Translational Regulation by an Intramolecular Stem-Loop Is Required for Intermolecular RNA Regulation of the *par* Addiction Module^{∇†}

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The *par* stability determinant of *Enterococcus faecalis* plasmid pAD1 is the only antisense RNA-regulated addiction module identified to date in gram-positive bacteria. *par* encodes two small, convergently transcribed RNAs, designated RNA I and RNA II, that function as the toxin (Fst)-encoding and antitoxin components, respectively. Previous work showed that structures at the 5' end of RNA I are important in regulating its translation. The work presented here reveals that a stem-loop sequestering the Fst ribosome binding site is required for translational repression but a helix sequestering the 5' end of RNA I is not. Furthermore, disruption of the stem-loop prevented RNA II-mediated repression of Fst translation in vivo. Finally, although Fst-encoding wild-type RNA I is not toxic in *Escherichia coli*, mutations affecting stem-loop stability resulted in toxicity in this host, presumably due to increased translation.

It is now generally accepted that small noncoding RNAs play a significant role in the regulation of gene expression in bacterial cells. Regulatory RNAs were first identified on bacterial plasmids, where they were found to play roles in controlling plasmid copy number, stability, and conjugation (3, 14, 34). The majority of plasmid-encoded regulatory RNAs are *cis* acting, transcribed from the opposite strand of the 5' end of the target mRNA, and inhibit translation and/or prematurely terminate transcription by complementary base pairing. More recently, numerous chromosomally encoded small regulatory RNAs have been described (15). Unlike their plasmid counterparts, these RNAs are generally *trans* acting, being unlinked to their target genes and sharing only partial complementarity with them. While many of these regulators appear to control gene expression similarly to the plasmid systems, some have been found to be capable of positively regulating their targets either by interfering with intramolecular translational inhibitory structures (24, 25) or by RNA stabilization (26).

Two RNA-regulated, plasmid-encoded addiction modules, one on *Escherichia coli* plasmid R1 (10, 11) and the other on *Enterococcus faecalis* plasmid pAD1 (31, 32), have been described. Addiction modules (also called postsegregational killing systems or toxin-antitoxin modules) stabilize plasmids within host cell populations by programming for death any daughter cell that loses the plasmid. They are ubiquitous on low-copy plasmids native to both gram-negative and gram-positive bacteria and encode at least two components, a stable toxin and an unstable antitoxin (13, 20). Proper segregation of

plasmid DNA ensures continued production of the labile antitoxin and suppression of toxin activity or translation. Plasmid loss leads to degradation of the antitoxin and toxin activation or translation and cell death. The majority of addiction modules are proteic systems; both the toxin and the antitoxin components are proteins. Toxin activity is inhibited by antitoxin binding, and the antitoxin is susceptible to degradation by a specific cellular protease. In the *E. coli* R1-encoded *hok/sok* system and the *E. faecalis* pAD1-encoded *par* system, the antitoxin is a relatively unstable regulatory RNA that binds to the toxin mRNA and prevents translation. Addiction systems present special problems for antisense RNA regulation since the rapid degradation of the RNA-RNA complexes that occur in most negatively regulated systems (2, 5, 12, 22) would leave no toxin message to be translated once the plasmid is lost. In the *hok/sok* system (10), this problem is solved by the accumulation of a pool of a conformation of the *hok* mRNA that neither binds the Sok antisense regulator nor allows ribosome binding (29). This pre-mRNA is then slowly degraded from the 3' end, triggering a conformational switch to a Sok- and ribosome-binding form (8). If the plasmid is still present, Sok binds rapidly via a U-turn motif located within one of the loops of the *hok* target (9), and the complex is rapidly degraded by RNase III (12). If the plasmid is lost, the Hok toxin is translated because of the absence of the unstable Sok antisense RNA and the cell is killed.

The *par* solution to this problem is different. Unlike most plasmid-encoded regulatory RNAs, the *par* antitoxin, RNA II, is not strictly *cis* acting. Instead, RNA II is transcribed convergently to the toxin-encoding target, RNA I, as shown in Fig. 1A. The complementarity required for RNA I-RNA II interaction is derived from the shared bidirectional terminator and two direct repeats, DRa and DRb, which are transcribed in opposite directions (17). Interaction is initiated at a U-turn motif present in the terminator loop of RNA I (18), and interaction at DRa and DRb sequesters the translation initiation region of the toxin gene, *fst*, preventing ribosome binding and translation (16). Rather than accelerating the degradation of RNA I, interaction with RNA II results in the formation of a

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FIG. 1. (A) Schematic representation of the *par* stability determinant of plasmid pAD1. The promoters for RNA I and RNA II are indicated by black arrows at each end. The two RNAs are transcribed in opposite directions across direct repeats DRa and DRb (hatched arrows above and below the map) to a common bidirectional terminator (cross-hatched converging arrows on the map). The extent of the RNA I and RNA II transcripts is shown by labeled arrows under the map. The open reading frame, *fst* (hatched box), encodes the 33-amino-acid peptide toxin. (B) Secondary structures of RNA I and RNA II. The terminator region and the direct repeats (DRa and DRb) are shaded. The two 5' structures, the SL and the UH, of RNA I are boxed and labeled, as are the *fst* ribosome binding site (RBS) and initiation codon (I.C.). The two RNAs have three dispersed complementary segments. Interaction between the two RNAs is initiated at the U-turn motif (labeled as YUNR) present in the loop of the terminator of RNA I. This interaction is indicated by the arrow labeled A. The interaction then extends to the direct repeat sequences (interaction indicated by arrows labeled B) and prevents the translation of the toxin, Fst, since the translation initiation region of the toxin is overlapped by the interacting RNAs. For a more detailed model of interaction of RNA I and RNA II, refer to Fig. 8 of reference 18.

stable complex from which RNA II is slowly removed by a process that remains undefined (33).

While this approach solves one problem, it presents another. Since the RNA I-RNA II interaction initiates at the terminator stem-loop, binding of the ribosome to the Fst Shine-Dalgarno (SD_{Fst}) sequence must be prevented until the terminator is transcribed (16, 17, 18). Previous computer modeling and secondary-structure analysis demonstrated that the 5' end of RNA I contains two intramolecular structures, an upstream helix (UH) which extends nearly to the 5' end of the RNA and a small stem-loop, referred to as the SL, which sequesters SD_{Fst} (18; Fig. 1B). Since previous work has established that secondary structures interfere with translation initiation (4, 7, 28), we sought to determine if the UH and/or SL were responsible for suppression of Fst translation. We found that disruption of SL and not UH resulted in a dramatic increase in translation in vitro. Indeed, modifications to select single SL base pairs resulted in either complete suppression of *fst* translation or resistance to RNA II-mediated translational suppression in vivo.

Unexpectedly, we found that SL disruption led to Fst toxicity in *E. coli*, which was not observed with wild-type RNA I, presumably due to increased translation. This toxicity could not be prevented by provision of RNA II.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. *E. coli* strain DH5 α (Invitrogen) was used for constructing the RNA I mutants used in this study. *E. faecalis* strain OG1X was used to assess Fst toxicity and RNA II-mediated protection. OG1X is a streptomycin-resistant, gelatinase-negative derivative of OG1 (21). *E. coli* and *E. faecalis* were routinely cultured in Luria-Bertani (LB; 27) and Todd-Hewitt (Sigma) broths, respectively, at 37°C. Antibiotics (Sigma) were used at the following concentrations: ampicillin, 100 μ g/ml; chloramphenicol, 10 to 25 μ g/ml; spectinomycin, 100 μ g/ml; tetracycline, 10 μ g/ml. Isopropyl- β -D-thiogalactopyranoside (IPTG) at 0.033 mM (Sigma) and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) at 0.004% (Gold Biotech) were used for selection of pGEMTeasy clones.

Construction of RNA I mutants. The plasmids used and constructed in this study are shown in Table 1. Plasmid pBAD18 was graciously provided by Thomas Hill, University of North Dakota. The primers and probes used are listed in Table 2. PCR was performed with PCR Supermix Hi Fidelity (Invitrogen) according to the manufacturer's protocol. Plasmid isolation from *E. coli* was carried out with the Bio-Rad miniprep kit in accordance with the manufacturer's instructions. For *E. faecalis* clones, plasmid DNA was isolated by using the modified alkaline lysis prep (30). Restriction enzymes and T4 DNA ligase were obtained from New England BioLabs and used in accordance with the manufacturer's protocol. Transformation into *E. coli* was achieved with Subcloning Efficiency DH5 α chemically competent cells (Invitrogen) according to the manufacturer's instructions. For transforming *E. coli* strains with additional plasmids, cells were made chemically competent (27). Plasmid constructs were introduced into *E. faecalis* by electroporation (17).

pDAK606 or pDAK704 was used as template DNA for PCR construction of mutants containing the wild-type *fst* sequences, and pDAK734 was used as a

TABLE 1. Plasmids used and constructed in this study

Plasmid	Relevant phenotype	Antibiotic resistance marker(s) ^a	Reference or source
Vectors			
pAM401/pWM401	<i>E. coli</i> - <i>E. faecalis</i> shuttle vector	Cm, Tc	35
pBAD18	Arabinose-inducible expression vector	Ap	16
pDL278	<i>E. coli</i> - <i>E. faecalis</i> shuttle vector	Sp	23
pGEMTEasy	<i>E. coli</i> TA cloning vector	Ap	Promega
RNA I clones/mutant forms			
pDAK606	pDL278 containing wild-type <i>par</i> locus	Sp	33
pDAK609	pWM401 containing RNA II	Sp	32
pDAK611	pDL278 containing RNA II	Sp	33
pDAK704	pAM401 containing wild-type RNA I gene	Cm	33
pDAK734	pAM401 with 19-stop RNA I construct A under control of native promoter	Cm	33
pDAK749*	pAM401 with RNA I construct E under control of native promoter	Cm	This work
pDAK757	pAM401 with RNA I construct F under control of native promoter	Cm	This work
pDAK779	pAM401 with RNA I construct G under control of native promoter	Cm	This work
pDAK780	pAM401 with RNA I construct D under control of native promoter	Cm	This work
pDAK781	pAM401 with RNA I construct H (C to U) under control of native promoter	Cm	This work
pDAK782	pAM401 with RNA I construct H (C to G) under control of native promoter	Cm	This work
pDAK784	pBAD18 with promoterless RNA I construct A	Ap	This work
pDAK785	pBAD18 with promoterless RNA I construct C	Ap	This work
pDAK786	pAM401 containing RNA I with alanine-to-glycine substitution on 10th codon	Cm	Previous work (unpublished)
T7 pDAK734	pGEMTEasy with 19-stop RNA I construct A under control of T7 promoter	Ap	This work
T7 pDAK749*	pGEMTEasy with RNA I construct E under control of T7 promoter	Ap	This work
T7 pDAK762	pGEMTEasy with RNA I construct C under control of T7 promoter	Ap	This work
T7 pDAK901	pGEMTEasy with RNA I construct A under control of T7 promoter	Ap	17
T7 pDAK903	pGEMTEasy with RNA I construct B under control of T7 promoter	Ap	17

^a Ap, ampicillin; Cm, chloramphenicol; Sp, spectinomycin; Tc, tetracycline.

template DNA for PCR construction of mutants containing the *fst* 19-stop mutation. The mutants were constructed by a three-step site-specific mutagenic PCR approach. The primers (Table 2) were used at a final concentration of 200 nM. In the first step, a mutagenic primer was used as the 5' primer and 3' RNA I-XbaI was used as the 3' primer. The latter primer contains the 3' end of RNA I and an XbaI recognition site for cloning. This step produced a mutant product. In the second step, a second PCR was carried out with RNA I-Sal-475 as a 5' primer and a 3' primer overlapping the mutagenic primer in the nonmutational region. The former primer contains the 5' end of RNA I and a SalI recognition site for cloning. Thermal cycling conditions were as follows: 2 min at 94°C, followed by 35 repeats of 45 s at 94°C, 45 s at 42°C, and 1 min at 72°C and a final extension for 10 min at 72°C. A fusion PCR was carried out to combine the above two PCR products (200 ng of each) with the end primers 3' RNA I-XbaI and RNA I-Sal-475 in order to obtain the desired RNA I mutant. The primers were added after two cycles of the fusion PCR in order to prevent the amplification of multiple PCR products due to traces of primers present in the template. For fusion PCR, thermal cycling conditions were as follows: 2 min at 94°C, followed by 35 repeats of 45 s at 94°C, 1 min at 55°C, and 1 min at 68°C. The fusion PCR product was cloned into pGEMTEasy and sequenced to confirm the presence of the desired mutation and the absence of extraneous mutations. The construct was then transferred into pAM401 at the XbaI and SalI sites. RNA II was provided in *trans* with construct pDAK609 or pDAK611, depending on the antibiotic resistance marker needed. For the construction of pDAK757, error-prone PCR was carried out as described by Cormack (6). The PCR product was cloned at XbaI and SalI sites in pAM401, and nontoxic constructs were screened by sequencing. The constructs for in vitro transcription were made as described by Greenfield and Weaver (17), with the templates and primers listed in Table 2.

Secondary-structure determination by Pb(II) probing. The in vitro transcripts were synthesized with T7 polymerase (Ambion MEGAscript kit) in accordance with the manufacturer's instructions. The transcripts were purified and end labeled, and Pb(II) probing analysis was carried out as previously described by Greenfield et al. (18).

In vitro translation. In vitro translation was carried out with purified transcripts as described previously (18), with the *E. coli* S30 extract system for linear template (catalog no. L1030; Promega) in accordance with the manufacturer's

instructions. [³⁵S]methionine was incorporated into the translation reaction mixture for detection. The in vitro translation products were resolved on a 16.5% Tris-Tricine gel. The gel was dried and exposed to an Amersham Biosciences storage phosphor screen and imaged with a Typhoon Imager, model 9410. Band volumes were quantitated with ImageQuant software.

Cloning of pBAD constructs. To make pDAK784, a promoterless version for wild-type RNA I was PCR amplified with pDAK704 as the template and primers 5'-KpnI WT and 3'-XbaI (Table 2) with XbaI and KpnI restriction sites. For pDAK785, T7 pDAK762 (C mutant) was PCR amplified with primers 5'-KpnI Disrupt and 3'-XbaI (Table 2), and for the alanine-to-glycine substitution at the 10th codon, a pAM401 construct with the desired mutation was used as the template and then amplified with primers 5'-KpnI Disrupt and 3'-XbaI (Table 2). These PCR products were cloned directly into pBAD18 at the XbaI and KpnI sites. The clones were sequenced with the pBAD sequencing primer. An overnight LB-ampicillin culture was diluted 1:50 in fresh LB without antibiotic and grown at 37°C with shaking at 200 rpm. L-Arabinose was added after 1 h of growth to a final concentration of 0.2%. Growth was monitored every hour for a change in optical density at 660 nm in a Milton Roy Spectronic 21D (Fisher Scientific) densitometer fitted for direct measurements of tubes with a 13-mm diameter. For viability counts, samples were taken at 0 and 30 min and 1 and 4 h after incubation, serial dilutions were made with phosphate-buffered saline, and aliquots were plated immediately onto LB plates. Viability was scored in CFU per milliliter after incubation at 37°C for 24 h. Northern analysis confirmed that the two transcripts showed equal levels of induction 1 h after the addition of arabinose (data not shown). RNA II was provided in *trans* by using a pDAK609 construct. Low arabinose concentrations (0.05, 0.1, and 0.15%), in addition to 0.2%, were used for these experiments.

RESULTS

Role of 5' structures in translational repression. Figure 2A depicts the configuration of the UH and SL structures, sequestering the 5' end and SD_{Fst}, respectively, in wild-type RNA I.

TABLE 2. Primers and probes used for in vivo constructs

Primer or probe ^a	Sequence	Specific use(s) in this study ^a
5'-KpnI Disrupt	5'-ATATATGCGGTACCGCGGCAGCTGTCCTCGATTGGA-3'	EP-pDAK785
5'-KpnI-GG	5'-ATATATGCGGTACCGCGGCAGCTCTCCTCGATTGGA-3'	EP-pDAK782
5'-KpnI-UG	5'-ATATATGCGGTACCGCGGCAGCTTGCCTCGATTG-3'	EP-pDAK781
5'-KpnI WT	5'-ATATATGCGGTACCGCGGCAGCTCGCCTCGAT-3'	EP-pDAK784
3' RNA I-Xba	5'-CGTCTAGATGAAAAAGCAATCCTACGGCGA-3'	EP-pDAK749*, -757, -762, -779, -780, -781, -782, -784, -785
3'-XbaI	5'-ATTGTGAATCTAGAAAAGCAATCCTACGGCGAA-3'	EP-pDAK781, -782, -784, -785
5'MP_A10G	5'-TGTTATTTGTGAAAGATTTAATGTCGTTGGTTATCGGACCAATCTTT-3'	MP-pDAK786
5'MP_C	5'-ATATAATTAATGCGGCAGCTGTCCTCGATTGGAGGTGT-3'	MP-pDAK762
5'MP_D	5'-TGCGGCAGCTGTCCTCGATTGGAGGGCTGTTATTGTGA-3'	MP-pDAK780
5'MP_E	5'-ATTGGCAGAAATTTCAAATTATGATATAATTAATGCTCGTCGTTGCCTCGATTGGAGGTGTTAT-3'	MP-pDAK749*
5'MP_F	5'-TCTAGATAAAAAGCAATCCTACGGCGAA-3'	MP-pDAK757
5'MP_G	5'-TAATGCGGCAGCTCACCTCGATTGGAGGT-3'	MP-pDAK779
5'MP-GG	5'-ATATATCGGTACCGCGGCAGCTGGCCTCGATTGGA-3'	MP-pDAK782
5'MP-UG	5'-TAATGCGGCAGCTTGCCTCGATTGGA-3'	MP-pDAK781
5'T7_C	5'-TGTAATACGACTCACTATAGGGCAGCTGTCCTCGATTGTG-3'	5' primer for T7 construct of mutant C
5'T7_D	5'-TGTAATACGACTCACTATAGGGCAGCTGTCCTCGATTGGAGG GC-3'	5' primer for T7 construct of mutant D
5'T7_E	5'-TGTAATACGACTCACTATAAGGGAATGCTCGTCGTTTCGCCTCG ATT-3'	5' primer for T7 construct of mutant E
5'T7_F	5'-TGTAATACGACTCACTATAAGGGCAGCTCGCCTCGATTGGA-3'	5' primer for T7 construct of mutant F
3'OP_C	5'-GCTGCCGCATTAATTATAT-3'	OP-pDAK762
3'OP_E	5'-GTGTAAACGAGTTTACACGCTT-3'	OP-pDAK749*
3'OP_F	5'-CGGGATCCAAATTGTTGAAGGTTTTATTATTAAATTGGCAGA ATTTCAAATTATGATATAATTAAT-3'	OP-pDAK757
3'OP-UG/GG	5'-ATTGGCAGAAATTTCAAATTATGATATAATTAATGCGG CAGCTCGCCTCGATTGGAGG-3'	OP-pDAK781, -782
3'RNA I-Sal	5'-ATGTGCGTCGACAAAAGCAATCCTACGGCGA-3'	EP-pDAK786
RNA I-Sal-475	5'-GTCGACGTGTAAACGAGTTTACACGCTTAGAAAAGATCATAA AACAGCTAGATAAAATTGTTGA AGGTTTTAT-3'	EP-pDAK749*, -762, -779, -780, -781, -782, -784, -785
SR-3	5'-AACCAACGACACTAAATCTTTCACAAATAACAC-3'	OP-pDAK786
SR-34	5'-ATGTGCGGATCCCAGCTAGATAAAATTGTTGAAGG-3'	EP-pDAK757
RNA I-specific probe	5'-ATAACCAACGACATTAATCTTTTCAC-3'	Probe for RNA I in Northern analysis
RNA II-specific probe	5'-TGTGTTATCTGTACGATTTAATGTCG-3'	Probe for RNA II in Northern analysis
Ec5SrRNA-specific probe	5'-GGAGACCCACACTACCATCGGCGC-3'	Probe for 5S rRNA- <i>E. coli</i> in Northern analysis
Ef 5SrRNA-specific probe	5'-AACAGGTGTATCCTTCTCGCTAT-3'	Probe for 5S rRNA- <i>E. faecalis</i> in Northern analysis
pAM401 sequencing	5'-GAGATTACGCGCAGACC-3'	SP for constructs cloned at XbaI and SalI sites on pAM401 vector
pBAD sequencing	5'-ATCCATAAGATTAGCGGATCCT-3'	SP for constructs cloned at XbaI and KpnI sites on pBAD18 vector

^a MP, mutagenic primer; OP, overlapping primer; EP, end primer; SP, sequencing primer.

Earlier results demonstrated that sequences in the 5' end of RNA I repressed ribosome binding and translation of the Fst toxin (16, 17). Since the original mutation (Fig. 2B) demonstrating these effects disrupted both the SL and the UH, it was unclear whether the SL was sufficient to suppress the translation or both structures were required for efficient translational repression. To resolve this issue, several mutations were introduced to alter the SL and UH structures (Fig. 2C to H) and their effect on the translation of Fst in vitro and/or in vivo was determined. The results of in vitro translation analyses are shown in Fig. 3A.

As observed previously (16), deletion of the 5' end of the

transcript (Fig. 2B), disrupting the SL and the UH, led to substantial derepression of Fst translation, greater than 20-fold higher translation than with the wild-type structure (A). Disruption of the UH (E) had no effect on translation levels compared to the wild type. Destabilization of the SL by changing two nucleotides at the base of the stem (C) resulted in an approximately 11-fold increase in translation relative to the wild type, not quite as high as in structure B but still significant. Restoration of the SL by introducing complementary mutations on the opposite side of the stem (D) restored translational repression. The in vitro structures of constructs C and E have been evaluated in comparison to wild-type construct A

TABLE 3. Transformation frequencies of RNA I SL mutants

Construct	Recipient strain	Transformation frequency ^a	Description of construct
pAM401	OG1X	2.11E + 04 ± 1.3E + 03	Vector control
pDAK757	OG1X	1.95E + 04 ± 3.7E + 03	Construct F
pDAK704	OG1X(pDAK611)	4.90E + 04 ± 2E + 03	Construct A, wild type
pDAK781	OG1X(pDAK611)	3.00E + 04 ± 2.5E + 03	Construct H, CG to UG
pDAK782	OG1X(pDAK611)	≤3.17	Construct H, CG to GG

^a The transformation frequency was measured as the number of transformants per microgram of DNA.

(see Fig. S1 in the supplemental material) by Pb(II) cleavage. It was evident that the UH of construct E was more accessible to Pb(II) cleavage compared to the UH of constructs A and C. The overall accessibility of SL to Pb(II) cleavage was similar for all three of the constructs evaluated. The rest of the structures showed a similar Pb(II) cleavage pattern, indicating that the mutations had not introduced any unwanted structural interactions elsewhere in the structure of the RNAs.

In structure F, a noncanonical U · G base pair was mutated to form a C · G base pair. This mutant was constructed because the same U-to-C mutation was isolated in a screen for RNA I mutants that were nontoxic in vivo. Even though the basal production level and stability of the mutant RNA I were indistinguishable from the wild type (data not shown), a construct bearing this mutation could be introduced into *E. faecalis* in the absence of RNA II at a frequency similar to that of the empty vector (Table 3). The mutant C · G base pair resulted in a change in free energy of SL from −7.7 kcal/mol to −9.6 kcal/mol. Since de Smit and van Duin (7) had previously calculated that for every −1.4-kcal/mol change in free energy of Shine-Dalgarno sequestering structures, below a threshold

of −6 kcal/mol, expression drops 10-fold, it seemed likely that the nontoxicity of this mutation was due to the increased stability of SL. As shown in Fig. 3B, even with increased amounts of protein loaded and increased exposure times sufficient to clearly observe Fst production from the wild-type RNA I, no visible Fst was produced from structure F RNA I in vitro. Although the C · G base pair was strong enough to prevent the translation of Fst in vivo and in vitro, a U · A base pair (G) at the same location was still toxic in vivo. The mutant U · A base pair results in a change in free energy of the SL from −7.7 kcal/mol to −8.1 kcal/mol, perhaps not sufficient to significantly affect translation in vivo.

While these results do not completely rule out a role for the UH in translational repression of Fst, it is clear that SL plays the primary role. Based on comparison of the effects of constructs B and C on translation, the UH has, at most, a twofold inhibitory effect on translation. However, this effect may also be due to complete disruption of the SL in structure B. Furthermore, translation of Fst appears to be fine-tuned by the sequence of SL, as demonstrated by the importance of the

FIG. 2. Schematic representation of wild-type and mutant UH and SL structures at the 5' end of RNA I. SD_{Fst} is indicated in bold letters and is indicated by arrowheads. Panel A shows the wild-type architecture and sequence. The underlined nucleotides represent the mutations introduced. Panels B to H represent the mutant RNA I forms used in this study. Panel B, 5' deletion mutation removing the upstream sequence of both the UH and the SL. Panel C, mutant SL in which the two bases at the bottom of the stem have been replaced with noncomplementary bases. Panel D, mutant SL with bases complementary to C restoring the stem. Panel E, mutant UH with altered bases replacing the upstream sequence of the helix. Panel F, G · C base pair replacing the G · U base pair of the SL. Panel G, A · U base pair replacing the G · U base pair at the base of the SL. Panel H, single-base changes at the base of the SL. Arrows in panel H indicate the mutations introduced resulting in a G · G base pair or a U · G base pair replacing the C · G base pair at the base of the SL. Secondary structures of mutants C and E were determined to ensure that the base changes did not alter the structure of RNA I in unexpected ways. For secondary-structure gels, see Fig. S1 in the supplemental material.

FIG. 3. Effects of UH and SL mutations on translation of Fst *in vitro*. Equal masses of purified RNAs were translated *in vitro*, and equal volumes of the *in vitro* translation products were resolved on a 16.5% Tris-Tricine gel as described in Materials and Methods. The gels shown are representative of more extensive comparisons. (A) Comparison of protein production by various mutants. Lanes are labeled with the alphabetic designation of the structure according to Fig. 2. The value below each lane represents the fold difference in the amount of Fst produced relative to the wild type. (B) Effect of the G-C substitution in structure F on Fst production. A threefold greater volume of the protein was loaded than in panel A, and exposure was lengthened in order to better visualize the difference between Fst production from structure F and wild-type structure A. Lanes are labeled as in panel A.

U · G base pair in allowing sufficient translation for toxicity *in vivo*.

Role of SL in facilitating RNA II repression of Fst translation. The above results support a model of Fst translational regulation in which the SL functions to suppress translation long enough to allow RNA II to interact with the transcript at the terminator loop. If true, this model predicts that mutations that disrupt the SL, like structure C in Fig. 2, will be toxic even

in the presence of RNA II because translation will begin before the RNA I terminator stem-loop required for interaction with RNA II is transcribed. To test this prediction, construction of an RNA I gene under the control of the native promoter was attempted. However, such constructs could not be established in *E. coli* without a secondary mutation in the *fst* coding region, suggesting that *fst* might be toxic in *E. coli* under conditions that enhance expression. To test this possibility, plasmid pDAK785 was constructed, wherein the SL mutation without the wild-type promoter was cloned under the control of the tightly regulated P_{BAD} promoter (19). A control strain, pDAK784, containing the promoterless wild-type gene fused to P_{BAD} was also constructed. L-Arabinose induction of the mutant transcript, but not the control, led to growth inhibition of the host strain (Fig. 4). Growth inhibition was accompanied by a 10-fold decrease in viability after 1 h of induction (data not shown). To ensure that toxicity was specific for Fst and not merely the result of overproduction of a foreign peptide, a glycine substitution was engineered into the 10th codon of Fst. Mutagenesis showed that this alanine-to-glycine substitution creates a nontoxic peptide in *E. faecalis* (unpublished observation). Arabinose induction of this peptide in *E. coli* did not affect growth (data not shown).

To address the original question of whether translation of the SL mutant form could be repressed by RNA II, RNA II was provided *in trans* on pDAK609 in an attempt to protect the *E. coli* host from arabinose induction of Fst. No protection was observed (Fig. 4). Introduction of the SL mutant form with the wild-type promoter into *E. coli* cells in which pDAK609 had previously been established was also attempted. Transformants were obtained, but all either had a wild-type SL or some other

FIG. 4. Growth inhibition due to increased translation of Fst in *E. coli*. Promoterless versions of the wild-type and mutant C (Fig. 2C) RNA I genes were cloned under the control of the tightly regulated promoter P_{BAD} . L-Arabinose was added to a final concentration of 0.2% after 1 h of growth. Symbols: ●, uninduced wild-type RNA I construct; ○, induced wild-type RNA I construct; ▲, uninduced mutant C RNA I construct; △, induced mutant C RNA I construct; ■, induced mutant C with pWM401 (vector control for the RNA II gene); ×, induced mutant C in the presence of pDAK609. The reason for the slightly improved growth of cells in the presence of pWM401 and pDAK609 is not clear, although it is clearly not due to the presence of RNA II. The error bars represent the standard deviation for each data point. OD 600nm, optical density at 600 nm.

mutation that would be expected to inactivate Fst or destabilize RNA I.

Since the double mutation of structure C could not be constructed with the native promoter, in order to address the role of SL in *E. faecalis*, two single-nucleotide substitutions affecting only one SL base pair were constructed (Fig. 2H). Both mutations altering the G · C base pair at the base of SL were successfully constructed in *E. coli*. A construct with a change from a G · U to a U · U base pair in SL could not be established under the control of the native promoter in *E. coli*. As expected, like pDAK704, which contains wild-type RNA I, neither pDAK782 containing the G · G pair nor pDAK781 containing the G · U pair could be introduced into *E. faecalis* containing the empty vector, pDL278. However, as observed previously, pDAK704 could be readily introduced into *E. faecalis* cells containing RNA II-encoding plasmid pDAK611 (Table 3). In contrast, no transformants were obtained with pDAK782. Transformation frequencies with pDAK781 were consistently ~40% lower than those obtained with pDAK704, and this difference was statistically significant, with a *P* value of less than 0.0001 by the single-factor analysis-of-variance test. DNA was isolated from the transformants and confirmed to still contain the mutation by sequencing. Similar frequencies of transformation of all three constructs into *E. coli* were obtained, indicating that the DNA preparations were of equal quality.

DISCUSSION

RNA secondary structures, in particular, structures at the 5' end of transcripts, have been implicated in controlling both RNA stability and translation in a variety of systems. Effects of secondary structures on translation are mediated through inhibition of ribosome binding by sequestering the Shine-Dalgarno sequence, which provides complementarity to the 16S rRNA of the 30S ribosomal subunit (1, 4, 28). This work shows that RNA I, the Fst toxin-encoding RNA of the *E. faecalis* plasmid pAD1 *par* addiction module, contains a structure at the 5' end, designated the SL, which suppresses Fst translation. This structure is required to permit the antitoxin function of RNA II in vivo, presumably by suppressing translation long enough to allow essential interactions at the 3' end of each RNA. Furthermore, increased Fst translation due to disruption of the SL leads to toxicity in *E. coli*, suggesting that the Fst target may be broadly conserved in bacterial cells.

Previous results demonstrated that the interaction between RNA I and RNA II is initiated at a U-turn motif present in the terminator loop of RNA I, begging the question of how translation is suppressed until the terminator is transcribed (18). The results presented here suggest that this is accomplished by the SL structure, which consists of a 6-bp stem, sequestering SD_{Fst}, and a 5-bp loop. Disruption of the SL by altering the complementarity of 2 bp at the base of the SL resulted in an 11-fold increase in in vitro translation, and complementary mutations on the other side of the stem restored translational repression. In contrast, disruption of the UH had no effect on translation, suggesting that the SL is sufficient for this purpose. Based on the working model (18), the UH would not be expected to form until the entire coding sequence for Fst has been transcribed. The only possible scenario wherein the UH

could affect Fst translation would be the formation of a metastable structure between the 5' sequence of the UH and some other downstream sequence and/or structure. However, mutation of the 5' sequence of the UH had no effect on Fst translation and no structural alterations were observed, suggesting that this is not the case. The effect of the SL on translation appears to be poised between delaying translation and shutting it off completely, since the replacement of a critical G · U base pair with a G · C pair resulted in undetectable translation in vitro and loss of toxicity in vivo.

This model of SL function, repression of Fst translation pending RNA II interaction, predicts that mutations disrupting SL should be toxic even in the presence of RNA II. RNA II did not protect *E. coli* cells from arabinose induction of the version of RNA I with the SL disrupted. Nor did the presence of RNA II in *trans* allow the introduction of the mutant form with a 2-bp SL disruption into *E. coli*. Two mutant forms with single-base-pair SL disruptions which replaced the terminal G · C base pair of the stem with either a G · U pair or unpaired G · G were constructed in *E. coli*. While the former mutant form allowed RNA II suppression, transformation efficiencies were lower, suggesting that suppression might be less efficient. The latter mutant form could not be introduced into *E. faecalis* cells, suggesting that RNA II could not prevent translation. This suggests that the SL is important for suppressing *fst* translation until RNA II has a chance to interact with RNA I. It should be noted, however, that the *trans* configuration described here does not perfectly mimic the natural context of the *par* RNAs. Further investigation is required to determine if copy number or *cis*-versus-*trans* effects impact this feature of *par* regulation.

In the plasmid R1-encoded *hok/sok* addiction module, an intramolecular translational inhibitory stem-loop is also required to delay translation until transcription of the *hok* toxin message is complete (29). In this system, however, the full-length *hok* message does not interact with the *sok* antitoxin RNA and processing of the *hok* message from the 3' end is required to activate both translation and *sok* interaction (8). In contrast, full-length *par* RNA I interacts readily with RNA II and there is no evidence that processing intermediates play a role in either translation or RNA-RNA interaction (18).

Finally, the increased translation of Fst in mutants with a disrupted SL resulted in toxicity in *E. coli*. This suggests that the target for Fst may be highly conserved across the gram-positive/gram-negative phylogenetic boundary; however, Fst may have a lower affinity for the gram-negative than the gram-positive version of the target or in some other way be less effective in inhibition.

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