Antisense RNA, Fur, Iron, and the Regulation of Iron Transport Genes in Vibrio anguillarum*

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The negative regulation of the expression of iron transport genes fatA and fatB in Vibrio anguillarum is mediated by a chromosomally encoded Fur protein and a plasmid pJM1-derived antisense RNA (RNAα), which is preferentially expressed under iron-rich conditions. In this work, we characterized the RNAα promoter region, and by using promoter fusion and rifampin experiments we were able to demonstrate that iron regulates RNAα synthesis posttranscriptionally by stabilizing RNAα half-life rather than enhancing transcription initiation. The Fur protein is also essential for RNAα synthesis at the transcription initiation level, independently of the iron status of the cell. From experiments assessing the relative contribution of Fur and RNAα, we were able to show that RNAα may indeed play an important role on the negative regulation of the expression of the iron transport genes under physiological conditions.

The ability to acquire iron from the very limited free iron sources in the tissues and fluids of mammalian hosts is an important component of bacterial virulence (1, 2). Highly virulent strains of the fish pathogen Vibrio anguillarum possess a very efficient plasmid-mediated high affinity iron uptake system, which can compete for the complexed iron with the host high affinity iron-binding proteins such as transferrin, lactoferrin, and ferritin (2, 3). There are two components of this plasmid-mediated iron uptake system. One is the siderophore anguibactin, the actual iron scavenger; the other is an energy-coupled iron transport system that processes the iron-anguibactin complex (2, 4, 5). The iron transport region of pJM1 is a 650-nucleotide RNA, which is encoded in the fatB coding region in the complementary strand and is preferentially expressed under iron-rich conditions. We recently constructed a plasmid in which RNAα is under the control of an external promoter (19). By using this construct we were able to show that the presence of RNAα led to a dramatic reduction of FatA and FatB expression, probably by interaction between the polycistronic RNAα mRNA and RNAα, which may result in a modification of the secondary structure of this polycistrionic mRNA (19, 20).

This change appears to enhance processing upstream of the RNAα coding region, resulting in a concomitant inhibition of FatA synthesis and a degradation of the fatB region in this mRNA (19, 20). Since that work was carried out by using fusions of RNAα with external promoters, very little was known about its physiological role in the iron repression of the pJM1 system and in the mechanism by which iron or other products regulate its synthesis.

In this work we demonstrate that RNAα indeed plays an important biological role on the negative regulation of the iron transport genes fatA and fatB under physiological conditions and show that the Fur protein is essential for RNAα transcription initiation, while iron plays a role in increasing the RNAα stability.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The bacterial strains and plasmids used in this study are listed in Table I.

Media and Growth Conditions—E. coli strains were grown at 37 °C in Luria broth or on Luria broth solidified with 1.5% (w/v) agar (Difco). V. anguillarum strains were grown at 26 °C in Trypticase soy broth supplemented with 1% (w/v) sodium chloride (TSBS). Trypticase soy agar supplemented with 1% (w/v) sodium chloride (TSAS), or M9 minimal medium (3) supplemented with 0.2% casamino acids (Difco). Iron-limiting conditions were established by adding the iron chelator ethylenediamine-di(o-hydroxyphenylacetic) acid (EDDA) (Sigma) to a final concentration of 0.5–1 μM in M9 minimal medium. Iron-rich conditions were obtained by adding 100 μg/ml ferric ammonium citrate to M9 minimal medium. Antibiotics were added to the culture medium at the following concentrations: ampicillin, 500 μg/ml; chloramphenicol (CM), 30 μg/ml.

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1 The abbreviations used are: EDDA, ethylenediamine-di(o-hydroxyphenylacetic) acid; bp, base pair(s); CAT, chloramphenicol acetyltransferase; ELISA, enzyme-linked immunosorbent assay; CAP, catabolite gene activator protein; IRE, iron-regulatory elements.
Isolation of Plasmids, Restriction Endonuclease Analysis, Transformation, and Conjugation—Plasmid DNA was prepared by the method of Birnboim and Doly (21). Restriction endonuclease digestion of DNA was performed under the conditions recommended by the supplier (Life Technologies, Inc.). Transformation was carried out as described by Cohen et al. (22). Conjugation was carried out as described by Tdmasky et al. (23).

RNA Isolation and RNase Protection Experiments—Overnight cultures of V. anguillarum in M9 minimal medium with the appropriate antibiotics were diluted 50-fold into 50 ml of fresh M9 minimal medium plus either 0.5–1 μg EDTA or 100 μg/ml ferric ammonium citrate and grown to an optical density of 0.4–0.6 at 600 nm. Cells were chilled on ice and pelleted by centrifugation. Total RNA was extracted using the hot phenol method as described by von Gabain et al. (24). RNase protection assays were carried out as described previously (20). The fata-specific riboprobe was made from plasmid pMET13.1, linearized with ClaI, and transcribed with T3 RNA polymerase. The fata-specific riboprobe was prepared by the method of Birnboim and Doly (21). The RNA was isolated and transcribed with T7 RNA polymerase. The RNA-α specific riboprobe was prepared from plasmid pHK–SAI–CL, linearized with Sall, and transcribed with T3 RNA polymerase. The RNAra specific riboprobe was used as an internal control and prepared from plasmid pQC3.5, linearized with Sall, and transcribed with T7 RNA polymerase.

RNA Stability Studies—Overnight cultures of V. anguillarum in M9 minimal medium with the appropriate antibiotics were diluted 50-fold into two flasks each containing 400 ml of fresh M9 minimal medium, one with the addition of 1 μg/ml EDTA, the other with the addition of 100 μg/ml ferric ammonium citrate. Cultures were grown to an optical density of 0.4–0.6 at 600 nm. Rifampicin was then added at a final concentration of 200 μg/ml, and 40 mM aliquots were removed from the culture at various times (0, 0.25, 0.5, 0.75, 1, 2, 4, 8, and 12 min), and immediately placed in dry ice with ethanol. Total RNAs were prepared by the hot phenol method as described by von Gabain et al. (24). The level of RNAra was measured by RNase protection experiments using an RNA-α specific riboprobe.

Site-directed Mutagenesis of the RNAra Promoter—A 189-bp HindII–Sau3AI DNA fragment containing the promoter region of RNAra was cloned in plBluescript SK+ to generate pQC7, which was used as the template to perform site-directed mutagenesis. The specific mutations were generated by using the Muta-Gen Phagemid kit (Stratagene) and synthetic mutagenic oligonucleotides. Mutations were confirmed by DNA sequencing using the dideoxy chain-termination method (25) and the Sequencing Kit (U.S. Biochemical Corp.) with the T3 or T7 sequencing primers. The mutated HindII–Sau3AI fragment was redone back to pKK232–8 to generate mutated RNAra promoter-cat fusion plasmids and transferred by conjugation to V. anguillarum H775–3 as described previously (23). The transconjugant strains were tested for the minimal inhibitory concentration for chloramphenical resistance and the level of chloramphenical acetyltransferase (CAT) was determined by the ELISA method.

Hydroxyamine Mutagenesis and Selection of Active Promoter Mutants in the Absence of V. anguillarum Fur—10 μg purified plasmid pQC6.1 DNA was mutagenized with hydroxyamine as described by Rose and Fink (26). Mutated DNA was transformed into E. coli HB101 and then conjugated to a Fur-deficient V. anguillarum 775MET11 and selected for the chloramphenical resistance (Cmr) phenotype on TSAS plates containing 5 μg/ml of Cm and 5 mM of MnCl2. DNAs were then purified from Cmr colonies, and the 189-bp HindII–Sau3AI insert was cloned in plBluescript SK+ to perform sequencing analysis. The mutated HindII–Sau3AI DNA fragment was recloned back to pKK232–8 to exclude the possibility of mutated vector sequences. The promoter activity was assessed by the cat mRNA level as detected by using a RNase protection assay with a cat-specific riboprobe.

CAT ELISA—V. anguillarum strains were cultured in TSBS or M9 minimal medium under either iron-rich or iron-limiting conditions for 16–18 h. 1.5 ml of the culture was microcentrifuged and used to prepare cell lysates and perform the ELISA assay using the CAT ELISA kit (Boehringer Mannheim) following the supplier’s instructions.

RESULTS

Identification and Mutational Studies of RNAra Promoters—We have previously mapped by primer extension three possible transcription start points for RNAra, Ra, Rb, and Rc (27). In order to more precisely define the promoter region, we subcloned a 390-bp Sau3AI DNA fragment containing the transcriptional start point Ra and its promoter region, pa. We also subcloned a 189-bp HindII–Sau3AI fragment containing the transcriptional start points Rb plus Rc and their promoter regions pb and pc, since their respective –10 and –35 regions overlap. These fragments were cloned into plasmid vector pKK232–8, which has a promoterless chloramphenical acetyltransferase gene (cat). The cloning orientation of the fragments containing the putative RNAra promoter sequences was confirmed by sequencing analysis. Plasmids were conjugated into V. anguillarum H775–3, and promoter strength was assessed by either the CAT levels or by the chloramphenical resistance phenotype. We found that the major promoter activity is associated with the fragment carrying the promoters pb and pc (designated pb’/pc) in plasmid pQC6.1. This plasmid confers to V. anguillarum resistance to 10 μg/ml of Cm on TSAS plates, while the subclone carrying the pa promoter region, pQC6.2, only shows weak promoter activity (cells harboring pQC6.2 are sensitive to 5 μg/ml of Cm on TSAS plates) (Fig. 1). To discriminate which promoter (pb or pc) is active in pQC6.1, we redone the 189-bp HindII–Sail fragment into plBluescript SK+ to gen-
Elisa method. Mutations in the termine promoter activity by measuring CAT levels using the GCT to TgcA GGCg (and p

The mutagenic bases in the highly conserved bases in the pKK232–8 and conjugated into p

mutants b1 and c). Combining the mutations of both mutant and of only 1.4-fold for the p

ditions (19, 20, 27). This enhancing effect of iron on the steady

erate, by site-directed mutagenesis, a series of mutations on the −10 and/or −35 regions of pb/c. Base pair substitutions were made on nucleotides considered to be among the most highly conserved bases in the E. coli −10 and −35 sequences. The mutated HindI–SalI fragment was donned back into pK232–8 and conjugated into V. anguillarum H775–3 to determine promoter activity by measuring CAT levels using the ELISA method. Mutations in the −10 regions of pb (AAATGG GT to AgAGG GcGc) and pc (TAAACT to CcAACG), respectively, resulted in a reduction in promoter activity of −8-fold for the pb mutant and of only 1.4-fold for the pc mutant (Fig. 2, see mutants b1 and c). Combining the mutations of both −10 regions of pb and pc resulted in a 73-fold reduction of the promoter activity (Fig. 2, see mutants b1/c and b2/c). These results suggest that both promoters pb and pc must act synergistically. It is of interest that mutations in the −35 regions of pc did not appreciably change the promoter activities (Fig. 2, see mutant c2).

Iron Regulation of the RNAα Promoter—We have previously shown that RNAα is preferentially found under iron-rich conditions (19, 20, 27). This enhancing effect of iron on the steady state levels of RNAα could be due to regulatory mechanisms occurring at either the transcriptional initiation or posttranscriptional level. To identify whether the RNAα promoter is regulated by iron, we first measured the highly active RNAα pb/c promoter activity under iron-rich and iron-limiting conditions. We used the cat fusion plasmid pQC6.1 in V. anguillarum 775 and determined CAT by the ELISA method. Table II shows that the promoter pb/c is fully active and that there is no significant difference of promoter activity under iron-rich and iron-limiting conditions. However, it is still possible that there is a cis element required for iron regulation of the RNAα promoter that is missing in the pQC6.1, since it contains only 64 nucleotides upstream of the start point Rb. Therefore, we generated other RNAα-cat fusion constructs containing 454–1800 bp upstream of the start point Rb (Fig. 1) and conjugated them into the V. anguillarum 775 strain. It is clear from Table II that the promoter activities from all of these fusion con-

TABLE II
Regulation of CAT activity from RNAα-cat fusion constructs in V. anguillarum 775

| Construct | CAT activity (AU/ml) | (Fe/EDDA ratios) |
|-----------|---------------------|------------------|
| pQC6.1    | 0.87 ± 0.20         |                  |
| pQC6.4    | 0.76 ± 0.12         |                  |
| pQC6.5    | 0.76 ± 0.15         |                  |

*Values shown were obtained from CAT activity under iron-rich conditions normalized to that under iron-limiting conditions.

structs are not iron-regulated. The same results were obtained by measuring cat mRNA level of the V. anguillarum 775 harboring these RNAα-cat fusion constructs grown under either iron-rich or iron-limiting conditions (Fig. 7B and data not shown). These results demonstrate that the promoter activity of RNAα is not regulated by iron.

Influence of the Iron Status of the Cell on the Stability of RNAα—In the previous section we presented results demonstrating that the RNAα promoter is not activated by increasing iron concentrations. However, there is a dramatic increase in the steady state amount of RNAα when cells are grown under iron-rich conditions. It is possible, therefore, that the iron concentration of the cell plays a role in maintaining the steady state levels of RNAα posttranslationally rather than at the transcription initiation level. To investigate this possibility, we studied the half-life of RNAα in the presence of rifampin under iron-rich and iron-limiting conditions. RNAs were prepared from rifampin-treated cultures of V. anguillarum 775, harboring the pb M1 plasmid, grown under either iron-rich or iron-limiting conditions. RNAα levels were determined by RNase protection assay using a riboprobe for specific recognition of RNAα (Fig. 3A). The results, shown in Fig. 3A, demonstrate that the half-life of RNAα is about 1 min under iron-rich conditions. Conversely, under iron limitation, most of the RNAα is already degraded by the 0 time point. RNAα was also detected at 30 s under iron-limiting conditions for an unexplained reason. These results indicate that high iron concentrations, either directly or indirectly, must play an important role in protecting RNAα from degradation. In order to further confirm that the regulation of RNAα by iron is mostly at the posttranscriptional level, we investigated the stability of RNAα synthesized from pb HC-A122, a construct that encodes an active RNAα that lacks the first 100 nucleotides from the 5' end as well as its indigenous promoter. In this construct, RNAα is synthesized under the control of a foreign promoter, the tetra-

**FIG. 2. Mutational analysis of the RNAα promoter.** Possible transcriptional start points Rb and Rc for RNAα are indicated. The putative −10 and −35 elements for their corresponding promoter, pb and pc, are underlined. Site-specific mutations introduced into this sequence are shown below the wild type (wt) sequence with arrows. The expression of CAT activity, measured by the ELISA method, from the wild type as well as the mutated RNAα promoters pb and pc in V. anguillarum H775–3 is also indicated. ND, not determined.
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Fig. 3. Decay of RNAα under iron-rich and iron-limiting conditions. At various times after transcription inhibition with rifampicin (200 μg/ml), total RNAs were isolated from log-phase culture of V. anguillarum 775 (A) or V. anguillarum 775 harboring pl HC-A122 (B) grown under either iron-rich (100 μM ferric ammonium citrate) or iron-limiting (1 μM EDDA) conditions. RNAα levels were detected by a RNase protection assay, using a riboprobe for specific recognition of RNAα. 0 indicates that total RNA was prepared from cells immediately before treatment with rifampicin. 0 indicates that total RNA was prepared from cells immediately after treatment with rifampicin.

cycline resistance gene promoter, and thus we are sure that there is no regulation at the initiation level. RNAs were prepared from a strain of V. anguillarum 775 harboring pl HC-A122, and RNAα levels were determined by a RNase protection assay using the same RNAα-specific riboprobe as that used in Fig. 3A. As shown in Fig. 3B, the half-life of RNAα is less than 2 min under iron-limiting conditions, whereas under iron-rich conditions, the level of RNAα is only slightly decreased up to 12 min. This result confirms that the regulation of RNAα by iron is mostly at the posttranscriptional level by enhancing the stability of RNAα. It is of interest that, although conserving the increased stability under iron-rich conditions, the RNAα synthesized under the control of the tetracycline promoter is more stable as a whole than that synthesized under the control of the indigenous promoter, under both iron-rich and iron-limiting conditions. It is possible that the 5' fusion of vector sequences may result in a hybrid RNAα with a more stable secondary structure, albeit not so different as to change the ancestral requirement for increasing iron concentrations for overall stability.

Regulation by Fur of Antisense RNAα Synthesis and Iron Transport Gene Transcription—We have identified a chromosome-encoded Fur protein in V. anguillarum 775 that plays a role in the negative regulation of the synthesis of iron transport proteins and catechol production (18, 19). Since the expression of the iron transport genes fatA and fatB is also negatively regulated by RNAα, while RNAα expression is regulated by iron, it was of interest to determine whether Fur intervenes in the regulation of RNAα synthesis under iron-rich conditions. Therefore, we measured the synthesis of RNAα as well as the synthesis of fatA and fatB-specific mRNAs as a function of the iron concentration in a fur mutant strain of V. anguillarum 775MET11 that is deficient in the synthesis of Fur protein. Total RNA was isolated from V. anguillarum 775 and 775MET11 cultured in M9 minimal medium under either iron-rich or iron-limiting conditions. The RNA levels were determined by RNase protection assays with probes complementary to the sense RNA in the fatA and fatB regions and to RNAα (Fig. 4). We found that RNAα could be detected in the wild type strain 775 preferentially under iron-rich conditions, while RNAα could not be detected either in iron-rich or iron-limiting conditions in the Fur-deficient mutant 775MET11, even after very long exposures of the x-ray film, in which the signal for RNAα in the wild type strain had been greatly intensified (Fig. 4 and data not shown). These results strongly suggest that the Fur product is essential for RNAα synthesis independently of the iron concentration of the cell. Conversely, the fatA and fatB specific mRNA was constitutively expressed. This result demonstrates that Fur regulates the expression of fatA and fatB genes at the transcriptional level. However, under iron-limiting conditions, the mRNA levels of these iron transport genes were considerably higher in the Fur- and RNAα-deficient mutant as compared with that in the wild type strain (Fig. 4, compare lane 4 with lane 2 and lane 9 with lane 7). These differences in levels are inversely related to the levels of RNAα found in the mutant and the wild type strain. The same results have been obtained from experiments in which the aroC probe was used as an internal control, since aroC transcription is independent of the iron status of the cell (Ref. 28 and data not shown). The fact that the expression of the iron transport genes fatA and fatB is greatly enhanced in the absence of RNAα suggests that RNAα may play an important role in the negative regulation of these iron transport genes under physiological conditions.

In order to further determine the contribution of Fur and RNAα on the negative regulation of the iron transport genes, we used a construct, pl HC-A122, that synthesizes the RNAα independently of the iron status of the medium. By introducing this construct into the Fur-deficient V. anguillarum strain 775MET11, we examined the expression of iron transport genes by RNase protection, using a riboprobe specific for the fatB region (Fig. 5). Total RNAs were isolated from V. anguillarum 775 and 775MET11 with or without harboring pl HC-A122, cultured in M9 minimal medium under either iron-rich or iron-limiting conditions. We found that RNAα is constitutively expressed at a high level in both the Fur-deficient mutant and in the wild type strain harboring pl HC-A122 (Fig. 5A), while fatB-specific transcripts were greatly degraded in
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Total RNA was isolated from V. anguillarum wild type strain 775 and Fur-deficient mutant strain 775MET11 harboring (+) and not harboring pl HC-A122 (−), grown under either iron-rich (+) or iron-limiting (−) conditions. The presence or absence of Fur is indicated with + or −, respectively. The riboprobe for specific detection of the aroc transcript was added to the hybridization buffer as an internal control. Specific transcripts for RNAa, fatB, and aroc were detected by RNase protection, using riboprobes for specific recognition of transcripts in the fatB or aroc region and RNAa, respectively. Panel A, detection of RNAa levels. RNAa and aroc-specific transcripts are indicated with arrows. Lane 1, 775MET11 (EDDA); lane 2, 775MET11 (FeCl3); lane 3, 775 (pHC-A122) (EDDA); lane 4, 775 (pHC-A122) (FeCl3); lane 5, 775MET11 (pHC-A122) (EDDA); lane 6, 775MET11 (pHC-A122) (FeCl3); lane 7, free aroc riboprobe; lane 8, free RNAa riboprobe. Panel B, detection of fatB-specific mRNA levels. fatB and aroc-specific transcripts are indicated by arrows. Lane 1, 775MET11 (EDDA); lane 2, 775MET11 (FeCl3); lane 3, 775 (pHC-A122) (EDDA); lane 4, 775 (pHC-A122) (FeCl3); lane 5, 775MET11 (pHC-A122) (EDDA); lane 6, 775MET11 (pHC-A122) (FeCl3); lane 7, free aroc riboprobe; lane 8, free fatB riboprobe.

Fig. 5. Regulation of fatB-specific transcript by V. anguillarum Fur in strains harboring constitutive high levels of RNAa. Total RNA was isolated from V. anguillarum wild type strain 775 and Fur-deficient mutant strain 775MET11 harboring (+) and not harboring pl HC-A122 (−), grown under either iron-rich (+) or iron-limiting (−) conditions. The presence or absence of Fur is indicated with + or −, respectively. The riboprobe for specific detection of the aroc transcript was added to the hybridization buffer as an internal control. Specific transcripts for RNAa, fatB, and aroc are detected by RNase protection, using riboprobes for specific recognition of transcripts in the fatB or aroc region and RNAa, respectively. Panel A, detection of RNAa levels. RNAa and aroc-specific transcripts are indicated with arrows. Lane 1, 775MET11 (EDDA); lane 2, 775MET11 (FeCl3); lane 3, 775 (pHC-A122) (EDDA); lane 4, 775 (pHC-A122) (FeCl3); lane 5, 775MET11 (pHC-A122) (EDDA); lane 6, 775MET11 (pHC-A122) (FeCl3); lane 7, free aroc riboprobe; lane 8, free RNAa riboprobe. Panel B, detection of fatB-specific mRNA levels. fatB and aroc-specific transcripts are indicated by arrows. Lane 1, 775MET11 (EDDA); lane 2, 775MET11 (FeCl3); lane 3, 775 (pHC-A122) (EDDA); lane 4, 775 (pHC-A122) (FeCl3); lane 5, 775MET11 (pHC-A122) (EDDA); lane 6, 775MET11 (pHC-A122) (FeCl3); lane 7, free aroc riboprobe; lane 8, free fatB riboprobe.

those strains harboring the pl HC-A122 plasmid independently of whether they had a deficiency in Fur (Fig. 5B). These results confirmed not only the important role of RNAa in the negative regulation of the iron transport genes but also that Fur is essential for RNAa synthesis, possibly required in an interaction with the promoter region of RNAa, which in turn activates transcription.

Fur Is Essential for RNAa Transcription Initiation—In the previous section we showed that Fur is essential for the expression of RNAa. In order to determine whether Fur regulates RNAa synthesis at the transcription initiation level, we conjugated pOC6.1, in which the RNAa promoter pb/c is fused to the cat gene, into the Fur-deficient V. anguillarum strain 775MET11. Promoter activity was measured by CAT ELISA and RNase protection of cat mRNA. Fig. 6A shows that the RNAa promoter (pb/c) activity was 7–12-fold less as compared with that in the wild type strain harboring pOC6.1. The similar result was obtained from RNase protection studies of cat mRNA (Fig. 7B). These results demonstrated that Fur either directly or indirectly is essential for initiation of transcription of RNAa. Inspection of Figs. 6A and 7B also shows that this Fur-mediated regulation is independent of iron. It is possible that an interaction between Fur and the DNA upstream of the RNAa coding sequences is necessary for this transcription initiation activity. Therefore, sequences upstream of RNAa were searched for homology to the E. coli Fur box. Fig. 6B shows the comparison of the putative Fur box with the consensus sequence for the E. coli Fur box. Only 10 out of 19 nucleotides were identical to the E. coli Fur consensus sequence. This result is not surprising, since the Fur requirement for RNAa transcription initiation is independent of the iron concentration of the cell and the Fur box is recognized by the complex Fur-Fe2+.

To further investigate the mechanism of Fur action on the
control of the RNA\textsubscript{a} transcription initiation, we first investigated whether the RNA\textsubscript{a} promoter is active in the E. coli Fur-deficient strain BN4020. The RNA\textsubscript{a} promoter-cat fusion plasmid pQC6.1 was transformed into the isogenic strains of E. coli Fur-proficient BN402 and Fur-deficient BN4020, and we examined whether the transformants were Cm\textsuperscript{r}. It was found that pQC6.1 endowed both BN402 and BN4020 with a resistance to 30 \( \mu \text{g/ml} \) Cm in Luria agar plates, indicating that the RNA\textsubscript{a} promoter pb/c is active in E. coli independently of Fur. This result also suggests that if activation of RNA\textsubscript{a} transcription in V. anguillarum requires Fur, which acts to facilitate the interaction of RNA polymerase with the RNA\textsubscript{a} promoter, then this requirement is not necessary with the E. coli transcription machinery. Alternatively, if Fur activates RNA\textsubscript{a} transcription via repression of an intermediate repressor in V. anguillarum, then this repressor does not exist in E. coli.

Next, we intended to isolate promoter mutations that did not require Fur, which could be interpreted as indicating a positive regulatory function for Fur, as is the case for the catabolite gene activator protein (CAP). Plasmid pQC6.1 DNA was mutagenized with hydroxylamine. Mutated pQC6.1 DNA was first transformed into E. coli HB101 and then conjugated into V. anguillarum Fur-deficient 775MET11. The activated RNA\textsubscript{a} promoter pb/c mutants in 775MET11 were selected on TSAS plates containing 5 \( \mu \text{g/ml} \) Cm and 5 \text{mM} \text{of MnCl}_2. 50 Cm\textsuperscript{r} colonies were isolated. 10 of them were selected to clone the 189-bp promoter insert in pBluescript SK\textsuperscript{+} to perform sequencing analysis. A single mutation, from TAGGCT to TAGACT, in the −10 region of promoter pb was found in all 10 clones (Fig. 7A). To ensure that the mutation phenotype resided only within the promoter insert, the 189-bp insert was recloned into pKK232−8 that had not been exposed to the mutagen, creating plasmid pQC-HA6.1. This plasmid was then transferred to V. anguillarum 775MET11 by conjugation. RNAs were prepared from V. anguillarum strains 775 harboring pQC6.1, 775MET11 harboring pQC6.1, and 775MET11 harboring pQC-HA6.1, cultured in M9 minimal medium under either iron-rich or iron-limiting conditions. The cat mRNA level was determined by a RNase protection assay, using a riboprobe for specific detection of the cat transcript. It was found that the mutated promoter pb/c of pQC-HA6.1 in 775MET11 had much higher activity than the wild type promoter pb/c of pQC6.1 in 775 and 775MET11 (Fig. 7B). This result indicates that this mutation in the promoter region greatly affected RNA polymerase binding to the promoter region, thus enhancing RNA polymerase function significantly. It also suggests that, if Fur is directly required for activation of transcription of RNA\textsubscript{a}, then this requirement is not necessary when the single nucleotide from TAGGCT to TAGACT, in the −10 region of promoter pb, was mutated. This nucleotide change could lead to a conformational change of DNA, thus promoting the interaction of RNA polymerase with the RNA\textsubscript{a} promoter region independently of the presence of Fur. A similar kind of mutations (class III mutations) in the −10 region of the lac promoter that facilitated lac expression in the absence of activator catabolite gene activator protein (CAP)-cAMP complex have also been isolated in E. coli (29). Alternatively, the mutation might have affected a putative repressor binding site, thus greatly increasing promoter activity.

**DISCUSSION**

Antisense RNA has been demonstrated to play a number of regulatory roles including the control of transcription, translocation, plasmid copy number and plasmid killing as well as a role in the control of plasmid replication and in the lysis/lysogeny cycle (30, 31). However, very little is known about the mechanisms of regulation of the expression of antisense RNA. RNA\textsubscript{a} is the first antisense RNA that was reported to play a role in the repression of the expression of iron transport genes (27). The expression of RNA\textsubscript{a} has been shown to be regulated by iron (19, 20, 27), and three possible transcription start points have been mapped at Ra, Rb, and Rc (27). By subdosing a fragment containing promoter regions corresponding to each transcription start point upstream of the cat gene, we were able to demonstrate in this work that the major promoter activity is associated with the region that we designated pb/c. Mutagenesis analysis of each −10 region of pb and pc allowed us to identify that both pb and pc are active. RNA\textsubscript{a} is mainly detected under iron-rich conditions; therefore, we attempted to understand the nature of this differential behavior by studying the promoter activity of various RNA\textsubscript{a}-cat gene fusion plasmids under iron-rich and iron-limiting conditions. Our results demonstrated that iron does not regulate RNA\textsubscript{a} transcription initiation. It became clear, however, by studying the half-life of RNA\textsubscript{a}, that iron regulates RNA\textsubscript{a} synthesis at the posttranscriptional level by affecting RNA\textsubscript{a} stability regardless of whether RNA\textsubscript{a} synthesis is under the control of the indigenous or of an external promoter. However, the mechanism of the stabilization by iron of the RNA\textsubscript{a} half-life is not clear. It is known that in eukaryotic mRNAs, iron-regulatory elements (IREs) can modulate the expression of the transferrin receptor and ferritin genes posttranslationally dependent on the location of IRE within the context of the transcript. Binding of the iron-regulatory protein to IRE at the 5′ end of a ferritin mRNA inhibits translation initiation by blocking ribosome binding, while binding of the iron-regulatory protein to IRE at the 3′ end of a transferrin receptor mRNA stabilizes this mRNA by blocking nuclease access to mRNAs (32). The specific secondary structure of a hairpin with a terminal hexaloop of the sequence CAGUG(U/C) is an important feature of IREs (32). We have found by sequencing analysis that in RNA\textsubscript{a} there are several stem-loop structures at the 3′ end as well as in other regions of the RNA\textsubscript{a} transcript, and the stem-loop structures at the 3′ end are also present on a truncated RNA\textsubscript{a} molecule that lacks the first 100 nucleotides at the 5′ end but that still has inhibitory functions. This truncated RNA\textsubscript{a} molecule, synthesized under the control of an external promoter, is also stabilized by iron. Therefore, these secondary structures may play an important role in RNA\textsubscript{a} stability. One of the possible mechanisms of iron stabilization of RNA\textsubscript{a} is that there is a protein factor that is only able to bind RNA\textsubscript{a} in the presence of iron. This binding prevents RNA\textsubscript{a} from ribonuclease attack either by a secondary structure change of RNA\textsubscript{a} or by a steric hindrance effect of this protein factor.

Previous work, using a construct in which RNA\textsubscript{a} synthesis was under the control of a strong external promoter, the tetracycline resistance gene promoter, showed that a high level of RNA\textsubscript{a} leads to a reduced expression of the fatA and fatB genes (19, 20). However, direct evidence of the physiological role of RNA\textsubscript{a} under the control of its own promoter was lacking. Therefore, we initiated experiments to understand the contribution of RNA\textsubscript{a} in the control of the expression of the iron transport genes under physiological conditions. We first found that RNA\textsubscript{a} was not synthesized in a Fur-deficient strain of V. anguillarum. By using the RNA\textsubscript{a} promoter pb/c-cat gene fusion studies we were able to demonstrate that the Fur requirement was at the RNA\textsubscript{a} transcription initiation level. This Fur regulation was independent of the iron concentration of the cell. It was of interest that the lack of RNA\textsubscript{a} in the Fur-deficient mutant led to a dramatic enhancement in the levels of the fatA and fatB transcripts (Fig. 4), strongly suggesting that

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\(^2\) Q. Chen and J. H. Crosa, unpublished results.
RNA α may play an important role in the negative regulation of the expression of the iron transport genes fatA and fatB under physiological conditions.

The question is, of course, how Fur regulates the initiation of transcription of RNA α. Fur is a pleiotropic protein that regulates many iron-regulated and some non-iron-regulated genes (11, 33, 34). In most cases, Fur acts as a transcriptional repressor with Fe³⁺ as a cofactor. Recently, a number of studies indicated that Fur could also act as an activator (35, 36). For example, sodB gene expression is positively regulated by Fur, possibly via a direct DNA-protein interaction (35). Since no Fur box has been found in the sodB gene, it is possible that a different mechanism might be involved in the positive regulation of the sodB gene by Fur (35). Our results in this work showed that Fur is essential for RNA α synthesis at the transcription initiation level, yet only a very poor Fur box has been found in the RNA α promoter region. Several possibilities come to mind with respect to the mechanisms of Fur action on RNA α synthesis. First, Fur could serve as an activator via a direct interaction with the RNA α promoter; second, Fur could be a repressor of another unidentified factor, which would be an actual repressor for the promoter region of RNA α; the presence of Fur would lead to repression of this additional repressor gene, resulting in expression of RNA α. Several other scenarios, in which Fur could play a role as an activator of other genes that may be involved in RNA α expression, are also possible. In the effort to distinguish these possibilities, we have isolated, by hydroxylamine mutagenesis of the RNA α promoter, 50 RNA α promoter mutants that are active in the V. anguillarum Fur-deficient strain. All of the mutants mapped at a single nucleotide within the –10 region of the promoter pb, from TAGGCT to TAGaCT, in 10 mutants that had been sequenced. This mutation greatly enhanced the promoter activity in the Fur-deficient strain compared with the wild type promoter activity (37–39). However, certain types of mutations were mapped within the –10 region of the promoter pb, which is the rate-determining step for the wild type lac promoter and which is also influenced by the CAP-cAMP complex. Therefore, the fact that we were able to isolate RNA α promoter mutants similar to class III mutations of the lac promoter suggests that Fur could have a function similar to that of CAP for the lac system, in the activation of RNA α expression. We also found that the activation of RNA α transcription initiation is independent of Fur in E. coli, possibly because of the existence of a different transcription machinery, i.e. the RNA polymerase in E. coli may not require Fur to activate transcription initiation of RNA α. The absence of a “Fur box” at the RNA α promoter region and the fact that the regulation of RNA α by Fur is independent of iron indicate that a unique mechanism must be involved in this regulation in which Fur interaction with DNA may occur by binding with sequences different from the “Fur box.” However, these experiments did not exclude the possibility that Fur could also function as a repressor of another gene that encodes a repressor of RNA α synthesis or as an activator of another activator for RNA α synthesis. Further experiments to understand the mechanism of Fur in the control of the synthesis of RNA α are currently under way.

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