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Transcription of the *Salmonella* Invasion Gene Activator, *hilA*, Requires HilD Activation in the Absence of Negative Regulators

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Salmonella enterica **serovar Typhimurium causes human gastroenteritis and a systemic typhoid-like infection in mice. Infection is initiated by entry of the bacteria into intestinal epithelial cells and is mediated by a type III secretion system that is encoded by genes in *Salmonella* pathogenicity island 1. The expression of invasion genes is tightly regulated by environmental conditions such as oxygen and osmolarity, as well as by many bacterial factors. The *hilA* gene encodes an OmpR/ToxR family transcriptional regulator that activates the expression of invasion genes in response to both environmental and genetic regulatory factors. HilD is an AraC/XylS regulator that has been postulated to act as a derepressor of *hilA* expression that promotes transcription by interfering with repressor binding at the *hilA* promoter. Our research group has identified four genes (*hilE*, *hha*, *pag*, and *ams*) that negatively affect *hilA* transcription. Since the postulated function of HilD at the *hilA* promoter is to counteract the effects of repressors, we examined this model by measuring *hilA*::Tn*5lacZY* expression in strains containing negative regulator mutations in the presence or absence of functional HilD. Single negative regulator mutations caused significant derepression of *hilA* expression, and two or more negative regulator mutations led to very high level expression of *hilA*. However, in all strains tested, the absence of *hilD* resulted in low-level expression of *hilA*, suggesting that HilD is required for activation of *hilA* expression, whether or not negative regulators are present. We also observed that deletion of the HilD binding sites in the chromosomal *hilA* promoter severely decreased *hilA* expression. In addition, we found that a single point mutation at leucine 289 in the C-terminal domain of the α subunit of RNA polymerase leads to very low levels of *hilA*::Tn*5lacZY* expression, suggesting that HilD activates transcription of *hilA* by contacting and recruiting RNA polymerase to the *hilA* promoter.**

Salmonella enterica serovar Typhimurium is the causative agent of a self-limiting gastroenteritis in humans and a typhoid-like infection in mice that serves as a model of *S. enterica* serovar Typhi infections in humans. *Salmonella* infections are caused by the ingestion of contaminated food or water, after which the bacteria are able to colonize the small intestine and invade intestinal enterocytes and M cells of the follicle-associated epithelium of Peyer's patches (9, 30, 41). Invasion is facilitated by a type III secretion system that allows the bacteria to secrete effector proteins into the eukaryotic host cell. The cumulative action of secreted effectors leads to engulfment of bacteria into the host cell (19). Subsequently, host-adapted strains of *Salmonella* gain access to underlying tissue to grow within the lymphatic system and spread to the liver and spleen, where unchecked growth leads to enteric fever and death (19, 29, 32).

More than 25 genes needed to encode the invasion machinery are located on the 40-kb *Salmonella* pathogenicity island 1 (SPI-1) at centisome 63 on the chromosome of serovar Typhimurium (reviewed in reference 12). These genes encode transcriptional regulators, type III secretion system components, chaperones, and secreted effectors. Other secreted effector proteins (SigD/SopB, SopE, and SopE2) are encoded elsewhere on the chromosome of *Salmonella* (6, 21, 22, 24, 45, 49). *Salmonella* has evolved a highly complex regulatory scheme to control the expression of invasion genes. Environmental conditions such as growth phase, pH, oxygen tension, and osmolarity regulate expression of *hilA*, an OmpR/ToxR type transcriptional regulator that is a central node in the regulatory network (4, 5). HilA binds to the *prg* and *inv* promoters on SPI-1 to activate transcription of these operons (4, 5, 34). Expression of *invF* leads to induction of the *sic*/*sip* operon, *sigD*/*sopB*, and *sopE* (4, 5, 11, 13, 34, 47). A mutation in *hilA* results in reduced expression of genes within the *prg*, *inv*, and *sic*/*sip* operons, as well as drastically reduced invasion of cultured epithelial cells, invasion of M cells, and mouse virulence (4, 41).

Because of the crucial role HilA plays in invasion gene expression, much work has been done to study its regulation. Work from our laboratory has identified mutations in several genes, including *hilE*, *pag*, *ams*, and *hha*, that increase chromosomal *hilA*::Tn*5lacZY* expression, suggesting that they encode negative regulators of *hilA* (16, 17). Mutations in *hilE* and *pag* fall within previously undescribed genes that are *Salmonella* specific and do not contain typical DNA-binding or protein interaction motifs. RNase E is encoded by *ams*, and Hha is a small histone-like protein that is able to bind to the *hilA* promoter (17, 38). Lon protease has also recently been described as a negative regulator of *hilA* and invasion gene ex-

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pression (46). Several groups have identified mutations in genes that result in decreased expression of *hilA*, indicating that *csrAB*, *sirA*/*barA*, *pstS*, *fadD*, *envZ*, *fliZ*, *hilC*/*sirC*/*sprA*, *fis*, and *hilD* have roles in *hilA* expression (1, 2, 27, 36, 42, 48). HilD, an AraC/XylS type transcriptional regulator, is a particularly important regulator of *hilA* expression, since a mutation in *hilD* results in a 14-fold decrease in chromosomal *hilA*::Tn*5lacZY-080* expression and a 53-fold decrease in invasion of HEp-2 cells (42). It is believed that HilD directly regulates *hilA* expression since it has been shown to bind to *hilA* promoter sequences (40, 43). Currently, HilD is postulated to act as a derepressor of *hilA* expression, since HilD is needed to allow $P_{hilA\text{-}lacZY}$ expression from a plasmid reporter (42). However, when sequences upstream of the −39 position in the *hilA* promoter, termed the upstream repressing sequence (URS), were deleted from pLS31, the *hilA* reporter plasmid, HilD was no longer required for high-level *hilA-lacZY* expression (42). These data led to the proposal that HilD modulates *hilA* expression by counteracting the effect of a negative regulator(s) that may repress *hilA* by binding at the URS. According to this model, HilD would act as a derepressor and not as a typical activator of transcription, which is thought to be the common method of regulation by members of the AraC/XylS family (20, 37, 42).

In order to examine this model of HilD activity at the *hilA* promoter, we were interested in determining the effect of a *hilD* mutation in strains of *Salmonella* containing mutations in negative regulators of *hilA*. We reasoned that, if HilD is a derepressor of *hilA* expression, then deleting negative regulators of the *hilA* gene would increase *hilA* expression in the absence of HilD. Therefore, we created single, double, and triple mutations in the negative regulator elements *hilE*, *hha*, *pag*, and/or *ams* in a *hilA-lacZY* chromosomal reporter strain with or without functional HilD. Surprisingly, our results indicate that functional HilD is required for *hilA* expression even when known negative regulators are deleted. Additional experiments were performed that suggest further that HilD provides an essential activating function for *hilA*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in the present study are shown in Table 1. Bacteria were routinely grown in Luria broth (LB; Gibco-BRL) containing the appropriate antibiotics at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 25 μg/ml; tetracycline, 25 μg/ml; and chloramphenicol, 20 μg/ml. *S. enterica* serovar Typhimurium strains used in β-galactosidase assays were grown under activating conditions for *hilA* expression by inoculating 3 μl of a stationary-phase culture into 3 ml of LB (1% NaCl) and incubating the culture statically overnight at 37°C until an optical density at 600 nm of 0.4 to 0.5 was reached, which corresponds to ~4.5×10^8 CFU/ml (28, 41).

Plasmid construction. Plasmid pJB5 was created by digesting pJB1 (48) with *Hin*dIII and *Not*I enzymes to remove the last 256 bp of the *hilD* coding region. The *lacZY* genes were removed from plasmid pGEM-TlacZY as a *Hin*dIII-*Not*I fragment and ligated into pJB1 at the *Hin*dIII and *Not*I sites, creating a single-copy plasmid *hilD-lacZY* transcriptional fusion. This plasmid contains 1,039 bp upstream of the putative translation initiation codon of *hilD*. Plasmid pJB3 was created by amplifying the *hilD* open reading frame including an upstream ribosome-binding site from *Salmonella* chromosomal DNA with primers BamHI3′hilD and NsiI5′hilD (primer sequences are in Table 2). The amplified product was digested with *Bam*HI and *Nsi*I and ligated into the single-copy cloning vector pZC320 (44) digested with *Bam*HI and *Nsi*I, such that *hilD* expression is driven by the *lac* promoter. Restriction digestions, DNA ligations, bacterial electroporations, and PCR amplifications were done under standard conditions and according to standard protocols.

Creation of defined mutations. The *hilD*::*cam*, Δ*hha*, Δ*hilE*, and ΔURS mutations were created in the SL1344 chromosome or in pLS31 by using the procedure described by Datsenko and Wanner (14). Briefly, PCR primers were synthesized with 50 bp of homology to the 5′ and 3′ ends of the *hilD* gene, the *hha* gene, the *hilE* gene, and sequences from positions −314 to −68 in the *hilA* promoter. Each upstream primer also contains priming site 2 for pKD3 or pKD4, and each downstream primer contains priming site 1 for pKD3 or pKD4 (14). The primers are named hilD5FRT, hilD3FRT, hha5W, hha3W, hilE5W′, hilE3W′, −314hilA, and −68hilA. PCR amplification was performed with plasmid pKD3 as the template for the *hha*, *hilE*, and *hilD* primers and plasmid pKD4 as the template for the URS primers to yield the expected fragments of 1.1 kb for pKD3 and 1.6 kb for pKD4. The linear PCR fragments were purified and electroporated into SL1344 or into DH12S pLS31 carrying pKD46, and mutants were selected on L-chloramphenicol plates when pKD3 was used as the template plasmid or on L-kanamycin plates when pKD4 was used as the template plasmid. Several chloramphenicol-resistant, ampicillin-sensitive (Cam^r Amp^s) or kanamycin-resistant, ampicillin-sensitive (Kan^r Amp^s) colonies were purified and found by PCR to have the transformed fragment recombined into the *hilD* gene, the *hha* gene, the *hilE* gene, or the sequence from positions −314 to −68 upstream of *hilA*. The Cam^r gene within the *hha* or *hilE* chromosomal sequence or the Kan^r gene within the URS chromosomal sequence was excised by introduction of plasmid pCP20 (temperature-sensitive replicon, Amp^r), which expresses the *flp* recombinase gene after thermal induction. Colonies that were Cam^s Amp^s or Kan^s Amp^s were shown to have excised the Cam^r or Kan^r gene by PCR, and therefore these colonies carry a complete deletion of the *hha* gene, the *hilE* gene, or the sequence from −314 to −68 upstream of *hilA*.

P22-mediated transduction. Antibiotic-resistant gene insertions were moved between strains by transduction with P22 HT int− as previously described (15). Transductants were selected on LB agar containing the appropriate antibiotics and 10 mM EGTA to prevent reinfection by P22.

β-Galactosidase assays. β-Galactosidase assays were performed with bacterial cultures by the method of Miller (39).

Introduction of *rpoA* mutations into SL1344. Unmarked *rpoA151*, *rpoA153*, *rpoA154*, and *rpoA155* point mutations were moved from LT2 into SL1344 by P22-mediated transduction of the *zhb-1624*::Tn*10*Δ marker that is 50% linked to *rpoA* mutations (Tn*10*Δ is a mini-Tet element that lacks the transposase sequences) (33). P22 lysates were made from several tetracycline-resistant (Tet^r) SL1344 *zhb-1624*::Tn*10*Δ colonies and used to transduce Tet^r into LT2 *pepT*::MudJ. The *pepT*::MudJ reporter in LT2 has a red colony phenotype on MacConkey agar; however, each *rpoA* mutation causes a decrease in *pepT*::MudJ expression such that colonies appear white or light pink on MacConkey agar. Lysates from SL1344 that gave rise to LT2 *pepT*::MudJ transductants that were white or light pink on MacConkey agar (~50%) were assumed to come from strains that harbored the *rpoA* mutation of interest. Subsequently, these SL1344 *rpoA* mutant strains were grown on Bochner media containing 24 μg of fusaric acid/ml to select for the loss of Tet^r associated with *zhb-1624*::Tn*10*Δ (8). The *pepT*::MudJ reporter or the *hilA*::Tn*5lacZY* reporter was then transduced into Tet^s SL1344 strains containing each *rpoA* mutation.

RESULTS

Individual or multiple mutations in *hilE*, *hha*, *pag*, or *ams* do not result in high-level expression of the *hilA* promoter in the absence of HilD. Since experiments by Schechter et al. suggested that HilD is not needed for expression of a $P_{hilA\text{-}lacZY}$ reporter plasmid when *hilA* promoter sequences upstream of position −39 are deleted, it was proposed that HilD is a derepressor needed to counteract the effects of negative regulators that utilize the URS to repress the *hilA* promoter (42). To investigate this unique model of regulation by HilD, we determined the effect of a *hilD* mutation in strains of *Salmonella* that also contained mutations in the negative regulators Hha, HilE, Pag, and RNase E. Work in our laboratory indicates that Hha regulates the *hilA* promoter directly, due to its ability to bind to the *hilA* promoter upstream of position −39 (17). It is unclear how HilE, Pag, or RNase E functions to cause negative regulation of the *hilA* promoter; however, they may directly or indirectly modulate *hilA* expression by interactions at the pro-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype[a]	Source or reference
Strains		
E. coli DH12S	*mcrA* Δ(*mrr-hsdRMS-mcrBC*)	Gibco-BRL
S. enterica serovar Typhimurium SL1344 and derivatives		
SL1344		50
EE658	SL1344 with *hilA*::Tn*5lacZY-080*, Tet^r Cam^r	5
TF80	*hha*::*kan* Kan^r	17
BJ2121	*hilE*::Tn*5* Kan^r	This work
BJ2186	*ams*::Tn*5* Kan^r	This work
BJ2398	EE658 with *hilD*::*cam*, Tet^r Cam^r	This work
BJ2400	EE658 with *pag*::Tn*5 hilD*::*cam*, Tet^r Cam^r Kan^r	This work
BJ2401	EE658 with *ams*::Tn*5 hilD*::*cam*, Tet^r Cam^r Kan^r	This work
BJ2477	EE658 with Δ*hha*, Tet^r	This work
BJ2478	EE658 with Δ*hilE*, Tet^r	This work
BJ2532	EE658 with Δ*hilE* Δ*hha*, Tet^r	This work
BJ2537	EE658 with Δ*hilE* Δ*hha hilD*::*cam*, Tet^r Cam^r	This work
BJ2540	EE658 with Δ*hilE* Δ*hha ams*::Tn*5 hilD*::*cam*, Tet^r Cam^r Kan^r	This work
BJ2541	EE658 with Δ*hilE* Δ*hha pag*::Tn*5 hilD*::*cam*, Tet^r Cam^r Kan^r	This work
BJ2542	EE658 with Δ*hilE* Δ*hha ams*::Tn*5*, Tet^r Kan^r	This work
BJ2544	EE658 with Δ*hilE ams*::Tn*5*, Tet^r Kan^r	This work
BJ2546	EE658 with Δ*hha ams*::Tn*5*, Tet^r Kan^r	This work
BJ2549	EE658 with Δ*hilE hilD*::*cam*, Tet^r Kan^r	This work
BJ2551	EE658 with *pag*::Tn*5*, Tet^r Kan^r	This work
BJ2552	EE658 with *ams*::Tn*5*, Tet^r Kan^r	This work
BJ2554	EE658 with Δ*hha hilD*::*cam*, Tet^r Cam^r	This work
BJ2560	EE658 with ΔURS *hilD*::*cam*, Tet^r Cam^r	This work
BJ2561	EE658 with ΔURS, Tet^r	This work
BJ2567	EE658 with Δ*hha pag*::Tn*5 hilD*::*cam*, Tet^r Cam^r Kan^r	This work
BJ2568	EE658 with Δ*hha ams*::Tn*5 hilD*::*cam*, Tet^r Cam^r Kan^r	This work
BJ2569	EE658 with Δ*hilE pag*::Tn*5 hilD*::*cam*, Tet^r Cam^r Kan^r	This work
BJ2570	EE658 with Δ*hilE ams*::Tn*5 hilD*::*cam*, Tet^r Cam^r Kan^r	This work
BJ2584	*pag*::Tn*5* Kan^r	This work
BJ2738	*zhb1624*::Tn*10*Δ *rpoA151*, Tet^r	This work
BJ2740	*zhb1624*::Tn*10*Δ *rpoA155*, Tet^r	This work
BJ2741	*zhb1624*::Tn*10*Δ *rpoA153*, Tet^r	This work
BJ2742	*zhb1624*::Tn*10*Δ *rpoA154*, Tet^r	This work
BJ2775	*rpoA151 hilA*::Tn*5lacZY*, Tet^r	This work
BJ2776	*rpoA155 hilA*::Tn*5lacZY*, Tet^r	This work
BJ2777	*rpoA153 hilA*::Tn*5lacZY*, Tet^r	This work
BJ2778	*rpoA154 hilA*::Tn*5lacZY*, Tet^r	This work
BJ2783	*pepT7*::MudJ, Kan^r	This work
BJ2784	*rpoA151 pepT7*::MudJ, Kan^r	This work
BJ2786	*rpoA155 pepT7*::MudJ, Kan^r	This work
BJ2787	*rpoA153 pepT7*::MudJ, Kan^r	This work
BJ2788	*rpoA154 pepT7*::MudJ, Kan^r	This work
BJ2827	*rpoA155* pJB5, Amp^r	This work
S. enterica serovar Typhimurium LT2 derivatives		
TN2262	*leuBCD485 pepT7*::MudJ *rpoA151* Kan^r Tet^r	33
TN3567	*leuBCD485 pepT7*::MudJ *zhb-1624*::Tn*10*Δ *rpoA151* Kan^r Tet^r	Charles Miller
TN3569	*leuBCD485 pepT7*::MudJ *zhb-1624*::Tn*10*Δ *rpoA155* Kan^r Tet^r	Charles Miller
TN3570	*leuBCD485 pepT7*::MudJ *zhb-1624*::Tn*10*Δ *rpoA153* Kan^r Tet^r	Charles Miller
TN3571	*leuBCD485 pepT7*::MudJ *zhb-1624*::Tn*10*Δ *rpoA154* Kan^r Tet^r	Charles Miller
Plasmids		
pJB3	*hilD* expressed from *lac* promoter in pZC320	This work
pJB5	P*hilD-lacZY,* Amp^r	This work
pKD3	FRT chloramphenicol template, Amp^r Cam^r	14
pKD4	FRT kanamycin template, Amp^r Cam^r	14
pKD46	Red recombinase, Amp^r	14
pCP20	FLP recombinase, Amp^r	14
pLS31	P*hilA-lacZY* reporter plasmid, Tet^r	42
pΔURS	URS deleted from pLS31, Tet^r	This work

[a] FRT, FLP recognition target.

TABLE 2. Sequences of primers used in this work

Primer	Sequence
hilD5FRT	5′-GTAGGATACCAGTAAGGAACATTAAAATAACATCAACAAAGGGATAATATGGTGTAGGCTGGAGCTGCTTC-3′
hilD3FRT	5′-ATTTTAATAAAAATCTTTACTTAAGTGACAGATACAAAAAATGTTAATGGTCATATGAATATCCTCCTTA-3′
hha5W	5′-ATCCTGTTAGTTTGTCTTGTTAAAAATTATTACAATCATAGGTAGAATTTGTGTAGGCTGGAGCTGCTTC-3′
hha3W	5′-CGAGGAGGCAGATAACACCTGCGTGTTCTCTAAAAAGTAATGTAGCGTGACATATGAATATCCTCCTTA-3′
−314hilA	5′-CGCTTGTTAGCTTTCTGCCAGGCATACCTCCTCTCTTCCTCCTGATATCGAGTGTAGGCTGGAGCTGCTTC-3′
−68hilA	5′-TTTTTGGGGTGTAAATGCTGCTTATTATATCTTCATGGTTAATGGTCTGATCATATGAATATCCTCCTTA-3′
BamHI3′hilD	5′-CGCGGATCCTTTACTTAAGTGACAGATACA-3′
NsiI5′hilD	5′-CCAATGCATCAACAAAGGGATAATATGGAA-3′
hilE5W′	5′-GTTATAGCAGATTGTCGGTATTTAATCTGGTATACAGAGACACCAACGAACATATGAATATCCTCCTTA-3′
hilE3W′	5′-ATTTCGCTATACAGCATCGCCCACTGCGAGTCCGCAAGCTTGTTTTGTCCGTGTAGGCTGGAGCTGCTTC-3′

posed URS. We expected that if HilD acts as a derepressor to relieve the effects of negative regulators at the *hilA* promoter, then strains containing mutations in *hilD* and negative regulatory elements would have significantly higher *hilA* expression than would be observed in a strain with a mutation in *hilD* alone. In other words, strains carrying mutations in negative regulatory elements should express *hilA* in the absence of HilD.

Figure 1A shows *hilA*::Tn*5lacZY* expression in strains containing mutations in negative regulators alone or in combination with the *hilD*::*cam* mutation. The wild-type strain expressed 421.5 Miller units of *hilA*::Tn*5lacZY* β-galactosidase activity when grown in activating high-osmolarity, low-oxygen conditions. The presence of individual Δ*hha*, Δ*hilE*, *ams*::Tn*5*, or *pag*::Tn*5* mutations in the strain increased *hilA*::Tn*5lacZY* expression 3.6-, 2.7-, 2.9-, or 6.0-fold, respectively. However, when the *hilD*::*cam* mutation was also present in the strains, individual mutations in negative regulators were unable to increase *hilA*::Tn*5lacZY* expression beyond the level seen in a *hilD* mutant alone. Therefore, mutations in *hilE*, *hha*, *pag*, or *ams* are unable to relieve repression of the *hilA* promoter in the absence of a functional *hilD* gene.

Since neither HilE, Hha, Pag, nor RNase E is solely responsible for repression of *hilA*::Tn*5lacZY* in the absence of HilD, we considered the possibility that these negative regulators function cooperatively to repress *hilA* expression. We created mutants with double and triple mutations in negative regulators, with or without a *hilD*::*cam* mutation, to determine whether mutations in two or more negative regulators could relieve repression of *hilA* in the absence of HilD. Double or triple mutations in *hilE*, *hha*, and *ams* have an additive effect, since each combination of mutations approximately doubled *hilA*::Tn*5lacZY* expression compared to the effect of single mutations in negative regulators (Fig. 1B). An exception is the *hha ams* double mutant, which exhibits only a 4.5-fold increase in *hilA*::Tn*5lacZY* expression, compared to increases of 3.6- or 2.9-fold for individual mutations in *hha* or *ams*, respectively. We were unable to create stable double or triple negative regulator mutants that contained *pag*::Tn*5*, since these strains lost the *hilA*::Tn*5lacZY* reporter at a high rate. It is interesting that strains containing *hilE hha* double mutations, *hilE ams* double mutations, or *hilE hha ams* triple mutations all approach 2,500 Miller units of *hilA*::Tn*5lacZY* expression, which is the same level reached by a strain containing a single mutation in *pag* (Fig. 1A).

Because of the large increases in *hilA*::Tn*5lacZY* expression

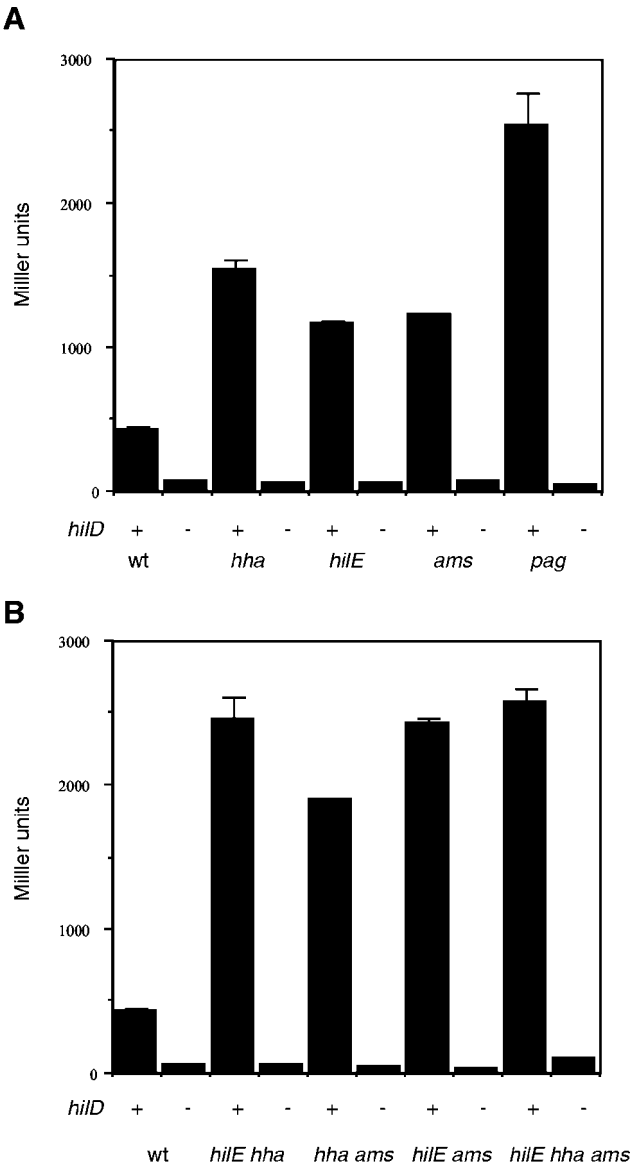

FIG. 1. Single or multiple mutations in negative regulators do not relieve repression of *hilA* in the absence of *hilD*. (A) Effect of single mutations in *hha*, *hilE*, *ams*, and *pag* on the expression of a serovar Typhimurium *hilA*::Tn*5lacZY* reporter in the presence or absence of *hilD*. (B) Effect of multiple repressor mutations on *hilA*::Tn*5lacZY* chromosomal expression in the presence or absence of *hilD*. All bacterial cultures were incubated statically in LB with 1% NaCl under oxygen-limiting conditions to induce expression of the *hilA*::Tn*5lacZY* reporter. Data are representative of at least three independent experiments.

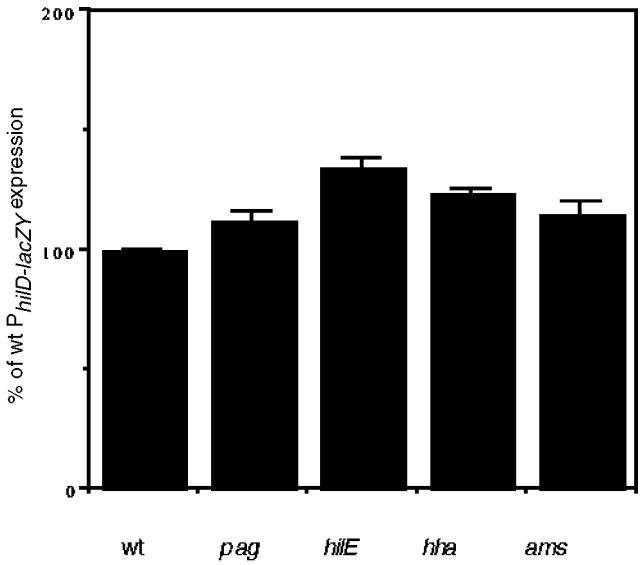

FIG. 2. Effect of *hha*::Tn*5*, *hilE*::Tn*5*, *ams*::Tn*5*, or *pag*::Tn*5* mutations on $P_{hilD\text{-}lacZY}$ expression from pJB5 in serovar Typhimurium. β-Galactosidase activity for the *hilD-lacZY* reporter was quantitated as a percentage of the β-galactosidase activity of the reporter in wild-type *Salmonella*, which was set at 100%. Bacterial cultures were incubated statically in LB with 1% NaCl under oxygen-limiting conditions. Data are representative of at least three independent experiments.

observed for double or triple negative regulator mutants, we expected that some combination of mutations would derepress the *hilA* promoter to the extent that HilD derepressing activity would be unnecessary. However, upon introduction of the *hilD*::*cam* mutation into each of these strains, we found that *hilA*::Tn*5lacZY* expression was always very low, similar to levels observed in a *hilD* mutant alone (Fig. 1B). Interestingly, we also observed that we could easily introduce the *pag*::Tn*5* mutation into various strains when the *hilD*::*cam* mutation was already present. Double or triple negative regulator mutants that included the *pag*::Tn*5* mutation expressed ~40 Miller units of *hilA*::Tn*5lacZY* expression when HilD was absent (data not shown). These results seem inconsistent with the idea that HilD simply negates the function of negative regulators of *hilA* (i.e., derepression), since HilD is required to obtain wild-type levels of *hilA* expression even in the absence of several negative regulators. An alternative possibility is that the negative regulators modulate *hilD* expression, whereas another unidentified negative regulator is able to completely repress the *hilA* promoter in the absence of HilD, although this seems unlikely since many searches for negative regulators have been conducted. However, we examined this possibility by determining the effects of negative regulator mutations on $P_{hilD\text{-}lacZY}$ expression from plasmid pJB5 in *Salmonella* (Fig. 2). We found that mutations in *hilE*, *hha*, *ams*, or *pag* caused virtually no difference in *hilD-lacZY* expression, suggesting that these regulators do not function by altering the expression of *hilD*.

Deletion of the *hilA* URS from the chromosome results in very low *hilA*::Tn*5lacZY* expression. To further investigate the model of HilD regulation at the *hilA* promoter, we sought to determine the effect of a chromosomal URS deletion on *hilA*::Tn*5lacZY* expression. We deleted a region of the native sequence of the *hilA* promoter from −314 to −68 in the *hilA*::Tn*5lacZY* chromosomal reporter strain, leaving 84 bp of unrelated DNA in its place (14). These sequences are the same as those deleted in a $P_{hilA\text{-}lacZY}$ plasmid reporter that resulted in high levels of HilD-independent *hilA* expression (42). In contrast to the reported $P_{hilA\text{-}lacZY}$ plasmid results when URS was deleted from pLS31, removal of chromosomal URSs resulted in very low *hilA*::Tn*5lacZY* expression (Fig. 3A). In fact, the expression levels were similar to those observed in the *Salmonella* strain lacking a functional *hilD*. Introduction of the *hilD*::*cam* mutation into the ΔURS mutant did not reduce *hilA*::Tn*5lacZY* expression further.

The high levels of *hilA* expression observed by Schechter et al. (42) when URSs were deleted from pLS31 are significantly different from the low levels observed when the URS was deleted from the chromosome. One explanation for the large difference may be that an artificial promoter was created within the plasmid, or *hilA* expression may have been artificially activated to drive expression of the plasmid reporter when upstream sequences were deleted. In that case, we would expect that the ΔURS mutation, which leaves 84 bp of unrelated DNA in place of the URS from −68 to −314, could prevent the formation of an artificial promoter when created in pLS31, resulting in lower reporter expression. Therefore, we created the plasmid pΔURS, with the ΔURS mutation (this mutation is identical to that constructed in the *Salmonella* chromosome) in pLS31, to examine its effect on $P_{hilA\text{-}lacZY}$ expression in *Salmonella*. We found that $P_{hilA\text{-}lacZY}$ expression was ~5-fold lower from pΔURS than from pLS31 and that expression from pΔURS did not require the presence of HilD (Fig. 3B). This may indicate that the 84-bp insertion partially disrupts a cryptic artificial promoter or an artificial activation from the vector that has been brought closer to the *hilA* promoter. A less likely possibility is that low *hilA* expression from ΔURS $P_{hilA\text{-}lacZY}$ is due to some negative regulatory effect of the small fragment of DNA that has been left in place of upstream sequences in the *hilA* promoter.

The point mutation, L289F, in the αCTD of RNAP causes a severe decrease in *hilA* expression. The data that we have presented here seem most consistent with the idea that HilD functions as an activator of *hilA* expression rather than as a derepressor. Many transcriptional activators, including various AraC/XylS regulators, are postulated to contact RNA polymerase at the α C-terminal domain (αCTD) to stabilize its interaction at promoter elements, facilitating closed complex formation and subsequent transcription (23, 37). Since HilD is a member of the AraC/XylS family of regulators, we thought it was possible that HilD contacts RNA polymerase at the αCTD to activate transcription of *hilA*. Point mutations in serovar Typhimurium LT2 chromosomal *rpoA* have been isolated that result in amino acid changes in the αCTD of RNA polymerase. These mutations cause a decrease in the expression of the *pepT* gene and are believed to disrupt interactions between the αCTD and the *pepT* activator, OxrA (33). We transduced the *rpoA* point mutations *rpoA151*, *rpoA153*, *rpoA154*, and *rpoA155* by P22 transduction into the wild-type serovar Typhimurium strain SL1344 to determine their effects on the chromosomal *hilA*::Tn*5lacZY* reporter (Table 3). Control experiments confirmed that each mutation caused the expected decrease in the expression of *pepT7*::MudJ in strain SL1344, as had been pre-

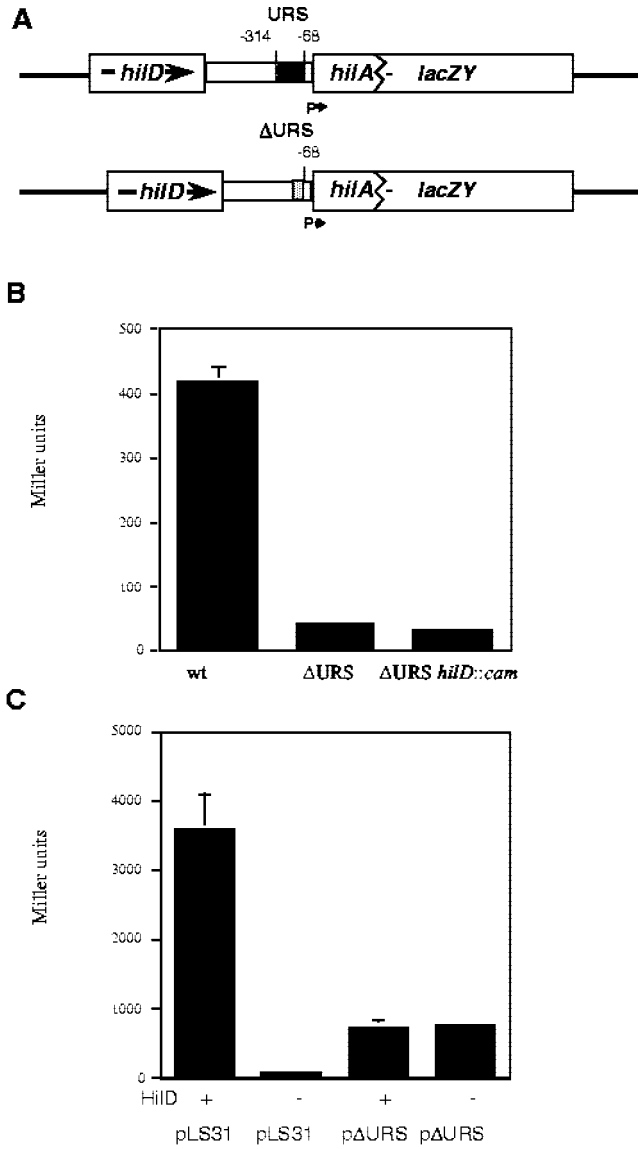

FIG. 3. Deletion of upstream promoter sequences results in an unactivatable *hilA* promoter. (A) Chromosomal or plasmid pLS31 sequences from positions −68 to −314 of the *hilA* promoter were replaced, by allelic exchange and deletion, with 84 bp of unrelated sequence to create a *Salmonella hilA*::Tn*5lacZY* reporter strain lacking URS sequences. (B) Expression of chromosomal *hilA*::Tn*5lacZY* was examined in the parent strain EE658 and the EE658 derivatives BJ2565 (ΔURS) and BJ2566 (ΔURS *hilD*::*cam*). (C) Effect of ΔURS mutation created in plasmid pLS31 in *Salmonella* in the presence or absence of *hilD*. β-Galactosidase expression was quantitated after growth in low-oxygen, high-osmolarity conditions. Data are representative of at least three independent experiments.

viously reported for strain LT2 (33). Interestingly, three of the mutations—*rpoA151*, *rpoA153*, and *rpoA154*—caused ~2-fold increases in *hilA* expression that were dependent on the presence of HilD. However, the *rpoA155* mutation, which changed leucine 289 to phenylalanine, caused a significant decrease in *hilA* expression, comparable to that observed in a strain containing a mutation in *hilD* alone.

One explanation for these results is that the *rpoA* mutation decreases *hilD* transcription. Control experiments indicated, however, that *hilD* from plasmid pJB5 was not significantly reduced (~1.3-fold) in the SL1344 *rpoA155* strain compared to its expression in SL1344 containing wild-type *rpoA* (1,236.5 ± 9.1 Miller units for SL1344 *rpoA155* compared to 1,662.9 ± 17.7 Miller units for wild-type SL1344). In addition, we found that the effect of the *rpoA155* mutation on *hilA* expression was not overcome by the expression of *hilD* from its own promoter or from the *lac* promoter on plasmids pJB1 and pJB3, respectively (Fig. 4). Plasmids pJB1 and pJB3 induce expression of $P_{hilA\text{-}lacZY}$ from pLS31 in *Escherichia coli*, similar to the high levels of *hilA* expression observed by Schechter et al. (42) in *E. coli* due to the expression of plasmid encoded *hilD*. *E. coli* (pLS31) containing parent plasmid pZC320 expresses 82.6 ± 4.3 Miller units of *hilA-lacZY* β-galactosidase activity, whereas *E. coli* (pLS31) containing pJB1 or pJB3 expresses 573.4 ± 5.4 or 1,093.9 ± 48 Miller units of *hilA-lacZY* β-galactosidase activity, respectively. A likely explanation for decreased *hilA* expression in the SL1344 *rpoA155* strain is disruption of an interaction between HilD and the αCTD of RNA polymerase that is critical for activation of *hilA* transcription.

DISCUSSION

In this study, we sought to better characterize *hilA* regulation by investigating the model of HilD derepression of the *hilA* promoter. HilD contains a C-terminal helix-turn-helix DNA-binding motif within a conserved 99-amino-acid domain characteristic of AraC/XylS family members (42). These transcriptional regulators are generally considered to activate transcription by direct contact and recruitment of RNA polymerase to their cognate promoters (7, 26, 37, 51). A current model for HilD function postulates a novel role for an AraC/XylS regulator by suggesting that HilD acts as a derepressor to counteract the effects of negative regulators that bind upstream of −39 on the *hilA* promoter. This model contrasts with more common functions of AraC/XylS regulators that act by contacting and recruiting RNA polymerase to activate transcription (42). To experimentally examine the HilD derepression model, we determined the effect of single or multiple mutations in the negative regulator elements *hha*, *hilE*, *pag*, and *ams* on *hilA* expression, with or without functional HilD. We reasoned that mutations in negative regulators that may bind to the URS should reduce the need for HilD and allow higher *hilA* expression when HilD is absent. In contrast, we found that in every combination of negative regulator mutants examined, *hilA* expression was very low in the absence of HilD.

The deletion of sequences upstream of −39 in a $P_{hilA\text{-}lacZY}$ plasmid reporter has been suggested to allow unregulated high-level expression of $P_{hilA\text{-}lacZY}$ in *Salmonella* and *E. coli* because sequences necessary for negative regulation of the promoter have been removed (42). However, our lab has noted that $P_{hilA\text{-}lacZY}$ expression from a *hilA* promoter deletion plasmid reporter can be variable, since its expression was much lower in *E. coli* strains HB101 and GS162 than in *Salmonella* or *E. coli* BW21355, the strain previously used by the Lee lab to demonstrate unregulated expression of the *hilA* promoter deletion plasmid (17). To further investigate the HilD derepressor model, we determined the effect of deleting URSs on *hilA* expression from the chromosome of *Salmonella*. The deletion

TABLE 3. Effect of *rpoA* point mutations on *hilA*::Tn*5lacZY* expression

Strain background (mutation)	Mean β-galactosidase activity (Miller units) ± SD with strain type[a]:		
	pepT7::MudJ	*hilA*::Tn*5lacZY*	*hilD*::*cam hilA*::Tn*5lacZY*
Wild type	718 ± 42	452.6 ± 14.6	43.2 ± 2.1
rpoA151 (G311E)	174.8 ± 6.7	886.0 ± 37.6	52.1 ± 6.3
rpoA153 (R317H)	390.0 ± 5.5	904.5 ± 88.3	33.4 ± 1.8
rpoA154 (W321stop)	342.6 ± 16	834.9 ± 41.8	43.8 ± 0.16
rpoA155 (L289F)	439.3 ± 48.2	42.5 ± 0.69	ND

[a] Effects of *rpoA* mutations on expression from *pepT7*::MudJ or *hilA*::Tn*5lacZY* chromosomal reporters in *Salmonella* sp. strain SL1344 with or without functional HilD are shown. All experiments were repeated at least three times, and the results of one representative experiment are shown. The *hilD*::*cam* mutation in the SL1344 *hilA*::Tn*5lacZY rpoA155* strain could not be constructed, and therefore expression of *hilA*::Tn*5lacZY* in this strain could not be determined. ND, not determined.

of native *hilA* chromosomal promoter DNA from −68 to −314 did not result in high-level *hilA*::Tn*5lacZY* expression, as predicted by the model. Rather, very low expression of *hilA* was observed in the ΔURS strain, a level of expression that was similar to the low-level expression of *hilA* in a *Salmonella* strain containing a mutation in *hilD*. It is interesting that HilD binds in two regions of the *hilA* promoter, from positions −179 to −231 and from positions −49 to −101, that are almost completely removed in the ΔURS mutant strains (43). Therefore, HilD binding sequences appear to be important for chromosomal *hilA* expression.

It is unclear why plasmid *hilA* regulation does not reflect chromosomal *hilA* regulation when upstream promoter sequences are removed. One possibility is that upstream deletions in the plasmid *hilA* promoter place a cryptic −35 region present in the parent plasmid near a cryptic −10 sequence to create an active artificial promoter. Another possibility is that the promoter deletions bring an activator protein binding sequence within the parent plasmid into close proximity with the native *hilA* promoter so that interactions with RNA polymerase are stabilized to allow $P_{hilA\text{-}lacZY}$ expression. Although a number of possible explanations can be proposed to explain the plasmid results, we feel that it is important to recognize that experiments performed with chromosomal reporters are usually the most reliable and trusted for measuring gene expression.

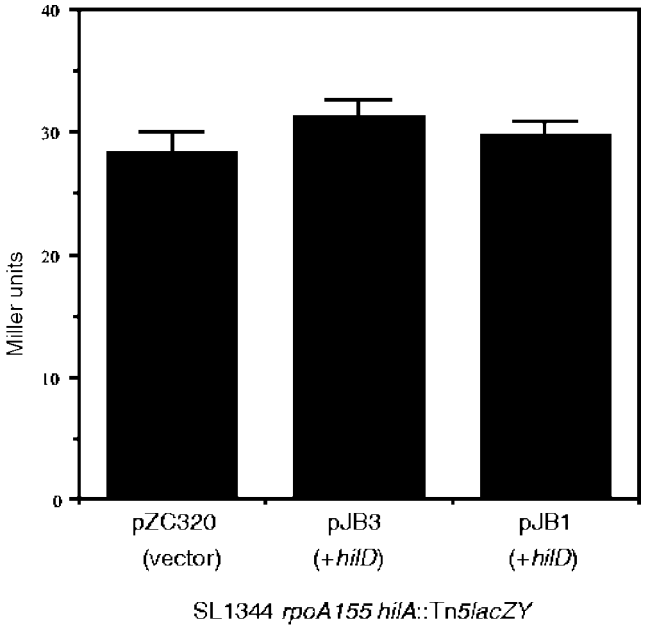

FIG. 4. Expression of *hilD* from the *lac* promoter on plasmid pJB3 does not increase *hilA*::Tn*5lacZY* in SL1344 *rpoA155*. Plasmid pJB1 or pJB3 or the parent vector, pZC320, was introduced into SL1344 *rpoA155 hilA*::Tn*5lacZY*, and β-galactosidase expression was quantitated from each strain after growth under low-oxygen, high-osmolarity conditions. Plasmid pJB1 expresses *hilD* from its own promoter, whereas plasmid pJB3 expresses *hilD* from the vector *lac* promoter. Data are representative of at least three independent experiments performed in triplicate.

Since HilD is required for wild-type levels of chromosomal *hilA* expression in the absence of several negative regulators and since HilD-binding sequences present in the URS appear to be important for *hilA* expression, we hypothesized that HilD is an activator of *hilA* transcription. In support of this idea, we found that a mutation in the αCTD of RNA polymerase that changes leucine 289 to phenylalanine results in reduced levels of *hilA* expression, a finding similar to that seen in a *hilD*::*cam* strain of *Salmonella*. The αCTD is thought to interact with many types of activators, including some of those within the AraC/XylS family, to allow transcription of various promoters (23, 37). In the αCTD, the first alpha helix and the loop between the third and fourth alpha helices are predicted to be DNA-binding determinants at promoter UP elements. However, leucine 289 is predicted to fall on the solvent-exposed face of the third alpha helix of the αCTD, such that it is unlikely to be involved in DNA binding and would be accessible for protein interactions (Fig. 5A and B) (18, 25). Residues in the third alpha helix, including leucine 289, have been shown to be important for activation of the P2 late promoter, and allele-specific suppressor mutations have been identified in the Ogr activator that overcome *rpoA* mutations to allow P2 late-promoter expression (3). Therefore, we propose that leucine 289 is important for a favorable interaction between the αCTD and HilD to occur, activating transcription of *hilA* (Fig. 5C and D). Recent unpublished data from our laboratory demonstrate that HilE is able to interact with HilD, suggesting that HilE repression of *hilA* may occur by inhibiting the ability of HilD to bind and recruit RNA polymerase to the *hilA* promoter.

It is possible that leucine 289 of the αCTD is responsible for mediating an interaction with an activator other than HilD at the *hilA* promoter, but we find this idea unlikely for several reasons. First, our data suggest that HilD is critical for activation of *hilA* expression. In addition, HilD binds to the *hilA* promoter in an area from position −49 to −101, which would

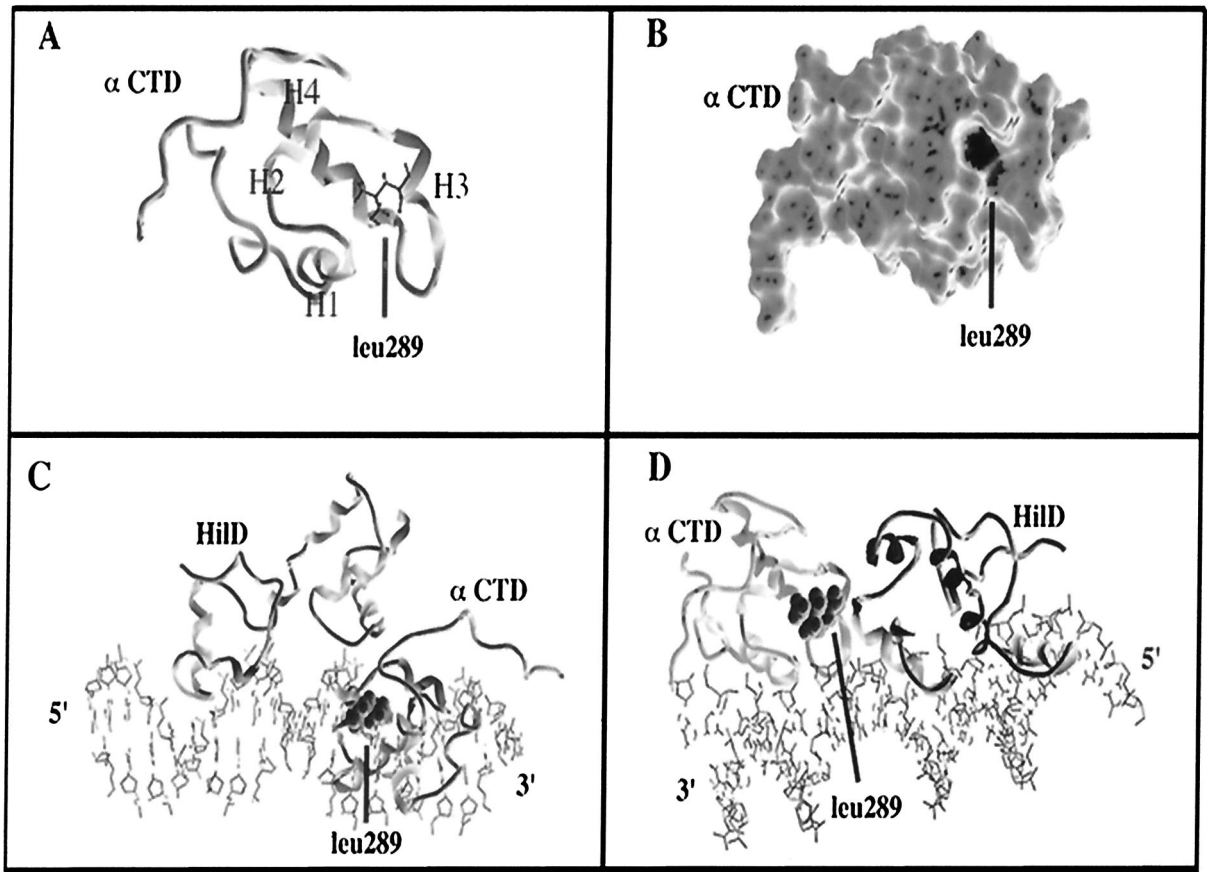

FIG. 5. Structural model of possible HilD interaction with the αCTD on promoter DNA. (A) Ribbon structure of the αCTD of RNA polymerase with leucine 289 highlighted and each alpha helix labeled as H1, H2, H3, or H4 (25). (B) Leucine 289 is predicted to be surface exposed on the αCTD of RNA polymerase. (C) Structural model of the possible interaction of HilD and leucine 289 of the αCTD of RNA polymerase, with DNA in the 5′-to-3′ direction. (D) A different view of the model presented in panel C, showing the 3′-to-5′ orientation of the DNA. Modeling was done by using Sybyl software (version 6.7; Tripos Associate, St. Louis, Mo.) on an O2 workstation (SGI, Mountain View, Calif.). Sybyl-Molcad was used to create panel B, which shows the solvent-exposed Connolly surface, by using the Connolly program to calculate the solvent-accessible surface of the molecule given the coordinates of its atoms (10). The Sybyl-Composer model was used to perform homology modeling for panels C and D to build structures for the CTD of HilD, from amino acids 211 to 309, based on the crystal structure of Rob, an AraC/XylS regulator that is homologous to HilD in its helix-turn-helix DNA-binding domain (31).

allow it to function as a typical class I transcriptional activator (23, 37). This makes HilD an excellent candidate for contacting the αCTD to recruit RNA polymerase to the *hilA* promoter. Several other positive regulatory elements of *hilA* expression have been identified. However, overexpression of HilD has been shown to overcome the effects of mutations in the positive regulators *fis*, *fadD*, *fliZ*, *pstS*, *sirA*, and *envZ*, suggesting that HilD is the positive regulator responsible for direct activation of the *hilA* promoter (35, 48). Interestingly, although overexpression of HilD is able to overcome the effects of mutations in other positive regulators of *hilA*, it does not overcome the effect of the L289F mutation in *rpoA*. These data suggest that the L289F mutation does not disrupt interaction with an activator of *hilA* expression whose loss can be compensated for by overexpression of HilD. Taken together, the data presented here indicate that HilD is an activator of *hilA* transcription and may function by contacting and recruiting the αCTD of RNA polymerase to the *hilA* promoter.

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