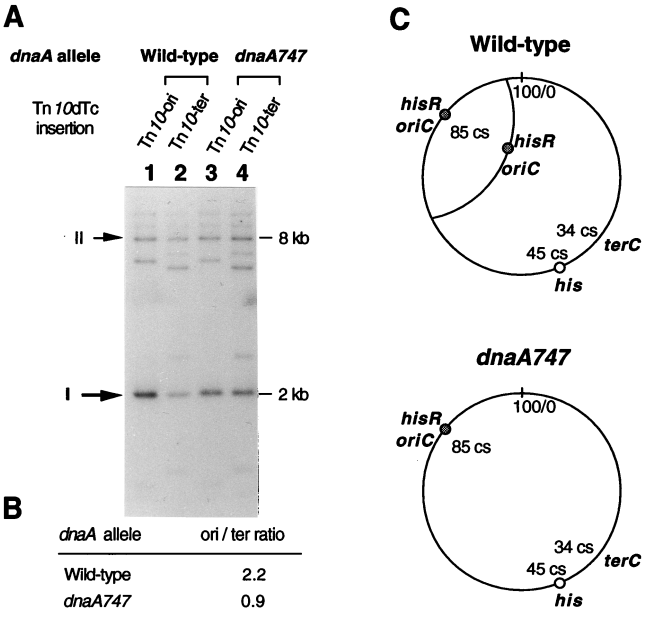

FIG. 1. Effect of the *dnaA747* allele on the relative gene dosage of *oriC*-proximal DNA sequences relatively to *terC*-proximal DNA sequences. Strains carry a chromosomal Tn*10*dTc insertion located either in the proximity of *oriC* (Tn*10*-ori; *zid-1257*::Tn*10*dTc) or in the proximity of *terC* (Tn*10*-ter; *zdi-6798*::Tn*10*dTc). (A) Southern blot analysis of *Hpa*I-digested chromosomal DNA hybridized with labeled pTS1 plasmid. Cells were grown overnight at 28°C in nutrient broth (NB), diluted 1 to 200, and incubated at 37°C in NB until an optical density at 600 nm of 0.4 was reached. Chromosomal DNA was extracted as described previously (3). DNA fragments were separated by electrophoresis on an agarose gel and transferred to a nylon membrane by capillarity under alkaline conditions (25). The membrane was hybridized to plasmid pTS1 DNA labeled with [α-^{32}P]CTP by nick translation (25). Plasmid pTS1 is a pUC18 (New England Biolabs) derivative carrying a 5.3-kb DNA insert which includes a Tn*10*dTc element (2.9 kb) and sequences from the region flanking the *argV* locus of *S. typhimurium* (4). In the experiment described above, pTS1 DNA hybridizes to a 2-kb fragment from the internal portion of Tn*10*dTc (band I) and to an 8-kb fragment from the *argV* region (band II). (The size of the latter fragment is reduced in a strain carrying an *argV* deletion [4]. Additional signals result from hybridization to the ends of Tn*10*dTc and to the Ap^r gene of the MudA element.) The strains used were MA786 (lane 1), MA923 (lane 2), MA785 (lane 3), and MA922 (lane 4). (B) Quantification of the relative gene dosage between *oriC*-proximal and *terC*-proximal Tn*10*dTc insertions. For each lane, the hybridization signal of band I was normalized to the signal of band II by using a 400S PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). The *oriC*/*terC* ratio was calculated for both $dnaA^+$ and *dnaA747* strains by dividing normalized signals corresponding to the Tn*10*-ori insertion by normalized signals corresponding to the Tn*10*-ter insertion. (C) Model in which the *hisR* locus is amplified relative to the *his* locus in a wild-type strain but not in the *dnaA747* mutant at 37°C. Reduction of the *hisR* gene dosage in a *dnaA* strain is responsible for deattenuation of *his* operon transcription. The positions of the different loci are indicated in centisomes.

Implications and conclusions. We propose a novel model for how *dnaA* alterations can affect gene expression and regulation. In this model, the DnaA protein does not act directly as a transcriptional regulator, but rather it influences transcription indirectly, through its role in DNA replication initiation, by modulating the relative copy number of a regulator gene and its target. The *his* operon might not be the only example of this form of control. In *E. coli*, expression of another biosynthetic operon regulated by attenuation, the *trp* operon, has been shown to be higher in a *dnaA* mutant (1). This effect was explained by postulating a direct role for the DnaA protein in the attenuation mechanism; however, no obvious DnaA boxes are found in the *trp* attenuator region, as already noticed by Messer and Weigel (21). From the analogies between the *trp* and *his* regulatory systems, we believe that the model proposed above can apply to the *trp* operon as well. Like $tRNA^{His}$, the $tRNA^{Trp}$ is encoded by a unique gene, *trpT*, located near *oriC*, whereas the *trp* operon is located near *terC*, suggesting that the copy ratio between *trpT* and the *trp* operon changes as a result of variations in DNA replication initiation frequency. In *E. coli*, histidine and tryptophan are both recognized by unique isoacceptor tRNAs encoded by single-copy genes (17). Conceivably, the increased ploidy of $tRNA^{His}$ and $tRNA^{Trp}$ genes that results from the multiplicity of replication forks might be important for optimizing translational rates in fast-growing cells. The lack of effects of *dnaA* mutations on two additional attenuation-controlled operons tested, the *thr* operon (1) and *leu* operon (our unpublished data), is consistent with threonine and leucine being amino acids recognized by multiple tRNA species encoded in several loci scattered around the chromosome (17).

The chromosomal gene dosage gradient flattens out as the growth rate decreases (10, 28). Therefore, one predicts that some deattenuation of *his* operon transcription should occur in slow-growing bacteria, even if histidine is supplied to the medium. The basal levels of *his* operon expression are indeed higher in poor medium relative to rich medium (reference 33 and our unpublished data); however, this difference was shown to be independent of the *his* attenuator and mainly results from ppGpp-mediated stimulation of the *his* P1 promoter (29). This suggests that, under nutrient-limited conditions, excess *hisR* gene dosage with respect to the *his* operon is not required for full attenuation. Perhaps the smaller demand for histidine in protein synthesis in bacteria growing in an unsupplemented medium causes histidyl-$tRNA^{His}$ levels to be high enough to ensure *his* attenuation regardless of the ploidy of the *hisR* gene.

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