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Differential mRNA Stability Controls Relative Gene Expression within the Plasmid-Encoded Arsenical Resistance Operon

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The arsenical resistance (*ars*) operon of the conjugative plasmid R773 encodes an ATP-driven anion extrusion pump, conferring bacterial resistance to arsenicals. The operon contains a regulatory gene, *arsR*, and three structural genes, *arsA*, *arsB*, and *arsC*. The hydrophilic ArsA and ArsC proteins are produced in large amounts, but the hydrophobic ArsB protein, an integral membrane polypeptide, is synthesized in limited quantities. Northern (RNA-DNA) hybridizations provide evidence that the inducible operon is regulated at the level of transcription. The genes were transcribed in the presence of an inducer (arsenite) as a single polycistronic mRNA with an approximate size of 4.4 kilobases (kb). This transcript was processed to generate relatively stable mRNA species: one of 2.7 kb, encoding the ArsR and ArsA proteins, and a second of 0.5 kb, encoding the ArsC protein. Segmental differences in stability within the polycistronic transcript are proposed to account for the differential expression of the *ars* genes. In addition, analysis of the mRNA structure at the 5′ end of *arsB* suggests a potential translational block to the synthesis of this membrane protein.

The arsenical resistance (*ars*) operon of resistance plasmid R773 confers resistance to arsenite, arsenate, and antimonite on *Escherichia coli* cells by the synthesis of an anion pump (23). This unique oxyanion-translocating ATPase, induced by the presence of its substrates, mediates their active extrusion from cells with energy derived from ATP (18, 22, 23, 28). Thus, resistance results from a lowering of the intracellular concentration of the toxic oxyanion.

A 4.3-kilobase (kb) *Hin*dIII fragment from R factor R773 was cloned into the vector pBR322 to produce a recombinant plasmid which produces constitutive resistance to arsenicals (17). Analysis of the nucleotide sequence of this fragment reveals three structural genes: *arsA*, *arsB*, and *arsC* (6). From the genetic evidence (7, 22) and from the nucleotide sequence (6), the oxyanion pump was predicted to be composed of a complex of the 63-kilodalton ArsA and the 45.5-kilodalton ArsB proteins. The 16-kilodalton ArsC protein appears to act as a modifier subunit and is not necessary for arsenite resistance or transport (22). The ArsA protein was purified from the cytosol and shown to be an oxyanion-stimulated ATPase (23). The hydrophobic ArsB protein has been identified as an inner membrane protein by creation of a gene fusion of the *arsB* gene with *lacZ* (26). It can be visualized as a [^{35}S]methionine-labeled membrane protein when made in a T7 expression vector but is not present in amounts sufficient to be visible as a Coomassie blue- or silver-stained band after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, even though the operon is transcribed in high amounts by using the T7 expression system (31).

Recently, the regulatory gene, *arsR*, has been identified on a 0.73-kb *Eco*RI-*Hin*dIII fragment contiguous with the 4.3-kb *Hin*dIII fragment on the plasmid R773 (M. J. D. San Francisco, C. L. Hope, J. B. Owolabi, L. S. Tisa, and B. P. Rosen, submitted for publication). The recombinant plasmid pWSU1, constructed by cloning the 5.0-kb *Eco*RI-*Hin*dIII fragment into pBR322, confers inducible arsenite, arsenate, and antimonite resistance on *E. coli*. The nucleotide sequence of the *arsR* gene has been determined, and its product, the ArsR protein, has been identified.

Although the ArsA and ArsC proteins are produced in large amounts and in proportion to the number of plasmid copies of the operon, neither the level of resistance nor the rate of extrusion of arsenicals is increased with plasmid copy number (B. P. Rosen, unpublished data). The lack of gene dosage effect appears to stem from poor expression of the *arsB* gene (26), which limits the assembly of the ArsA-ArsB complex (32). The regulation of the operon was investigated to understand the mechanism(s) responsible for the disproportionate levels of the gene products.

In this report we present evidence that the induction of the *ars* operon is at the transcriptional level. The steady-state levels of operon-length *ars* transcript increase in a linear manner in response to increasing inducer (arsenite) concentration. There is selective degradation of the *arsB* segment of the initial transcription product. From consideration of the Northern (RNA) blot data and analysis of the intercistronic region between *arsA* and *arsB*, differential expression of the *ars* genes is proposed to result from segmental differences in stability within the polycistronic *ars* operon. Thus, the production of the intrinsic membrane component of the oxyanion pump is limited by posttranscriptional events.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. Strains of *E. coli*, bacteriophages, and plasmids used in this study are described in Table 1. DNA probes for RNA analysis were prepared by subcloning regions of the *ars* operon. The 0.73-kb *Eco*RI-*Hin*dIII fragment from pWSU1 and 1.2-kb *Eco*RI-*Hin*dIII fragment from M13mCMC49-3d1-22 replicative form DNA were individually cloned into the *Eco*RI- and *Hin*dIII-digested pBluescript vector. The sizes and identities of the inserts were verified by restriction mapping. The resulting plasmids, pBluescript-730 and pBluescript-1200, respectively, were digested with *Kpn*I and *Xba*I, and appropriate fragments were subcloned into *Kpn*I- and *Xba*I-digested M13mp18, to give M13mp18-730 and M13mp18-1200, respectively. M13mp18-625 was prepared by ligating a 625-base-pair *Bam*HI-*Hin*dIII fragment from M13mCMC6-

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TABLE 1. List of strains, plasmids, and phages

Strain, plasmid, or phage	Genotype or description	Source or reference
HB101	F⁻ *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*	14
TG1	K-12 Δ(*lac-pro*) *supE* F′ *traD36 proAB lacI*^{q} Δ*lacZM15*	Amersham Corp.
pWSU1	*ars* operon (*arsRABC* genes) cloned into *Eco*RI- and *Hin*dIII-digested pBR322, Ap^r	This laboratory
M13mp18	*lacp lacZ′*	34
pBluescript	2.97-kb phagemid derived from pUC19, Ap^r	Stratagene
M13mCMC6	M13mWB2349 clone containing the *ars* structural genes in the opposite orientation for transcription	6
M13mCMC6-1d6-34	*Bal*31 deletion clone of M13mCMC6 containing the *arsC* gene in the opposite orientation for transcription	This laboratory
M13mCMC6-3d6-38	*Bal*31 deletion clone of M13mCMC6 containing the *arsB* and *arsC* genes in the opposite orientation for transcription	This laboratory
M13mCMC49	M13mWB2348 clone containing the *ars* structural genes	6
M13mCMC49-3d1-22	*Bal*31 deletion clone of M13mCMC49 containing the first half of the *arsA* gene	This laboratory
M13mp18-1200	1.2-kb *Eco*RI-*Hin*dIII fragment from M13mCMC49-3d1-22 (containing the first half of the *arsA* gene) inserted into *Eco*RI- and *Hin*dIII-digested pBluescript and subcloned into *Kpn*I- and *Xba*I-digested M13mp18	This study
M13mp18-730	730-base-pair *Eco*RI-*Hin*dIII fragment from pWSU1 (containing the *arsR* gene) inserted into *Eco*RI- and *Hin*dIII-digested pBluescript and subcloned into *Kpn*I- and *Xba*I-digested M13mp18	This study
M13mp18-625	625-base-pair *Hin*dIII-*Bam*HI fragment from M13mCMC-3d6-38 (containing the first half of the *arsB* gene) inserted into *Bam*HI- and *Hin*dIII-digested M13mp18	This study

3d6-38 into *Bam*HI- and *Hin*dIII-digested M13mp18. All M13 phage and pBluescript derivatives were grown in *E. coli* TG1, as previously described (16). Cells were grown in LB medium, M9 medium, or H medium (14). Selective media contained ampicillin (100 μg/ml).

Induction of *ars* mRNA and isolation of total RNA. Cells of *E. coli* HB101 containing pWSU1 were grown in LB medium with ampicillin at 37°C to early log phase. Culture samples (15 ml) were transferred to prewarmed flasks. Sodium arsenite was added to each flask in the indicated concentrations. One flask received no inducer. The time course was terminated by chilling the culture on ice. RNA was extracted from samples (15 ml) essentially as previously described (30). RQ1 DNase (Promega Biotec) was used to remove DNA.

Isolation and preparation of probe DNA. Single-stranded DNA was isolated from M13 phages and labeled by using the M13 universal probe primer (Bethesda Research Laboratories) as previously described (11). Labeled DNA was recovered by ethanol precipitation. Care was taken to prevent denaturation of the labeled probe DNA.

Northern blot hybridization. Northern blot analysis was performed by fractionation of RNA samples (10 μg per lane) on 1% agarose gels containing 2.2 M formaldehyde (14) followed by transfer to nylon membrane filters (Hybond N; Amersham Corp.). RNA size markers were purchased from Bethesda Research Laboratories and visualized on autoradiographs by using nick-translated lambda DNA as a probe.

RNA was fixed to the filters by baking at 80°C under vacuum for 2 h. The baked filters were prehybridized at 42°C for 4 to 6 h in a solution containing 5× Denhardt solution (9) and 5× standard saline citrate (SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) buffer (11). The prehybridization solution was replaced by a solution containing 1× Denhardt solution and 1×10^7 to 3×10^7 cpm of probe DNA. The filters were hybridized at 42°C for 24 h.

The filters were washed four times (15 min each time) with 2× SSC and 0.1% sodium dodecyl sulfate at room temperature and four times (15 min each time) at 50°C in 0.2× SSC and 0.1% sodium dodecyl sulfate. The filters were blot dried and exposed to Kodak XAR2 film for 1 to 3 h at room temperature. Radioactivity in specific lanes was quantified by using an AMBIS radioanalytic imaging system (AMBIS Systems, San Diego, Calif.).

Determination of half-life of transcripts. Early log phase cells were induced with 5 mM arsenite for 10 min. Further initiation of transcription was then blocked by addition of rifampin (0.2 mg/ml). Samples (15 ml) were withdrawn at different times, and total RNA was extracted. RNA was analyzed by Northern blot hybridization by using gene-specific probes. The decay rates of the specific transcripts were determined by quantitative radioanalytic imaging of the Northern blots.

RESULTS

Nature of the *ars* mRNA species. A genetic and restriction map of the *ars* operon of the *E. coli* resistance plasmid R773 is shown in Fig. 1. The operon was subcloned into the plasmid pBR322 as a 5.0-kb *Eco*RI-*Hin*dIII fragment to form the recombinant plasmid pWSU1. The regulatory gene, *arsR*, spans the region from nucleotides 124 to 480 from the

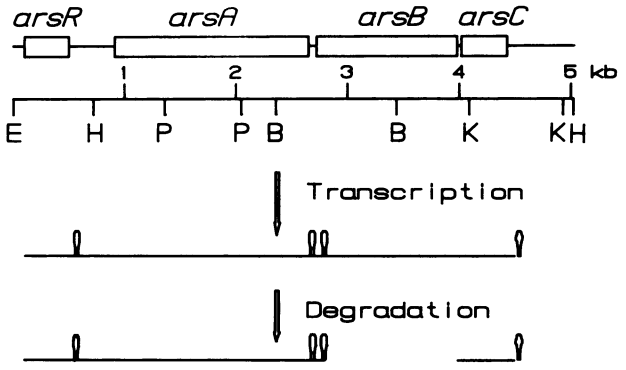

FIG. 1. Physical and genetic map of the R773 *ars* operon. The physical map of the operon is summarized from earlier work (17). Open reading frames in the DNA are indicated by boxes. The relevant predicted secondary structure in the RNA is indicated by hairpins. Restriction endonuclease sites: *B*, *Bam*HI; *E*, *Eco*RI; *H*, *Hin*dIII; *P*, *Pst*I; and *K*, *Kpn*I.

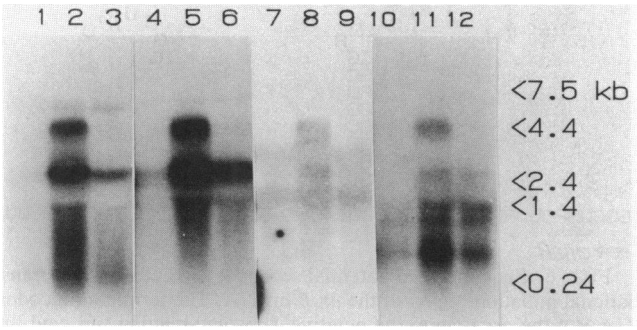

FIG. 2. *ars* mRNA species. Total RNA was extracted from HB101 containing pWSU1 induced or uninduced with 0.2 mM arsenite. RNA (10 μg) was fractionated on 1% agarose–formaldehyde gels, blotted onto nylon membrane, and hybridized with the following gene-specific M13 probes: lanes 1 through 3, *arsR*; lanes 4 through 6, *arsA*; lanes 7 though 9, *arsB*; lanes 10 through 12, *arsC*. Cultures were either not induced (lanes 1, 4, 7, and 10); induced for 5 min (lanes 2, 5, 8, and 11); or induced for 15 min (lanes 3, 6, 9, and 12). Molecular weight markers were an RNA ladder visualized by using nick-translated lambda phage DNA as a probe.

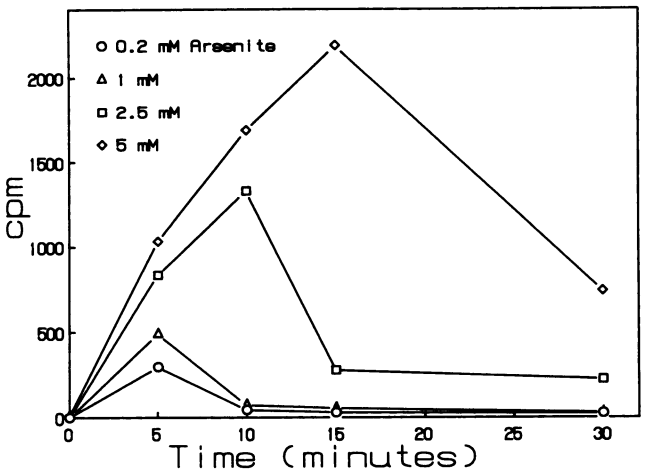

FIG. 3. Effect of inducer concentration on the time course of induction of the *ars* operon. HB101(pWSU1) was grown to log phase and induced with 0.2 mM (○), 1 mM (△), 2.5 mM (□), or 5 mM (◇) arsenite. RNA was isolated at the indicated times after the addition of inducer and used in Norther blot hybridizations with the *arsR* gene-specific probe (M13mp18-730). The amount of full-length (4,400-nucleotide) transcript was quantified with a radioanalytic imaging system and expressed in counts per minute (cpm).

*Eco*RI site. From nucleotides 482 to 512 is an inverted repeat capable of forming a stable hairpin structure. Between the end of the *arsR* gene and the start of the *arsA* gene are 390 base pairs of probably untranslated DNA. The *arsA* gene is followed by an intercistronic region (containing a potentially stable hairpin structure) and by the *arsB* (with a potentially stable hairpin beginning at the third codon) and *arsC* genes (6).

To identify the mRNA species derived from the *ars* genes, Northern blot hybridizations were carried out. Total RNA from HB101 cells containing pWSU1 induced with 0.2 mM arsenite was fractionated by electrophoresis on an agarose-formaldehyde gel, blotted onto a nylon membrane, and hybridized with DNA probes consisting of ^{32}P-labeled M13 recombinant DNA carrying single-stranded inserts complementary to *arsR*, *arsA*, *arsB*, or *arsC* mRNA (Fig. 2). No transcription was observed in the absence of inducer (Fig. 2, lanes 1, 4, 7, and 10). After 5 min of induction, a 4.4-kb mRNA species was observed by using each of the four gene-specific probes (Fig. 2, lanes 2, 5, 8, and 11). This full-length transcript disappeared by 15 min (Fig. 2, lanes 3, 6, 9, and 12). Both the *arsR* and *arsA* gene-specific probes revealed a second mRNA species of 2.7 kb (Fig. 2, lanes 2 and 5). The 2.7-kb species was frequently broad and sometimes resolved into several distinct bands. The *arsC* gene-specific probe hybrizided with a 0.5-kb mRNA species (Fig. 2, lane 11). Other processed transcripts ranging between 1.2 and 1.8 kb also hybridized with the *arsC*-specific probe. Although the amount of label incorporated into the *arsB* probe was low in this experiment, no *ars*-specific mRNA species smaller than the 4.4-kb full-length transcript was ever observed by using the *arsB* probe. The nonspecific bands observed in those lanes are most likely ribosomal RNA.

Quantification of *ars* mRNA induction. The Northern blot experiments shown in Fig. 2 suggest that the transcription of the *ars* operon is turned on rapidly. Operon-length mRNA was detectable as early as 5 min after induction, irrespective of hybridization probe. However, transcription of the operon was also transient, as demonstrated by the lack of a detectable level of operon-length transcript 15 min after induction. It is likely that translation of the transcript results in the synthesis of the oxyanion pump that functions to reduce the intracellular concentration of arsenite. A consequence of this interpretation is that the duration of the steady-state production of operon-length *ars* mRNA would increase with increasing concentration of inducer.

Cultures of *E. coli* HB101(pWSU1) were induced with varying concentrations of arsenite, and RNA was isolated from cultures removed at various time intervals. The *ars* mRNA species which hybridized with the *arsR* gene-specific probe in Northern blots were analyzed by using radioanalytic imaging. Operon-length mRNA levels increased in a linear manner in response to increasing arsenite concentration (Fig. 3). Maximal expression of this transcript occurred at 5 min in cultures induced with 0.2 and 1 mM arsenite and at 10 and 15 min in cultures induced with 2.5 and 5 mM arsenite, respectively.

The steady-state level of the various transcripts was determined by radioanalytic imaging of the blots to allow quantification of the radioactivity in each peak. In one typical experiment, the concentration of each species in the steady state was measured at 10 min after induction with 5 mM arsenite by using the *arsA* and *arsC* gene-specific probes. The amount of probe which hybridized to each species as a percentage of the total amount hybridized was 18% in the full-length 4.4-kb transcript, 39% in the 2.7-kb *arsRA* transcript, and 43% in the 0.5-kb *arsC* transcript. Thus, in the steady state, 20% of the mRNA is in the form of a full-length polycistronic species and 80% is in processed forms.

Stability of the *ars* mRNA species. To verify whether the different steady-state concentrations of the transcripts could be explained by their relative stabilities, the processing of the mRNA species as a function of time was determined by interrupting transcription with rifampin and observing their decay (Fig. 4). The half-life of each species was determined by radioanalytic imaging of the Northern blots hybridized with the various gene-specific probes. This revealed a clear difference in the rate of degradation of the transcripts (Fig.

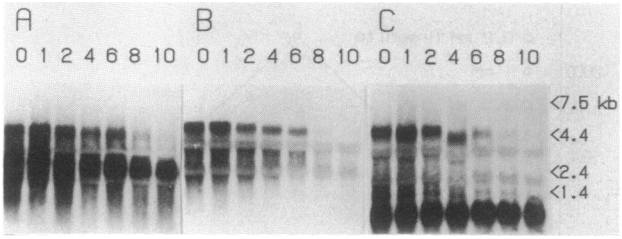

FIG. 4. Kinetics of *ars* mRNA decay. HB101(pWSU1) was induced with 5 mM arsenite for 10 min, and then rifampin was added to block transcription. RNA was isolated and used in Northern hybridizations with gene-specific M13 probes. Probes: A, *arsA*; B, *arsB*; and C, *arsC*. The time (in minutes) after addition of rifampin is indicated above each lane.

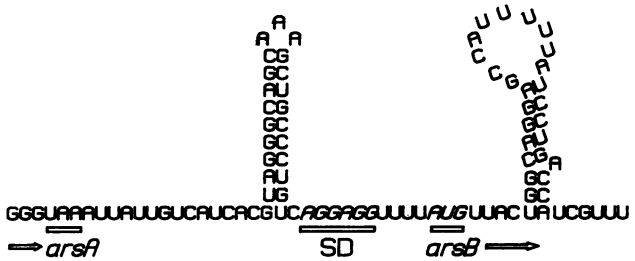

FIG. 6. Sequence and potential secondary structure of the translational initiation region of the *arsB* mRNA. The termination codon UAA of the *arsA* gene, the putative ribosome-binding site, and the AUG initiation codon of the *arsB* gene are indicated (6). The calculated free energies of formation of the two indicated potential secondary structures are calculated as −21.4 and −17.0 kcal/mol, respectively (5, 25).

5). The half-life of the 4.4-kb operon-length transcript was 4 min. The half-lives of the 2.7- and 0.5-kb transcripts were both quite long, in excess of 10 min.

DISCUSSION

The work reported here confirms that the *ars* genes are contained within a polycistronic operon. The operon is inducible, with expression regulated at the level of transcription. Recently, it was shown that transcription of the operon initiates 17 or 18 nucleotides upstream of the *arsR* gene (San Francisco et al., submitted). Upstream of these sites are the sequences GATACTT and TTGACTT, which are identical to the −10 and −35 sequences, respectively, of the A1 promoter of *E. coli* phage T7 (24). The role of the *arsR* gene product in regulation of the operon is presently unknown.

The organization of genes with related functions into a transcription unit favors their coordinated expression. In certain cases, however, genes are differentially expressed. This is made possible by several mechanisms, such as attenuation (35), transcription from multiple promoters (33), different translation efficiency (15), and differential rates of mRNA degradation (1, 3, 4, 12, 13, 21). Differential expression is especially prevalent when one or more of the gene products is an integral membrane protein (2, 8, 10, 20), as is the case with *arsB* (26). The molecular mechanism responsible for the decreased expression of these proteins is obscure. Our data demonstrate that segmental differences in stability within the polycistronic transcript of the *ars* operon contribute to differential expression of its genes in *E. coli*. The 4.4-kb transcript encodes the *arsR*, *arsA*, *arsB*, and *arsC* gene products and decays with a half-life of 4 min to generate 5′ and 3′ mRNA remnants. Because these 2.7- and 0.5-kb *ars* mRNA decay intermediates are relatively stable (with a half-life of about 10 min), they accumulate to a cellular concentration of 2 to 3 times that of the operon-length transcript.

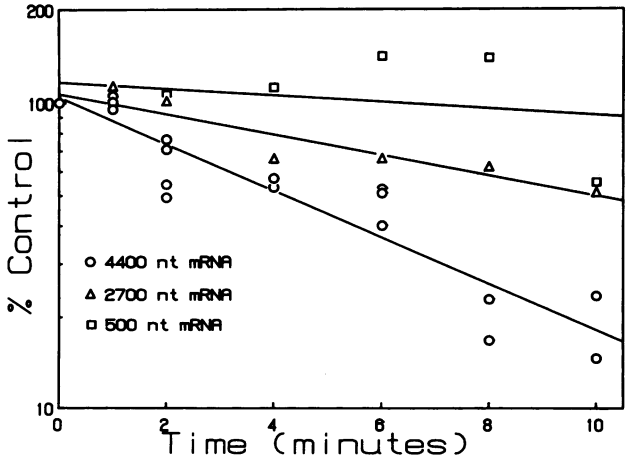

FIG. 5. Half-life of *ars* mRNA. The amounts of each probe hybridizing to each RNA species from the experiment shown in Fig. 4 were quantified by direct radioanalytic analysis of the hybridized filters by using the AMBIS imaging system. Each value was normalized to the amount of hybridization present at the time of rifampin addition. The lines represent least squares fit of the data. ○, 4.4-kb full-length transcript; △, 2.7-kb product; □, 0.5-kb product.

The manner in which *ars* mRNA decays is crucial to the differential synthesis of the proteins encoded by the operon. The 2.7-kb transcript contains both *arsR* and *arsA* but not *arsB*. From the nucleotide sequence, the size of both *arsR* and *arsA* genes and the untranslated region between them is slightly less than 2.7 kb. This suggests that the 3′ end of the 2.7-kb mRNA remnant is in the intercistronic region between *arsA* and *arsB*. An interesting feature of this region is the presence of a palindromic sequence of 10 base pairs that could lead to the formation of a stable hairpin in the corresponding transcript (Fig. 6). The incidence of equal proportions of *arsC*-specific transcript as *arsRA* transcript and the lack of any internal promoter before the *arsC* gene suggest that the hairpin at the end of *arsA* may function as a decay terminator. Hairpin structures that appear to impart stability to selected regions of polycistronic mRNAs have been identified in several other bacterial operons (1, 3, 19, 21, 29). The *arsB* region of the polycistronic transcript appears to decay more rapidly than the *arsRA* and *arsC* regions, which have the potential to form secondary structures at their 3′ termini. This implies that the polycistronic transcript is first cleaved at one or more sites well downstream of the intercistronic hairpin structure and that the exposed 3′ termini are degraded processively in the 3′ to 5′ direction until impeded by the secondary structure.

Analysis of the *arsB* translational initiation region also indicates that the secondary structure in the mRNA may limit translation of this gene (Fig. 6). There is a relatively stable hairpin beginning with the third codon. In addition, the second codon, UUA, is the most inefficiently utilized leucine codon in *E. coli* (27). We predict that the combination of these factors would result in uncoupling of transcription and translation, slowing ribosome movement past the

initiating codon of the *arsB* gene. In conclusion, our data indicate that the limiting quantities of the ArsB protein in the inner membrane of *E. coli* result both from a differential rate of degradation, which could lead to a very rapid loss of function of the *arsB* message, and from inefficient translational initiation of its mRNA.

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