JOURNAL OF BACTERIOLOGY, June 1997, p. 3519–3527 Vol. 179, No. 11
0021-9193/97/$04.00+0

Enhancing Transcription through the *Escherichia coli* Hemolysin Operon, *hlyCABD*: RfaH and Upstream JUMPStart DNA Sequences Function Together via a Postinitiation Mechanism

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Received 27 January 1997/Accepted 19 March 1997

***Escherichia coli hlyCABD* operons encode the polypeptide component (HlyA) of an extracellular cytolytic toxin as well as proteins required for its acylation (HlyC) and *sec*-independent secretion (HlyBD). The *E. coli* protein RfaH is required for wild-type hemolysin expression at the level of *hlyCABD* transcript elongation (J. A. Leeds and R. A. Welch, J. Bacteriol. 178:1850–1857, 1996). RfaH is also required for the transcription of wild-type levels of mRNA from promoter-distal genes in the *rfaQ-K*, *traY-Z*, and *rplK-rpoC* gene clusters, supporting the role for RfaH in transcriptional elongation. All or portions of a common 39-bp sequence termed JUMPStart are present in the untranslated regions of RfaH-enhanced operons. In this study, we tested the model that the JUMPStart sequence and RfaH are part of the same functional pathway. We examined the effect of JUMPStart deletion mutations within the untranslated leader of a chromosomally derived *hlyCABD* operon on *hly* RNA and HlyA protein levels in either wild-type or *rfaH* null mutant *E. coli*. We also provide in vivo physical evidence that is consistent with RNA polymerase pausing at the wild-type JUMPStart sequences.**

Escherichia coli hlyCABD operons encode four gene products required for the synthesis (*hlyA*), acylation (*hlyC*), and export (*hlyBD*) of a cytolytic exotoxin (32). Hemolysin determinants are generally found on large, transmissible plasmids in animal isolates of *E. coli* or within unique chromosomal inserts of human uropathogenic isolates (24, 34). *hlyCABD* operons of plasmid and chromosomal origins display 97% sequence identity within the protein-coding regions (14); however untranslated regions and sequences 5′ to the promoters are largely heterologous between the two groups (14, 17, 35).

The *E. coli* protein RfaH is required in vivo for enhanced elongation of transcription through a chromosome-borne *hlyCABD* operon (20), as well as in vitro for increased transcription through plasmid-borne *hlyCABD* genes when they are under the control of a heterologous promoter (1). RfaH also enhances steady-state levels of mRNA of several other loci, including a plasmid-borne *hlyCABD* operon (2), the *traY-Z* operon on the F plasmid (4), the *rplK-rpoC* gene cluster (27), and the *rfaQ-K* gene cluster (26, 28), as well as the homologous *rfa* operon in *Salmonella typhimurium* (5). Although the precise mechanism of action of RfaH is unknown, all of the available data support a model for RfaH involvement in enhanced transcriptional elongation.

Little is known about other *E. coli* genes potentially involved in RfaH-enhanced transcript elongation or their potential sites of action. However, there have been reports of common sequence elements in the noncoding regions of RfaH-enhanced operons. Hobbs and Reeves (15) recognized a 39-bp sequence in the noncoding regions upstream of nine bacterial gene clusters, in four genera, involved in production of various polysaccharides. They named the sequence JUMPStart (for just upstream of many polysaccharide-associated gene starts). In the canonical sequence, there are two occurrences of 5′GGTAG C3′, and the second half of the JUMPStart sequence is GC rich. Among all of the gene clusters identified, the orientation of the JUMPStart sequence is the same with respect to the direction of transcription. Stevens et al. (29) expanded the JUMPStart family to 13 members, including the *kps* and plasmid-borne *hlyCABD* operons, which encode secreted gene products associated with extraintestinal *E. coli* infection, and the *traY-Z* operon, all of which are dependent upon RfaH for wild-type expression. More recent surveys have identified over 20 members of the JUMPStart family (19, 25).

In a previous study of RfaH-enhanced hemolysin expression, we recognized a putative JUMPStart sequence within the noncoding region of a chromosomal *hlyCABD* operon (20). Data from that study support a model in which RfaH enhances transcript elongation within operons containing the JUMPStart sequences (20). In this study we report that deletions of two conserved subsequences within the JUMPStart consensus sequence reduced *hlyCABD* transcript elongation in a pattern similar to that seen when the *hlyCABD* operon is expressed in an *rfaH* null mutant of *E. coli*. We also report data that are consistent with a prolonged association of RNA polymerase (RNAP) with the JUMPStart sequence in vivo. The significance of these observations in the model of RfaH-enhanced transcript elongation is discussed.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* strains and plasmids used in this study are described in Table 1. The uropathogenic clinical isolate *E. coli* J96 is the source of the recombinant *hlyCABD* operon on pSF4000 (34).

Media and reagents. Cultures were grown in Luria-Bertani medium at 37°C with aeration in the presence of appropriate antibiotics (chloramphenicol, 12.5 μg/ml; kanamycin, 25 μg/ml) unless otherwise noted. SP6 and T3 polymerases, rRNasin, RQ1 DNase, *Taq* DNA polymerase, Klenow fragment, and nucleoside triphosphates were purchased from Promega. DNase I was purchased from Gibco-BRL. [γ-^{32}P]ATP was purchased from Amersham, and [α-^{32}P]UTP was purchased from DuPont. RPAII reagents were from Ambion. Mutagenic oligonucleotides were purchased from Genosys.

***hlyCABD* deletion mutagenesis.** The JUMPStart sequence within the *hlyCABD* leader region was mutagenized by deleting sequences from positions +126 to +137 (pWAM2240) or +149 to +159 (pWAM2245), relative to the start of transcription (20), by incorporation of a phosphorylated mutagenic oligonucle-

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TABLE 1. Recombinant plasmids and *E. coli* strains used in this study

Plasmid or strain	Properties or genotype	Source (reference)
Plasmids		
pSF4000	J96 *hlyCABD* operon in pACYC184	This laboratory (33)
pWAM2240	pSF4000 (Δ+126-+137)	This study
pWAM2245	pSF4000 (Δ+149-+159)	This study
pWAM744	1,386-bp pSF4000 *Hin*dIII-*Eco*RI fragment; contains *hlyBD* in opposite orientation to SP6 promoter in pGEMII; source of *hlyBD* +6143 to +6410 probe	This laboratory
pWAM764	2,151-bp pSF4000 *Pst*I fragment; contains a portion of *hlyA* in opposite orientation to SP6 promoter in pGEMI; source of *hlyA* +3738 to +3966 probe	This laboratory
pWAM1452	997-bp pSF4000 *Pst*I fragment; contains a portion of *hlyCA* in opposite orientation to T3 promoter in pBS+; source of *hlyCA* +779 to +1049 probe	This laboratory
pWAM2098	938-bp pSF4000 *Xma*I fragment; contains *hly* leader and a portion of *hlyC* in opposite orientation to T3 promoter in pBS+; source of *hly* leader +42 to +627 probe	This study
E. coli strains		
RZ4500	*lacZ*Δ*145*	P. Kiley (8)
WAM1925	RZ4500 *rfaH*::Tn*5 1.8*	This laboratory (20)

otide during PCR amplification of the *hlyCABD Xma*I insert in pWAM2098 (Table 1). The method of Michael (23) was followed exactly except that 100 pmol of each wild-type primer (T7 [5′TAATACGACTCACTATAGGGAGA3′] and T3 [5′ATTAACCCTCACTAAAGGGA3′] promoter regions flanking the *Xma*I insert in pWAM2098) and 500 pmol of one mutagenic primer (5′GGTTGATG ACTGTTAATTCCATCTGCATTAATATTAGC3′ [+128 to +176, with positions +149 to +159 deleted] or 5′GATTGCTAATGGTTTGTGTGTTAATTC CAGAAGGCGG3′ [+108 to +156, with positions +126 to +137 deleted]) were used in a final volume of 50 μl. (The positions of the deletions are indicated in Fig. 1.) The mutagenized *Xma*I fragments were cloned into the *hlyCABD* operon in pSF4000, replacing the wild-type 938-base *Xma*I fragment, to create pWAM2240 (positions +126 to +137 deleted) and pWAM2245 (positions +149 to +159 deleted). The newly cloned *Xma*I regions of *hlyCABD* were sequenced by using the fmol DNA Sequencing System (Promega).

Assay of HlyA protein levels. Cultures were grown to an optical density at 600 nm (OD_{600}) of 0.80. For comparative analyses of extracellular HlyA protein levels, culture filtrates were precipitated overnight with 10% (wt/vol) trichloroacetic acid. The dried precipitates were resuspended in 3× sodium dodecyl sulfate (SDS) sample buffer (Sigma)–0.5 M Tris, pH 8.8 (1:2). Samples were boiled for 10 min, separated by SDS–10% polyacrylamide gel electrophoresis, and stained with Coomassie brilliant blue. Signals were quantified by densitometric analysis with a Zeineh soft laser scanning densitometer. Three independent cultures of each test strain were used to compare levels of extracellular HlyA.

Generation of antisense RNA probes. Templates for in vitro transcription of antisense RNA probes were either PCR products or linearized recombinant plasmids. As a source of the +408 to +627 *hly* leader-5′*hlyC* template, DNA from pWAM2098 was amplified by PCR with a primer identical to the nontemplate strand of pSF4000 from positions +408 to +424 and the M13 reverse sequencing primer (New England Biolabs). As a source of the +779 to +1049 *hlyC* template, DNA from pWAM1452 was amplified by PCR with a primer identical to the nontemplate strand of pSF4000 from positions +779 to +795 and the M13 reverse sequencing primer. To generate base +3738 to +3966 *hlyA* and +6143 to +6410 *hlyBD* templates, recombinant plasmids pWAM764 and pWAM744 were linearized by restriction digestion as described previously (20). RNA probe synthesis was carried out as described previously (20). The locations of *hlyCABD* regions encoding RNA complementary to the antisense RNA probes are depicted in Fig. 3A and 4A.

Preparation of total cellular RNA and quantitation of *hlyCABD* mRNA. Cellular RNA was prepared from 15 ml of *E. coli* grown to an OD_{600} of 0.6 by the hot-phenol method of Emory and Belasco (11). Quantitative RNase protection analysis was performed as described previously (20) except that both RNase A (2.5 U/ml) and RNase T_1 (100 U/ml) were utilized in the digestion step. Dried gels were exposed to a Molecular Dynamics phosphor screen for 2 to 4 h and scanned with a PhosphorImager SI (Molecular Dynamics). Band intensities were analyzed by using the ImageQuaNT V4.2 program (Molecular Dynamics).

In vivo $KMnO_4$ footprinting. *E. coli* transformed with either pSF4000, pWAM2240, or pWAM2245 was grown overnight in M9-glucose supplemented with 0.5% Casamino Acids, 1 μg of thiamine per ml, and 25 μg of chloramphenicol per ml. The next morning, the cultures were diluted 1:100 into 10 ml of the same medium and grown to an OD_{600} of 0.6. At this time the cultures were split, rifampin was added to one flask to a final concentration of 200 μg/ml, and the cultures were incubated for an additional 5 min at 37°C. At this time $KMnO_4$ was added to all flasks to a final concentration of 10 mM and left for 15 s, after which the cultures were transferred to ice-cold STE buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA) in 30-ml Corex tubes on ice to quench the reaction. Plasmids were isolated and quantified as described previously (16). $KMnO_4$-reactive thymines in the nontemplate strands of *hlyCABD* DNA were mapped by primer extension of plasmid DNA from $KMnO_4$-treated cells as described previously (16). Primer extension products of the *hly* leader region were detected by autoradiography following exposure of dried gels to DuPont NEF-496 film for up to 7 days.

RESULTS

Deletion of two highly conserved regions of the JUMPStart sequence from a chromosomally derived *hlyCABD* operon. We and others have shown that the *E. coli* protein RfaH enhances *hlyCABD* mRNA expression both in vivo (20) and in vitro (1). Results from the in vivo study demonstrated that RfaH is not required for initiation of *hlyCABD* transcription from the native *hly* promoter or for stability of *hly* mRNA (20). The in vitro study showed that transcription of the *hlyCABD* genes under the control of a heterologous promoter is increased in the presence of purified RfaH (1). These data are consistent with a role for RfaH in enhanced elongation of transcription. RfaH also increases steady-state mRNA levels from several other gene clusters in *E. coli* and *S. typhimurium* (4, 5, 26–28).

JUMPStart is a conserved sequence within the untranslated regions of all RfaH-affected operons identified to date (15, 29). The function of the JUMPStart sequence is unclear. In this study, we tested the hypothesis that the JUMPStart sequence and RfaH function in the same pathway. We characterized two deletion mutations of the 5′ noncoding region of a *hlyCABD* operon derived from uropathogenic *E. coli* J96 in wild-type and null *rfaH* mutant backgrounds. The deletions were derived by PCR-based mutagenesis of the *hly* subclone pWAM2098. The mutagenized DNA was substituted for the wild-type sequence in the intact *hlyCABD* operon on pSF4000 (Table 1). Figure 1 depicts the alignment of the *hlyCABD* sequence in pSF4000 with the JUMPStart consensus sequence defined by Hobbs and Reeves (15). In the alignment of all known JUMPStart sequences, gaps frequently occur within the upstream subsequence and within the spacer between the two subsequences. No gaps have been identified within the downstream JUMPStart subsequence (18). The deletion in pWAM2240 removes the region from positions +126 to +137, including most of the first, moderately conserved JUMPStart subsequence. The deletion in pWAM2245 removes the region from positions +149 to +159, including the absolutely conserved 5′GGCGGTAG 3′.

We initially examined the effect of the deletions on hemolytic phenotypes of wild-type and *rfaH* null mutant colonies grown on a blood agar plate. Previous work showed that the

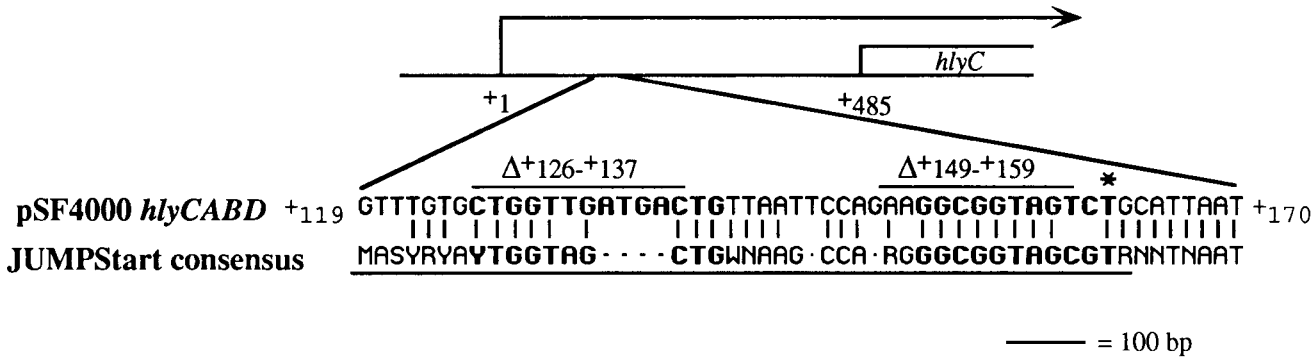


FIG. 1. Alignment of the *hlyCABD* leader region with the JUMPStart consensus sequence and locations of *hlyCABD* JUMPStart deletions. The top line of sequence depicts a portion (+119 to +170) of the untranslated leader region of the *hlyCABD* operon on pSF4000, which extends from the start of transcription (+1) to the start of translation of *hlyC* (+485). The bottom line of sequence depicts the JUMPStart consensus (underlined) and extended sequence as defined by Hobbs and Reeves (M, A or C; S, G or C; R, G or A; Y, C or T; W, T or A; N, A or C or G or T) (15). Gaps (–) were inserted to optimize the alignment. Vertical lines indicate sequence matches. Boldface indicates JUMPStart consensus subsequences as defined by sequence alignments (18). Δ+126-+137, bases deleted in pWAM2240; Δ+149-+159, bases deleted in pWAM2245. The asterisk denotes a T conserved among all JUMPStart sequences identified to date.

rfaH null allele significantly reduces the hemolytic-zone size (2, 19). When transformed into the wild-type *E. coli* RZ4500, both JUMPStart deletions conferred reduced zones of hemolysis compared to those produced by colonies transformed with the intact pSF4000 plasmid (19). Transformation of the JUMPStart deletions into the *rfaH* null mutant *E. coli* WAM1925 resulted in zone sizes similar to that conferred by the wild-type pSF4000 in the *rfaH* null mutant background. The observation that the effect of the JUMPStart deletions on zone size was not compounded by the *rfaH* null allele suggested that one is epistatic to the other or that RfaH and JUMPStart are part of the same functional pathway.

Effect of JUMPStart deletions on HlyA protein levels in wild-type and *rfaH* null mutant *E. coli*. To determine the effect of the JUMPStart deletions on extracellular HlyA expression, we compared the levels of extracellular HlyA precipitated from wild-type or *rfaH* null mutant *E. coli* culture filtrates by SDS-polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue. Deletion of the JUMPStart sequences resulted in 8- and 16-fold reductions in extracellular HlyA levels from *E. coli* harboring pWAM2240 and pWAM2245, respectively, compared to that from *E. coli* harboring the wild-type *hlyCABD* operon (Fig. 2A, compare wild-type signals [lanes 2 to 5] with signals from JUMPStart deletions pWAM2240 and pWAM2245 [lanes 6 and 7 and lanes 8 and 9, respectively]). As described previously (20), the *rfaH* null mutation alone confers an 8- to 16-fold reduction in extracellular HlyA compared to that of the wild-type *E. coli* (Fig. 2B, compare wild-type signal [lane 2] with *rfaH* null mutant signals [lanes 3 to 5]). When examined in an *rfaH* null mutant background, the JUMPStart deletions did not compound the effect of the *rfaH* mutation on extracellular HlyA levels (Fig. 2B, compare *rfaH* null mutant signals [lanes 3 to 5] with signals from JUMPStart deletion mutants in the *rfaH* null mutant background [lanes 6 to 9]). Therefore, the reductions in HlyA levels conferred by the JUMPStart deletions in *rfaH*$^+$ *E. coli* are similar to the reduction conferred by the null *rfaH* mutation on HlyA expression from either the intact pSF4000 (20) or JUMPStart deletion mutants.

JUMPStart deletions, like the *rfaH* null mutation, reduce steady-state *hlyCABD* mRNA levels. We previously demonstrated that the reduction in HlyA protein levels from *rfaH* null mutant *E. coli* correlates with decreased steady-state levels of *hlyCABD* mRNA (20). To see if the JUMPStart sequences, like RfaH, affect *hlyCABD* mRNA levels, we performed quantitative RNase protection analyses on total RNAs extracted from the wild-type and *rfaH* null mutant *E. coli* harboring pSF4000 or one of the two deletions. The antisense RNA probes used are shown in Fig. 3A, and the results of RNase protection assays are shown in Fig. 3B and C. The multiple +779 to +1049 probe signals (*hlyCA*) reflect the two RNA species that result from in vitro transcription of the template DNA (1, 18). Probes protected by *hlyC* mRNA also migrate faster than the control probes. The incomplete protection is due to in vivo processing of *hlyC* mRNA (20).

Deletion of either JUMPStart sequence resulted in a significant reduction in *hlyCABD* mRNA from wild-type *E. coli* (Fig. 3B and C, *rfaH*$^+$ lanes; compare wild-type signals with signals from JUMPStart mutants [note that the mutants shown in Fig.

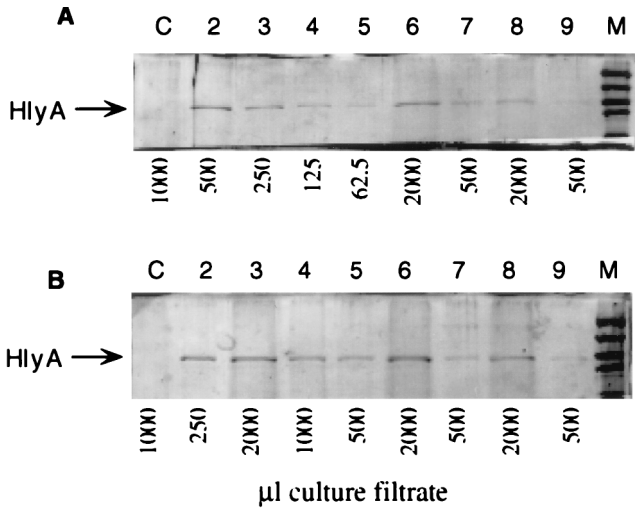
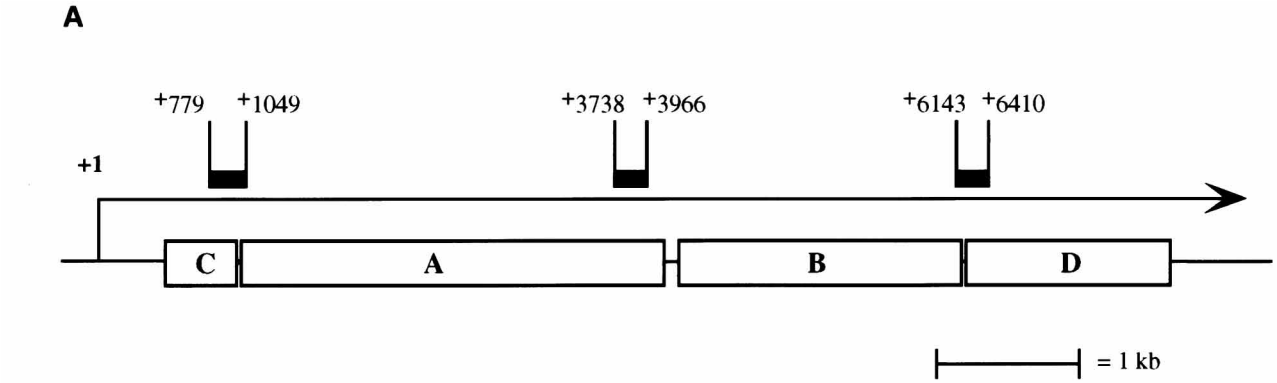


FIG. 2. Analysis of extracellular HlyA protein from wild-type (A) or *rfaH* null mutant (B) *E. coli* harboring either wild-type or JUMPStart-deleted *hlyCABD*. Culture filtrates of the different strains were concentrated, subjected to SDS-PAGE, and stained with Coomassie brilliant blue. The amount of filtrate analyzed is indicated below each lane. Material from *E. coli* containing pSF4000 (wild type) are in lanes 2 to 5 for panel A and lanes 3 to 5 for panel B; in both panels A and B, pWAM2240 (Δ+149-+159) are in lanes 6 and 7 and pWAM2245 (Δ+149-+159) are in lanes 8 and 9. To aid in the comparison, lane 2 in panel B contains material from wild-type *E. coli* RZ4500 harboring pSF4000. The C lanes contian supernatant from RZ4500 without any plasmid, and the M lanes contain broad-range molecular weight markers (Bio-Rad).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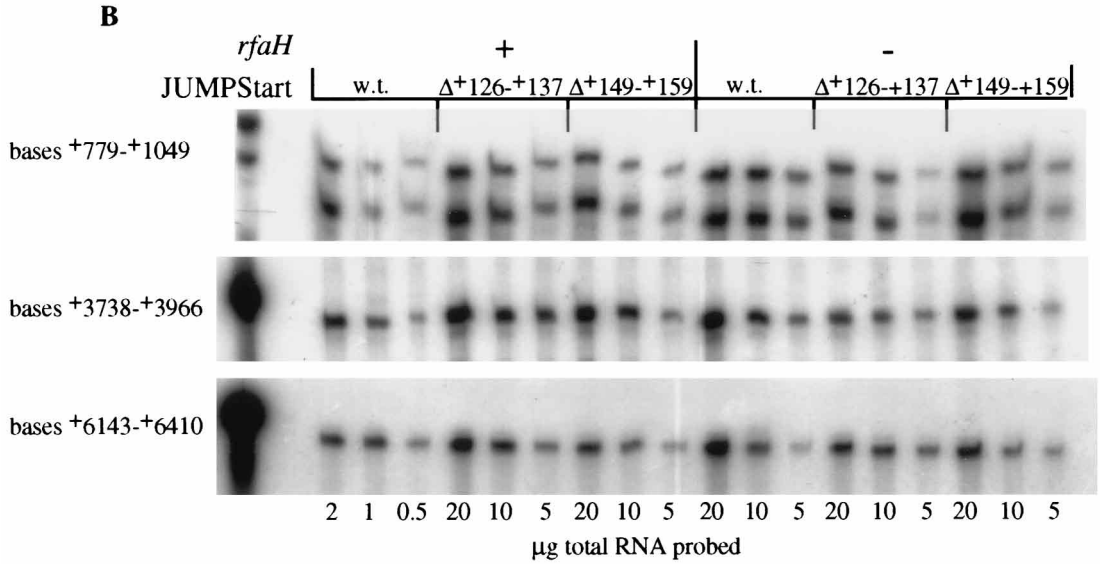
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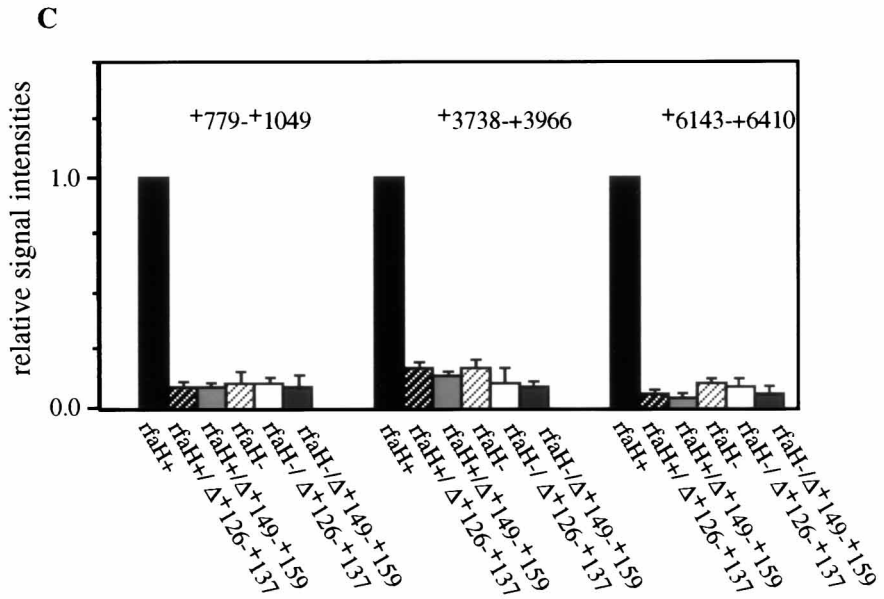
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relative signal intensities
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rfaH+
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rfaH-
rfaH-/ Δ+126-+137
rfaH-/Δ+149-+159

3B had 10-fold more total RNA probed]). The 3′ end of the *hlyC* message from pWAM2240 and pWAM2245 was reduced 10- and 11-fold, respectively, compared to RNA expressed from the intact *hlyCABD* operon. The level of *hlyA* message was reduced 6- and 7-fold, and the level of *hlyBD* message was reduced 14- and 20-fold, from the same constructs. The *rfaH* null allele alone reduced the 3′ end of the *hlyC* message 9-fold, *hlyA* mRNA 6-fold, and *hlyBD* mRNA 9-fold from the intact *hlyCABD* operon, compared to the wild-type strain (Fig. 3B and C, compare signals from pSF4000 in *rfaH*$^+$ versus *rfaH* null mutant *E. coli*). When examined in an *rfaH* null mutant background, the JUMPStart deletions reduced the 3′ end of the *hlyC* message 9- and 11-fold, *hlyA* mRNA 8- and 10-fold, and *hlyBD* mRNA 10- and 14-fold (Fig. 3A and B, compare pSF4000 signals in *rfaH*$^+$ *E. coli* with signals from JUMPStart mutants of *rfaH* mutant *E. coli*). These data indicate that expression of the JUMPStart deletion constructs in the *rfaH* null mutant strain of *E. coli* did not compound the effect on *hlyCABD* mRNA levels of either the JUMPStart deletions or the null *rfaH* allele alone. These data are consistent with the model that RfaH and JUMPStart are part of the same functional pathway.

Effect of the JUMPStart deletions and the *rfaH* null allele on steady-state levels of RNA from the *hly* leader and 5′ end of *hlyC*. Previous work showed that the *rfaH* null allele does not reduce leader and 5′*hlyC* mRNA expression from pSF4000 to the extent that it reduces levels of downstream *hlyCABD* mRNA (18, 20). We performed RNase protection analyses to compare levels of *hly* leader and 5′*hlyC* mRNA from the intact and JUMPStart-deleted *hlyCABD* operons in wild-type and *rfaH* null mutant *E. coli*. Figure 4A shows the location of an antisense probe to a region within the *hlyCABD* leader, extending into *hlyC*. The data in Fig. 4B depict the profile of the antisense probe following an RNase protection assay, as described in the legend to Fig. 3.

Deletion of the JUMPStart sequences resulted in two- and threefold reductions in *hly* leader-5′*hlyC* mRNA from wild-type *E. coli* (Fig. 4B and C; in *rfaH*$^+$ lanes, compare wild-type signals with signals from JUMPStart mutants [note that equal amounts of total RNA were assayed for each strain]). When examined in an *rfaH* null mutant background, the JUMPStart deletions expressed amounts of *hly* leader-5′*hlyC* mRNA similar to those expressed from those plasmids in wild-type *E. coli* (Fig. 4B and C, compare JUMPStart signals in *rfaH*$^+$ lanes and in *rfaH* mutant lanes). The *rfaH* null allele alone conferred less than a twofold reduction in *hly* leader-5′*hlyC* mRNA levels (Fig. 4B and C, compare pSF4000 signals in *rfaH*$^+$ lanes with pSF4000 signals in *rfaH* mutant lanes).

The data in Fig. 4 demonstrate that expression of the JUMPStart deletions in either wild-type or *rfaH* null mutant *E. coli* reduced *hly* leader and 5′*hlyC* mRNA to a lesser degree than they reduced downstream *hlyCABD* mRNA. The data also show that, similar to the effect on downstream *hlyCABD* mRNA, JUMPStart deletions in combination with the *rfaH* null allele did not result in a compounded reduction in *hly* mRNA expression beyond the effect of either mutation by itself. The <2- to 3-fold reduction in 5′*hly* RNA conferred by the null *rfaH* allele or the JUMPStart deletions may have been caused by premature transcript termination or increased mRNA decay in the *hly* leader region. The experiments presented in this work do not address the effect of the JUMPStart sequences on *hlyCABD* message decay, and we therefore cannot rule out a role for the JUMPStart sequences in downstream mRNA stability. However, our previous work demonstrated that RfaH is not required for *hly* mRNA stability (20). Although the precise nature of the JUMPStart effect remains to be determined, the two- to three-fold reduction in upstream *hly* RNA did not fully account for the reduction in extracellular HlyA levels from these strains. The RNase protection data presented in Fig. 3 and 4 favor the hypothesis that RfaH and JUMPStart are part of the same functional pathway, and these data are consistent with a model for the JUMPStart sequences in an RfaH-dependent elongation of *hly* transcription.

RNA polymerase may pause at the JUMPStart sequence. We and others have proposed a model for RfaH-enhanced gene expression at the level of transcription elongation (1, 4, 9, 13, 20). This work demonstrates that removal of either RfaH, JUMPStart sequences, or both leads to similar reductions in steady-state *hlyCABD* transcript levels in vivo, suggesting that they are part of the same functional mechanism. Recent in vitro experiments support this hypothesis, since removal of RfaH or the JUMPStart sequences reduces transcription of a hybrid construct containing the *hlyCABD* genes expressed from the *tac* promoter (1).

If transcriptional elongation of *hlyCABD* mRNA depends on alteration of the transcription machinery, including, minimally, RNAP, then pausing of RNAP during transcription may allow the necessary cofactors (*cis* and/or *trans*) to gain access to the transcription elongation complex. We used in vivo $KMnO_4$ footprinting to directly measure occupancy of the *hly* promoter and identify potential RNAP pause sites on the three different constructs described above. $KMnO_4$ reacts with thymine residues in melted DNA such as DNA opened in a transcription bubble (16). Cells transformed with plasmids containing either the intact *hlyCABD* operon or the Δ+126 to +137 or Δ+149 to +159 construct were pulsed for 15 s with $KMnO_4$. Some cultures were treated with rifampin to collect RNAP in open complexes on the promoter prior to $KMnO_4$ treatment. Plasmid DNA was isolated, quantified, and probed by primer extension on the nontemplate DNA strands.

Figure 5 shows $KMnO_4$-reactive thymines in the nontemplate strands of pSF4000, pWAM2240, and pWAM2245 from wild-type and *rfaH* null mutant *E. coli* during steady-state transcription (without rifampin) or following arrest of RNAP in the open complex at the *hlyCABD* promoter (with rifampin). Samples that were treated with rifampin reveal a highly reactive thymine in the open complex at the *hlyCABD* promoter at position +3 relative to the transcription start site. The samples that were not treated with rifampin reveal the steady-state level of promoter occupancy (+3) as well as a transcription-dependent reactive thymine just downstream of the JUMPStart se-

FIG. 3. RNase protection analysis of steady-state *hlyCABD* mRNA levels in wild-type or *rfaH* null mutant *E. coli*. Cultures were grown to an OD_{600} of 0.60. Extraction of total RNA, construction of antisense probes, and RNase protection analysis are described in Materials and Methods. (A) Map of *hlyCABD* operon antisense RNA probes. (B) Autoradiographs of radiolabelled antisense probes protected from RNase A and RNase T_1 digestion following hybridization to serial dilutions of total RNAs extracted from RZ4500 (*rfaH*$^+$) or WAM1925 (*rfaH*) harboring either pSF4000 (wild type [w.t.]), pWAM2240 (Δ+126-+137), or pWAM2245 (Δ+149-+159). The amounts of total RNA probed are indicated below the lanes. The first lane in each panel represents 10% of the amount of antisense probe used in each hybridization reaction. (C) Histogram of relative levels of *hlyCABD* mRNA. The slopes of signal intensity as a function of total RNA probed for each strain were calculated and graphed relative to that for *rfaH*$^+$ *E. coli* harboring pSF4000. The error bars represent the standard deviations of results from two separate RNA extractions for each strain. The three probes do not contain equal amounts of radioactivity, and the film exposure times differ for each probe. Therefore, comparisons of relative intensities among the different constructs cannot be made.

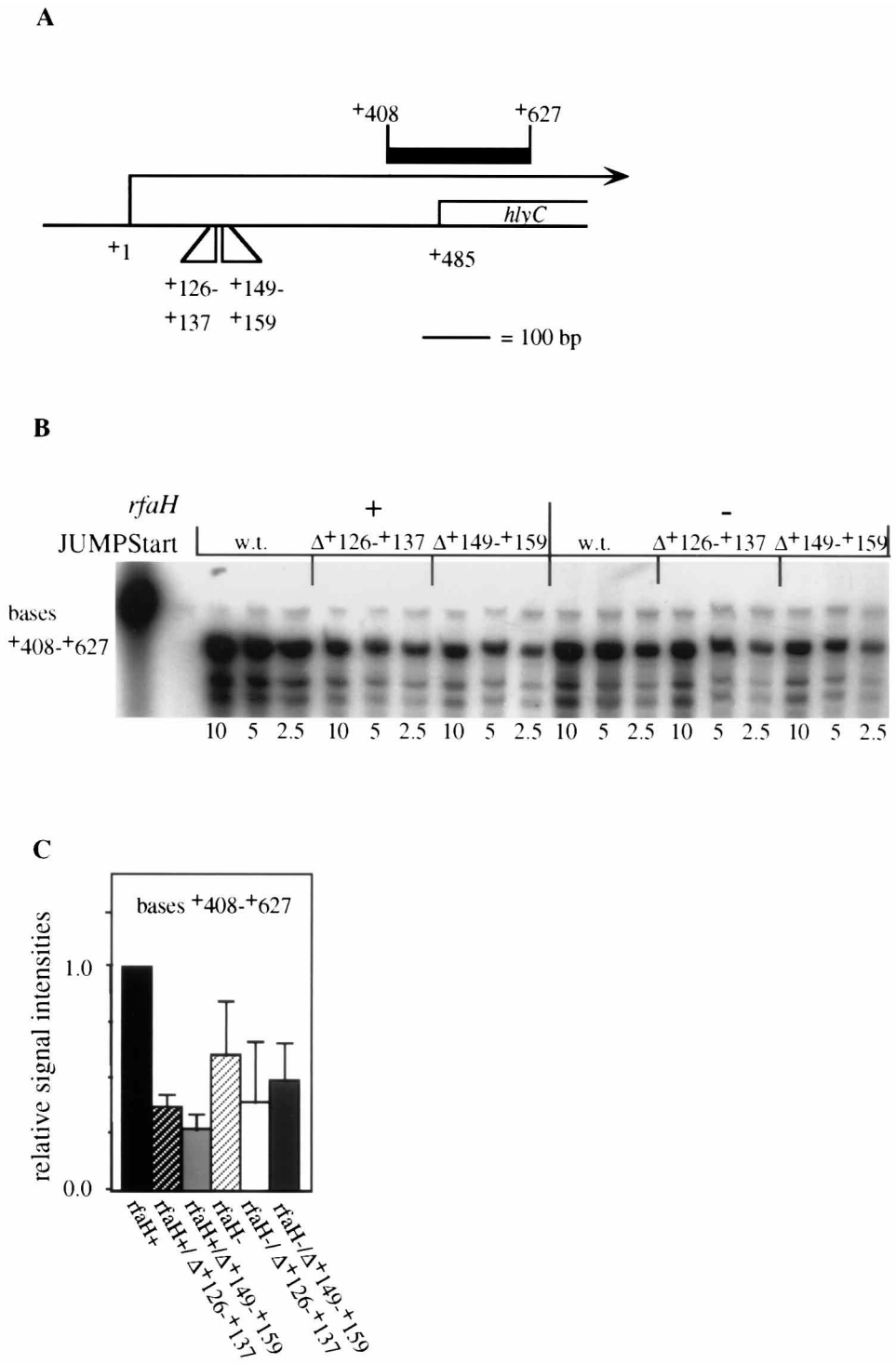

FIG. 4. RNase protection analysis of steady-state *hly* leader and 5′*hlyC* mRNA in wild-type or *rfaH* null mutant *E. coli*. See the legend to Fig. 3 for experimental details. (A) Map of *hly* leader-5′*hlyC* mRNA antisense RNA probe. (B) Autoradiograph of radiolabelled antisense *hly* probe protected from RNase A and RNase T_1 digestion following hybridization to serial dilutions of total RNAs extracted from RZ4500 (*rfaH*$^+$) or WAM1925 (*rfaH*) harboring either pSF4000 (wild type [w.t.]), pWAM2240 (Δ+126-+137), or pWAM2245 (Δ+149-+159). The amounts of total RNA probed are indicated below the lanes. The first lane represents 10% of the amount of antisense probe used in each hybridization reaction. (C) Histogram of relative levels of *hly* mRNA. The slopes of signal intensity as a function of total RNA probed for each strain were calculated and graphed relative to that for wild-type *E. coli* harboring pSF4000. The error bars represent the standard deviations of results from two separate RNA extractions for each strain.

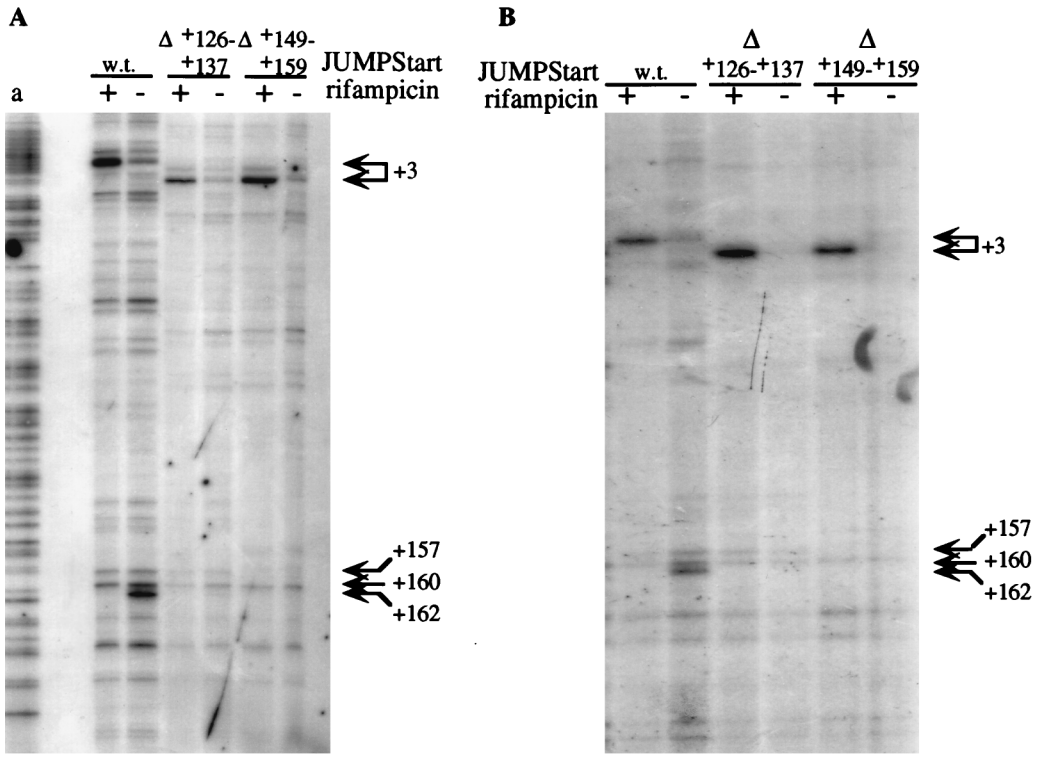

FIG. 5. Primer extension analysis of the nontemplate strand of *hlyCABD* leader DNA following in vivo treatment with potassium permanganate. pSF4000 (wild type [w.t.]), pWAM2240 (Δ+126-+137), and pWAM2245 (Δ+149-+159) were extracted from either wild-type *E. coli* RZ4500 (A) or *rfaH* null mutant *E. coli* WAM1925 (B) following treatment with $KMnO_4$ in the presence (+) or absence (−) of rifampin. All samples were extended with an oligonucleotide complementary to positions +271 to +288 of pSF4000. Lane a in panel A was generated by fmol (Promega) sequencing of pSF4000 DNA with the same oligonucleotide as in the footprinting reactions. The bands depict the locations of adenosine nucleotides in the template strand of the wild-type pSF4000 sequence. The numbering of permanganate-sensitive bases is relative to the start of transcription (+1 = pSF4000 base 311 from reference 12).

quences (+162). In Fig. 5A the lanes corresponding to pSF4000 with and without rifampin contain three times more DNA, and in Fig. 5B the lane corresponding to pSF4000 without rifampin contains two times more DNA, than the other lanes in order to demonstrate the locations and relative intensities of background signals compared to the +3 and +162 signals. All other lanes contain equal amounts of plasmid.

The data in Fig. 5 suggest several basic features of *hlyCABD* transcription. The +3 signal evident in the rifampin-treated samples confirms the location of the *hlyCABD* promoter identified by primer extension and deletion analysis in previous studies by our laboratory (20, 35). Quantitative comparisons of promoter signals on pSF4000 from wild-type and *rfaH* null mutant *E. coli* concur with previous data which demonstrated that RfaH is not required for initiation of *hly* transcription. Deletion of the JUMPStart sequences did not alter the site or extent of open complex formation at the *hly* promoter in either the wild-type or *rfaH* null mutant *E. coli*. Finally, a combination of the *rfaH* null allele and the JUMPStart mutations did not alter the pattern of *hly* open complex formation.

In addition to providing information about the *hly* promoter in the context of the *rfaH* null allele and the JUMPStart deletions, the $KMnO_4$ footprinting technique was useful in identifying the highly reactive thymine at position +162 of pSF4000. This base represents an absolutely conserved T, three bases 3′ to the downstream conserved 5′GGTAG3′ subsequence within every JUMPStart sequence identified to date (Fig. 1). This T is modified by $KMnO_4$ only in the absence of rifampin treatment, when RNAP is allowed to proceed beyond transcription initiation. Footprints of the nontemplate DNA strands of pWAM2240 and pWAM2245 from wild-type and *rfaH* null mutant *E. coli* demonstrate that the T at position +162 is not modified when the JUMPStart subsequences are deleted. In addition, the *rfaH* null mutation has no significant effect on the appearance of the signal at +162, indicating that it is not required for the hyperreactivity. $KMnO_4$ footprinting of the template strand of either pSF4000, pWAM2240, or pWAM2245 from wild-type or *rfaH* null mutant *E. coli* RZ4500 did not reveal any transcription-dependent hypersensitive thymines in the JUMPStart region of *hlyCABD* DNA (18).

DISCUSSION

The noncoding regions of all RfaH-affected operons contain all or a portion of a 39-bp conserved sequence termed JUMPStart (15, 29). We and others have shown that RfaH is necessary for wild-type levels of promoter-distal mRNA in these operons, both in vivo and in vitro, supporting a model for the involvement of RfaH in transcription elongation (1, 2, 20). One of the major problems in our understanding of RfaH-dependent transcription elongation is the identity of the sequence or structure element(s) in the DNA or RNA that permits RfaH activity. The JUMPStart sequences represent potential *cis*-acting sites involved in RfaH-enhanced transcript elongation.

Nieto et al. (25) reported that removal of a 35-base sequence 2 kb upstream of an *hlyCABD* operon derived from a large, transmissible plasmid results in decreased hemolysin expression in vivo. Contained within that deletion was a portion of the conserved JUMPStart sequence (5′GGCGGTAG3′). Fur-

ther work by that group demonstrated that removal of this sequence reduced transcription of the *hlyCABD* genes from a heterologous promoter in vitro (1). However, the location of this particular sequence relative to the native start of *hlyCABD* transcription was not demonstrated in their in vivo or in vitro systems.

In this study we examined precise deletion mutations of the JUMPStart consensus sequence within the untranslated leader sequence of a chromosomal *hlyCABD* operon, transcribed from the native *hly* promoter, for their effects on hemolysin expression from wild-type and *rfaH* null mutant *E. coli*. Our principal conclusions are that (i) like the *rfaH* null allele, the JUMPStart deletions result in reductions in hemolysin expression; (ii) the combination of JUMPStart deletions and the *rfaH* null mutation does not result in compounded reduction in hemolysin expression, and therefore the two are probably part of the same pathway; and (iii) presence of the JUMPStart subsequences confers transcription-dependent $KMnO_4$ hyperreactivity to a thymine in the nontemplate strand of *hlyCABD* DNA at position +162 (relative to the start of transcription). Overall, our results are consistent with a role for the JUMPStart sequences in *hlyCABD* transcript elongation, lending further support to the hypothesis that RfaH and the JUMPStart sequences contribute to the same mechanism of transcriptional elongation.

Previous studies demonstrated that RfaH enhances *hlyCABD* mRNA levels, with a greater effect on promoter-distal *hly* mRNA than on promoter-proximal mRNA (20). The data shown in Fig. 3 and 4 demonstrate that the JUMPStart deletion mutants have an effect on *hly* leader and *hlyCABD* mRNA levels similar to that of the *rfaH* null allele. The combination of the JUMPStart deletions and the *rfaH* null mutation does not result in a compounded decrease in *hlyCABD* mRNA levels. These data strongly suggest that RfaH and JUMPStart do not act independently but instead influence the *hlyCABD* transcript via a common pathway.

We used the technique of in vivo $KMnO_4$ footprinting to identify open complexes and potential pause sites in the wild-type *hlyCABD* operon and in the deletion mutants expressed in wild-type and *rfaH* null mutants of *E. coli* (Fig. 5). Conservation of the JUMPStart sequence suggests that it is recognized by one or more host factors, including RNAP. Following treatment with rifampin to prevent initiation of transcription by RNAP, we identified a hyperreactive thymine at position +3 of the nontemplate strand, consistent with RNAP in open complex formation at the *hlyCABD* promoter. These data confirmed the previously mapped *hlyCABD* transcription start site on pSF4000 (20). The location and intensity of the open complex signal at +3 were the same on either the wild-type or JUMPStart-deleted *hlyCABD* constructs in wild-type or *rfaH* null mutant *E. coli*. On the basis of previous observations (20), our $KMnO_4$ footprint analyses, and RNA analyses, we conclude that RfaH and the JUMPStart sequences are not required for initiation of *hlyCABD* transcription.

$KMnO_4$ treatment of wild-type and *rfaH* null mutant *E. coli* in the absence of rifampin, which provides a steady-state profile of unstacked *hlyCABD* DNA, identified a hyperreactive thymine at position +162 of the nontemplate strand in the wild-type operon but not in the corresponding position of either JUMPStart mutant. A less reactive thymine at position +160 on the nontemplate strand is present in the $KMnO_4$ profiles of the wild-type and the JUMPStart-deleted constructs. The reactive thymine at base +157 is present in the $KMnO_4$ profiles of the wild type and the Δ+126 to +137 construct, but it is not present in the Δ+149 to +159 construct because this base is deleted in this mutant. One possibility to explain the footprinting and RNA data is that the JUMPStart sequences may signal RNAP to pause in elongation, perhaps in the region of position +160 to allow modification of the transcription complex to a more processive form. Removal of the JUMPStart sequences would inhibit this modification step, allowing the polymerase to move more rapidly through this early region (resulting in loss of $KMnO_4$ hyperreactivity) but more slowly past putative downstream pauses and terminators. A similar situation exists in the bacteriophage λ Q transcriptional elongation system, where mutations in the promoter-proximal Q binding site (*qut*) do not support an RNAP pause at positions +16 and +17, allowing Q to modify RNAP (16). RNAP stalled at the JUMPStart sequence would allow *cis* or *trans* antitermination factors, such as RfaH, to modify RNAP to an elongation-competent form. This model is consistent with the footprinting and RNase protection data from both wild-type and *rfaH* null mutant *E. coli* because RfaH is not required for polymerase to pause at the JUMPStart sequences (position +162 hyperreactive thymine); however, it would be required for further modification leading to an elongation-competent complex.

What is the role of RfaH in the JUMPStart-dependent elongation of *hlyCABD* transcription? RfaH could act like N, or like one of the *E. coli* Nus factors, in the λ or *rrn* antitermination systems. In λ N-mediated antitermination, the *nut* site (RNA) and several *E. coli* Nus proteins collectively help N locate its proper site on the surface of RNAP and maintain this physical association throughout subsequent transcription (10). For example, NusA allows N to capture RNAP transcribing the *nut* site, leading to a termination-resistant complex (36). Transcription through the *E. coli rrn* operons is also enhanced by a cooperative effect of several *E. coli* Nus factors that recognize signals resembling *cis*-acting sequences in the λ phage antitermination systems but do not interact with an N or Q homolog (3). RfaH could interact with RNAP transcribing the JUMPStart sequences. Deletion of RfaH would not inhibit interactions of RNAP with the JUMPStart sequences (RNA, DNA, or both), but RNAP would not be efficiently modified without RfaH present.

RfaH has significant amino acid sequence similarity with NusG (1, 18), a protein shown in *E. coli* to be involved in both transcriptional termination and antitermination (6, 7, 21, 22, 30, 31). The role of NusG in transcription by *E. coli* RNAP is controversial. Under certain conditions, NusG can enhance termination by rho (21, 31), or it can enhance antitermination by N at both rho-dependent (21) and rho-independent (21, 31) terminators. NusG has been shown to directly increase the rate of RNAP elongation, which may help RNAP get through pause sites and prevent termination by rho (7). NusG has also been shown to bind directly and selectively to rho and weakly to RNAP, which may bridge the rho-RNAP gap to enhance rho-dependent termination (21). The homology of RfaH with NusG suggests that RfaH may interact with known transcription factors; however, without knowledge of the functional domains of NusG, our model is speculative. Experiments are in progress to determine whether RfaH, like NusG, is involved in the antitermination systems of bacteriophage λ.

In conclusion, we identified multiple elements within the JUMPStart consensus sequence that are required for wild-type levels of *hlyCABD* mRNA in vivo. We also demonstrated a transcription-dependent unstacking of DNA within the *hly* leader region that depends upon the presence of both JUMPStart subsequences, which may be the result of RNAP paused at this site. Our data support a mechanism for enhanced elongation of transcription that requires both RfaH and the JUMPStart sequences.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grant AI20323. J. A. Leeds was supported in part with funds from Cellular and Molecular Biology Training Grant GM07215 and by The University of Wisconsin Graduate School.

We thank members of our laboratory for helpful discussions. We thank Mark Kainz for help with $KMnO_4$ footprinting techniques, helpful discussions, and critical reading of the manuscript. We thank A. J. Bokal for help with PCR mutagenesis. We thank Mark Krebs for help with phosphorimaging techniques. We thank Jon Woods, Roberto Kolter, and Jon Beckwith for use of their equipment. We thank Bill Reznikoff for reminding us of Occam's razor.

REFERENCES

1. **Bailey, M. J. A., C. Hughes, and V. Koranakis.** 1996. Increased distal gene transcription by the elongation factor RfaH, a specialized homologue of NusG. Mol. Microbiol. **22:**729–737.
2. **Bailey, M. J. A., V. Koronakis, T. Schmoll, and C. Hughes.** 1992. *Escherichia coli* HlyT protein, a transcriptional activator of haemolysin synthesis and secretion, is encoded by the *rfaH*(*sfrB*) locus required for expression of sex factor and lipopolysaccharide genes. Mol. Microbiol. **6:**1003–1012.
3. **Berg, K. L., C. Squires, and C. L. Squires.** 1989. Ribosomal RNA operon antitermination: function of leader and spacer region Box B-Box A sequences and their conservation in diverse micro-organisms. J. Mol. Biol. **209:**345–358.
4. **Beutin, L., P. A. Manning, M. Achtman, and N. Willetts.** 1981. *sfrA* and *sfrB* products of *Escherichia coli* K-12 are transcriptional control factors. J. Bacteriol. **145:**840–844.
5. **Brazas, R., E. Davie, A. Farewell, and L. I. Rothfield.** 1991. Transcriptional organization of the *rfaGBIJ* locus of *Salmonella typhimurium*. J. Bacteriol. **173:**6168–6173.
6. **Burns, C. M., and J. P. Richardson.** 1995. NusG is required to overcome a kinetic limitation to Rho function at an intragenic terminator. Proc. Natl. Acad. Sci. USA **92:**4738–4742.
7. **Burova, E., S. C. Hung, V. Sagitov, B. L. Stitt, and M. E. Gottesman.** 1995. *Escherichia coli* NusG protein stimulates transcription elongation rates in vivo and in vitro. J. Bacteriol. **177:**1388–1392.
8. **Choe, M., and W. S. Reznikoff.** 1991. Anaerobically expressed *Escherichia coli* genes identified by operon fusion techniques. J. Bacteriol. **173:**6139–6146.
9. **Creeger, E. S., T. Schulte, and L. I. Rothfield.** 1984. Regulation of membrane glycosyltransferases by the *sfrB* and *rfaH* genes of *Escherichia coli* and *Salmonella typhimurium*. J. Biol. Chem. **259:**3064–3069.
10. **Das, A.** 1992. How the phage lambda *N* gene product suppresses transcription termination: communication of RNA polymerase with regulatory proteins mediated by signals in nascent RNA. J. Bacteriol. **174:**6711–6716.
11. **Emory, S. A., and J. G. Belasco.** 1990. The *ompA* 5′ untranslated RNA segment functions in *Escherichia coli* as a growth-rate-regulated mRNA stabilizer whose activity is unrelated to translational efficiency. J. Bacteriol. **172:**4472–4481.
12. **Felmlee, T., S. Pellett, and R. A. Welch.** 1985. The nucleotide sequence of an *Escherichia coli* chromosomal hemolysin. J. Bacteriol. **163:**94–105.
13. **Gaffney, D., R. Skurray, and N. Willetts.** 1983. Regulation of the F conjugation genes studied by hybridization and *tra-lacZ* fusions. J. Mol. Biol. **168:**103–122.
14. **Hess, J., W. Wels, M. Vogel, and W. Goebel.** 1986. Nucleotide sequence of a plasmid-encoded hemolysin determinant and its comparison with a corresponding chromosomal hemolysin sequence. FEMS Microbiol. Lett. **34:**1–11.
15. **Hobbs, M., and P. R. Reeves.** 1994. The JUMPStart sequence: a 39 bp element common to several polysaccharide gene clusters. Mol. Microbiol. **12:**855–856.
16. **Kainz, M., and J. Roberts.** 1992. Structure of transcription elongation complexes in vivo. Science **255:**838–841.
17. **Knapp, S., I. The, W. Wels, G. Michel, H. Tschape, J. Hacker, and W. Goebel.** 1985. Analysis of the flanking regions from different haemolysin determinants of *Escherichia coli*. Mol. Gen. Genet. **200:**385–392.
18. **Leeds, J. A.** 1996. Ph.D. thesis. University of Wisconsin—Madison, Madison.
19. **Leeds, J. A., and R. A. Welch.** Unpublished observations.
20. **Leeds, J. A., and R. A. Welch.** 1996. RfaH enhances elongation of *Escherichia coli hlyCABD* mRNA. J. Bacteriol. **178:**1850–1857.
21. **Li, J., S. W. Mason, and J. Greenblatt.** 1993. Elongation factor NusG interacts with termination factor ρ to regulate termination and antitermination of transcription. Genes Dev. **7:**161–172.
22. **Mason, S. W., J. Li, and J. Greenblatt.** 1992. Host factor requirements for processive antitermination of transcription and suppression of pausing by the N protein of bacteriophage λ. J. Biol. Chem. **267:**19418–19426.
23. **Michael, S. F.** Mutagenesis by incorporation of a phosphorylated oligo during PCR amplification. BioTechniques **16:**410–412.
24. **Muller, D., C. Hughes, and W. Goebel.** 1983. Relationship between plasmid and chromosomal hemolysin determinants of *Escherichia coli*. J. Bacteriol. **153:**846–851.
25. **Nieto, J. M., M. J. A. Bailey, C. Hughes, and V. Koronakis.** 1996. Suppression of transcription polarity in the *Escherichia coli* haemolysin operon by a short upstream element shared by polysaccharide and DNA transfer determinants. Mol. Microbiol. **19:**705–713.
26. **Pradel, E., and C. A. Schnaitman.** 1991. Effect of *rfaH* (*sfrB*) and temperature on expression of *rfa* genes of *Escherichia coli* K-12. J. Bacteriol. **173:**6428–6431.
27. **Ralling, G., and T. Linn.** 1987. Evidence that Rho and NusA are involved in termination in the *rplL-rpoB* intercistronic region. J. Bacteriol. **169:**2277–2280.
28. **Rehemtulla, A. S., K. Kadam, and K. E. Sanderson.** 1986. Cloning and analysis of the *sfrB* (sex factor repression) gene of *Escherichia coli* K-12. J. Bacteriol. **166:**651–657.
29. **Stevens, M. P., P. Hanfling, B. Jann, K. Jann, and I. S. Roberts.** 1994. Regulation of *Escherichia coli* K5 capsular polysaccharide expression: evidence for involvement of RfaH in the expression of group II capsules. FEMS Microbiol. Lett. **124:**93–98.
30. **Sullivan, S. L., and M. E. Gottesman.** 1992. Requirement for *E. coli* NusG protein in factor-dependent transcription termination. Cell **68:**989–994.
31. **Sullivan, S. L., D. F. Ward, and M. E. Gottesman.** 1992. Effect of *Escherichia coli nusG* function on λ N-mediated transcription antitermination. J. Bacteriol. **174:**1339–1344.
32. **Welch, R. A., M. E. Bauer, A. D. Kent, J. A. Leeds, M. Moayeri, L. B. Regassa, and D. L. Swenson.** 1995. Battling against host phagocytes: the wherefore of the RTX family of toxins? Infect. Agents Dis. **4:**254–272.
33. **Welch, R. A., E. P. Dellinger, B. Minshew, and S. Falkow.** 1981. Hemolysin contributes to virulence of extra-intestinal *Escherichia coli* infections. Nature (London) **294:**665–667.
34. **Welch, R. A., R. Hull, and S. Falkow.** 1983. Molecular cloning and physical characterization of a chromosomal hemolysin from *Escherichia coli*. Infect. Immun. **42:**178–186.
35. **Welch, R. A., and S. Pellett.** 1988. Transcriptional organization of the *Escherichia coli* hemolysin. J. Bacteriol. **170:**1622–1630.
36. **Whalen, W., B. Ghosh, and A. Das.** 1988. NusA protein is necessary and sufficient *in vitro* for phage λ *N* gene product to suppress a Rho-independent terminator placed downstream of *nutL*. Proc. Natl. Acad. Sci. USA **85:**2494–2498.