The 5’ end of two redundant sRNAs is involved in the regulation of multiple targets, including their own regulator

Maude Guillier and Susan Gottesman*

Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

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ABSTRACT

Small RNAs are widespread regulators of gene expression in numerous organisms. This study describes the mode of action of two redundant Escherichia coli sRNAs, OmrA and OmrB, that down-regulate the expression of multiple targets, most of which encode outer membrane proteins. Our results show that both sRNAs directly interact with at least two of these target mRNAs, ompT and cirA, in the vicinity of the translation initiation region, consistent with control of these targets being dependent on both Hfq and RNase E. Interestingly, these interactions depend on short stretches of complementarity and involve the conserved 5’ end of OmrA/B. A mutation in this region abolishes control of all OmrA/B targets tested thus far, thereby highlighting the crucial role of the OmrA/B 5’ end. This allowed us, by looking for mRNA sequences complementary to the OmrA/B 5’ end, to identify ompR as an additional direct target of these two sRNAs. Since the OmpR transcriptional regulator activates expression of both omrA and omrB genes, this newly identified control should result in an autoregulatory loop limiting the amount of OmrA/B sRNAs.

INTRODUCTION

Bacterial small RNAs (sRNAs) play major roles in the physiology of the cell. Not fully recognized until the last decade, now about 100 of these molecules have been identified in the Escherichia coli genome (1), which represents more than 2% of the total number of genes. These sRNAs are usually between 50 and 400 nt in length and in most cases do not encode any protein. In general, their synthesis is tightly regulated and, as a result, they are expressed under highly specific conditions. Once synthesized, sRNAs either positively or negatively regulate the expression of one or several target genes. Several major stress regulons have been shown to include at least one sRNA involved in the cellular stress response (2).

Bacterial sRNAs do not all follow the same mode of action; the most extensively studied group is the class of Hfq-binding sRNAs, comprising at least one-third of the experimentally verified E. coli sRNAs (3). These sRNAs usually base-pair with their target-mRNAs and regulate the translation and/or stability of the mRNA at the post-transcriptional level. In most cases, this regulation is negative and sRNAs inhibit ribosome binding by base-pairing in the vicinity of the target ribosome-binding site. Nevertheless, several examples of positive control by sRNAs have been reported that are generally based on the same mechanism: the pairing of the activator sRNA to its target mRNA inhibits the formation of an inhibitory secondary structure and thereby derepresses translation of the target gene (4–6).

Hfq is an RNA chaperone that was first identified for its role in the replication of the RNA phage Qβ, but now appears to have multiple roles for cellular RNAs (7). It was shown to stabilize several Hfq-binding sRNAs in vivo (8) and to enhance base-pairing between sRNA and target-mRNA in vitro (8–11); besides affecting sRNA accumulation, Hfq is also likely required for sRNA action in vivo (12), at least in Gram-negative bacteria. In addition, Morita et al. (13) showed that Hfq copurified with Hfq-binding sRNAs, such as SgrS or RyhB, and with the RNase E endonuclease. Hfq could therefore recruit a ribonucleic complex, which would account for the observed RNase E-dependent destabilization of a target-mRNA after its pairing with a regulatory sRNA (as in reference 14 for instance).

As the role of many sRNAs has started to be investigated, it appears that at least eight of these molecules, and probably more, downregulate the synthesis of outer membrane proteins (OMP) in E. coli and other enterobacteria (15–19). Two of these, OmrA and OmrB, are sRNAs encoded by adjacent genes on the E. coli chromosome.

*To whom correspondence should be addressed. Tel: +1 301 496 3524; Fax: +1 301 496 3875; Email: susang@helix.nih.gov

Present address: Maude Guillier, UPR 9073 du CNRS, Institut de Biologie Physico-chimique, 13 rue Pierre et Marie Curie, 75005 Paris, France.

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They are almost identical at their 5′ and 3′ ends, but have a distinct central region. We have previously shown that both OmrA and OmrB are made in response to the activation of the EnvZ-OmpR two-component system and that their production results in a decrease in the level of several OMPs, such as the OmpT protease and the CirA, FecA and FepA receptors for iron–siderophore complexes (20).

This study focuses on the mechanism by which OmrA/B sRNAs downregulate their targets. Our results show that a conserved short region at the 5′ end of OmrA/B directly base-pairs with ompT and cirA mRNAs in the translation initiation region (TIR). Interestingly, this 5′ end of OmrA/B is also involved in the regulation of their other targets, including the newly identified omR target. Since OmpR activates the transcription of omrA and omrB, these sRNAs should limit their own synthesis by directly down-regulating their activator.

MATERIAL AND METHODS

Bacterial strains

Strains used in this study are listed in Table 1. Unless otherwise indicated, cells were grown aerobically in LB medium at 37°C. When necessary, antibiotics were used at the following concentrations: 50 μg/ml ampicillin on plates or 150 μg/ml in liquid cultures, 25 μg/ml kanamycin and 10 or 25 μg/ml chloramphenicol. Mutant alleles of the rne gene (14) as well as fur::kan (20), fur::cm (21), OmrAB::kan (20) and hq::cm (22) were introduced by P1 transduction. For cloning procedures, PCR amplification was carried out using the Expand High Fidelity PCR system (Roche) and DH5α was used as the recipient strain.

Strains carrying ompT-lacZ or cirA-lacZ translational fusions were constructed as follows. DNA fragments corresponding to nt s–220 to +30 of either ompT or cirA followed by a Flag tag (sequence 5′-GACTACAAGGACGACGATGACAAG-3′) were cloned in frame with lacZ between the EcoRI and BamHI sites (for ompT) or between the EcoRI and SmaI sites (for cirA) of the pRS414 plasmid (23) to give plasmids pRSompT10 or pRScirA10, respectively. These plasmids were then crossed with λR468 bacteriophage and monolysogens were constructed as previously described (23). Mutations in these translational fusions were introduced in the pRSompT10 or pRScirA10 plasmid using the Quick-change II XL site-directed mutagenesis kit (Stratagene) and mutant monolysogens were then made as above.

Strains carrying variants of the ompT chromosomal copy (strains MG1281, MG1307 and MG1309) derive from a strain where a cat-sacB cassette was recombined in the ompT gene as previously described. An oligonucleotide carrying the Flag tag sequence or PCR products of a fragment of ompT DNA with the mut2 change either by itself or in combination with the Flag tag were then used to replace the cassette by selecting recombinants able to grow on sucrose plates. Candidates were checked for chloramphenicol sensitivity and the full ompT gene was sequenced. The malt::lacI allele was then introduced in two steps. First, a crp::cat mutation was P1 transduced into these strains. Then, after P1 transduction of malt::lacI, candidates were selected as clones that grew on minimal medium + 0.2% sorbitol, but not on minimal medium + 0.2% maltose.

Finally, the strain carrying a pBAD-ompR-lacZ fusion (or pBAD-ompRmut2-lacZ) was made by replacing the cat-sacB cassette of a pBAD-cat-sacB construct that was inserted upstream of lacZ gene in strain PM1205 (from P. Mandin, unpublished) by a DNA fragment carrying nts –123 to +30 of ompR (or ompRmut2).

Plasmids

Plasmids pBRplacOmrA, pBRplacOmrB, pBAD-OmrA and pBAD-OmrB have been described previously (20). mut2* and mut3* derivatives of pBRplacOmrA and pBRplacOmrB plasmids were made using the Quick-change II XL mutagenesis kit (Stratagene). After sequencing, the AatII-EcoRI fragment was subcloned into pBRplac to get rid of unwanted secondary mutations.

RNA extraction and northern blot analysis

RNA was extracted from cells in exponential phase as previously described (20). Typically, cells were grown in LB + ampicillin to an OD600 of about 0.3, and
synthesis of OmrA/B was induced using 100 μM IPTG (Figures 1A and B, 3B, 6C and 7B) or 0.2% arabinose (Figure 1C). RNA was harvested at the indicated time-points. For Figure 5, RNA was extracted from strain MG1189 transformed with different plasmids and grown in LB medium supplemented with ampicillin and 100 μM IPTG at the same time that samples were taken to assay the β-galactosidase activity, i.e. at an OD_{600} of about 0.4. After extraction, RNA was ethanol-precipitated, resuspended in DEPC-water and its concentration was estimated by measuring the OD at 260 nm.

Northern analysis was then performed as follows. When the level of OmrA, OmrB or SsrA was analyzed, 3 μg total RNA were separated on a 5 or 10% acrylamide TBE-urea gel (Biorad) and then transferred onto a nylon membrane as previously described (20). To analyze the level of other RNAs, a constant amount of total RNA (between 6.5 and 15 μg) was separated onto a 1% agarose gel in MOPS buffer and transferred to a nylon membrane by capillary action. Detection was carried out using biotinylated probes and the Brightstar Biodetect kit (Ambion). SsrA and ompA RNAs were used as negative controls. Sequences of probes specific to OmrA, OmrB, SsrA, ompT, cirA, fecA, fepA or ompA were previously published (20). The sequence of the ompR-probe is 5′Biotin-caggttggtgcaagagacagggtacgaagagcgtac3′. In Figures 5–7, wt and mutant OmrA or OmrB were detected with OmrAmut-probe (5′Biotin-caggttggtgcaagagacagggtacgaagagcgtac3′) or OmrBmut-probe (5′Biotin-caggttggtgcaagagacagggtacgaagagcgtac3′), respectively. When needed, membranes were stripped by boiling in SDS 0.5% for 10 min and could then be hybridized with another probe. Up to three different probes were used per membrane.

β-Galactosidase assays
Cells were grown in LB medium supplemented with ampicillin and 100 μM IPTG. Duplicate samples of 0.5 ml were removed in exponential phase (OD_{600} about 0.4), mixed with 0.5 ml of cold Z-buffer and their β-galactosidase activity was assayed as described (24). Results are the average of at least two independent experiments.

Western blot analysis
Cells were grown in LB medium containing ampicillin and IPTG at a final concentration of 100 μM. When the OD_{600} reached 0.4, proteins were precipitated with 5% TCA. The pellet was then washed with 80% acetone and resuspended in SDS-sample buffer (New England Biolabs), so that the concentration would correspond to 15 OD_{600}/ml. For detection of the OmpT-Flag protein, 10 μl of these samples were then subject to electrophoresis on 10% Bis–Tris NuPAGE Gel (Invitrogen) in MOPS buffer, transferred to a nitrocellulose membrane and flagged proteins were detected using the anti-Flag M2 monoclonal antibody–alkaline phosphatase conjugate (Sigma) following manufacturer’s instructions, combined with the Lumi-Phos chemiluminescent substrate (Pierce). For the detection of EFTu, 7.5 μl of samples diluted 100-fold were separated and transferred as above, and then probed using a 1:3000 dilution of anti-EFTu antiserum and the ECL detection kit (Amersham).

RESULTS

Degradation of ompT and cirA mRNAs in response to OmrA/B production requires the RNase E endonuclease

Since several targets of Hfq-binding sRNAs have been shown to be degraded by RNase E (14,25,26), this essential endonuclease was a good candidate for degrading OmrA/B targets. This was tested by looking at the effect of two different mutations of the enzyme on ompT and cirA mRNA levels after induction of OmrA or OmrB expression (Figure 1). The first mutant, rne-3071, is a thermosensitive allele carrying a single amino acid change in the N-terminal domain of the protein that affects the catalytic activity of the protein; the protein becomes inactive at elevated temperature and the strain is unable to grow (27). The second mutant, rne-131, carries a two nucleotide deletion resulting in a frameshift and the appearance of a premature stop codon (28). The resulting truncated protein lacks the domain involved in the interaction with other components of the degradosome; the C-terminal domain is not essential and the strain carrying this truncation can be grown at most temperatures (29).

Cells carrying either rne mutation and ectopically expressing OmrA or OmrB from an IPTG-inducible promoter were grown to exponential phase (OD_{600} > 0.3), transferred to nonpermissive temperature to inactivate the RNase E if needed (for the rne-3071 mutant, panel A) and the level of ompT and cirA mRNAs was followed by northern blot after induction of OmrA/B expression. In wild-type (wt) cells, at both 37°C and 43.5°C, degradation of ompT mRNA is visible as soon as 4 min after the induction of OmrA/B expression and most of the ompT message is gone by 10 min (Figure 1, top of panels A and B). Similar results were obtained with cirA mRNA; it was no longer detectable after 4 min of OmrA/B induction, while the control mRNA for ompA was unaffected. In the rne-3071 mutant, both ompT and cirA mRNAs were stabilized; no degradation was observed during the 10 min that followed induction (Figure 1A, bottom panel). RNase E endonucleolytic activity is therefore required for the degradation of at least these two target mRNAs of OmrA/B. Degradation of these targets was also decreased in the rne-131 mutant (Figure 1B), suggesting that the degradosome may play a role as well. In these experiments, accumulation of OmrA/B was unaffected by rne-3071 or rne-131 alleles and control of both ompT and cirA by these sRNAs was similar at 37°C and 43.5°C.

The requirement for endoribonuclease RNase III, that belongs to the same family as the eukaryotic Dicer endonuclease (30), was investigated as well, using a null allele of the rnc gene, encoding RNase III. Because we found that in the rnc− strain used (strain MG1272), both OmrA and OmrB were strongly expressed from pBRplac derivatives, even without induction (data not shown), the OmrA/B RNAs were ectopically expressed from a PBAD promoter using arabinose in this set of experiments.
Figure 1C). Under these conditions, the decrease in \textit{ompT} mRNA is not as striking as with expression from a \textit{P}_{lac} promoter, although the decrease in \textit{cirA} is complete (compare Figure 1B and C, top panels). The decrease in \textit{ompT} and \textit{cirA} mRNA levels after OmrA/B induction is at least as marked in both \textit{rnc}+ and \textit{rnc}/
\textit{C0} cells (Figure 1C), showing that RNase III is not required in the degradation of these targets of OmrA/B.

These results are consistent with findings for other Hfq-dependent sRNAs in \textit{E. coli}, in which RNase E but not RNase III was found to be primarily responsible for mRNA degradation (reference 14 for instance).

Repression of \textit{ompT} or \textit{cirA} translational fusions by OmrA/B differs greatly in extent

For many Hfq-dependent sRNAs, pairing with negatively regulated mRNAs overlaps or is near the RBS and translation initiation codon. Initial pairing predictions for OmrA and B and these regions yielded a short possible pairing for OmrA/B and \textit{cirA}, overlapping the RBS (Figure 2B). Nucleotides 2 to 24 of OmrA or OmrB, most of which are conserved between these two sRNAs (Figure 2A), are predicted to be involved in this interaction, in two stretches, one of 8 nt and the other of 12 nt. They would imperfectly base-pair with the nucleotides -35 to -10 of \textit{cirA} mRNA, i.e. in the vicinity of the \textit{cirA} TIR, which suggests that OmrA/B could inhibit ribosome binding to \textit{cirA} message. For \textit{ompT}, the longest stretch of predicted pairing is downstream from the start codon, at nt 12–20 of the translated region (Figure 2C); once again, this pairing is with the 5' end of OmrA and OmrB.

The effect of OmrA/B long-term expression on the expression of these fusions was then assayed by measuring the \textit{β}-galactosidase activity associated with either fusion after transformation with a plasmid overexpressing either sRNA. Because \textit{cirA} expression is Fur-repressed at the transcriptional level, activity of the \textit{cirA-lacZ} fusion was measured in a \textit{Δfur::cm} background.

As shown in Figure 3A and Table S1, OmrA and OmrB reduced the activity of \textit{cirA-lacZ} 10.6- and 5.5-fold, respectively under these conditions. Both OmrA and OmrB bind tightly to Hfq (31,32), and therefore were expected to depend on Hfq for function. The requirement for Hfq for control by OmrA/B was assayed on \textit{cirA-lacZ}
expression. In an hfq mutant strain, control of cirA-lacZ by OmrA/B was fully abolished (Figure 3A). This result confirms that the cirA-lacZ fusion mimics the major expectations for OmrA and OmrB regulation of cirA, i.e. strong negative regulation and dependence upon Hfq. Therefore, it seems likely that the fusion contains the necessary elements for Omr regulation.

In contrast, the effect of OmrA/B on the expression of ompT-lacZ was extremely weak (Figure 3A and Table S1). This result suggested that elements within the ompT transcript that are important for the full regulation by OmrA and OmrB are not present in this fusion, and therefore the regulation of ompT may differ somewhat from that of cirA. Because of this modest effect of OmrA/B on the translational fusion, the requirement for Hfq was analyzed by a direct examination of the levels of ompT mRNA after OmrA/B induction in an hfq mutant (Figure 3B). In contrast to a wt strain, no degradation of ompT mRNA was visible in this mutant for as long as 10 min after induction, clearly showing that control of ompT by OmrA/B is Hfq-dependent as well.

In the absence of Hfq, both OmrA and OmrB fail to accumulate as much as in wt cells (Figure 3B), which explains, at least in part, the need for Hfq in the control of their targets. Lower levels of Hfq-dependent RNAs are commonly seen in hfq mutants, due to rapid turnover of these sRNAs (14). However, in vitro experiments clearly demonstrate direct roles for Hfq in pairing of sRNAs and their targets (33). It seems likely that Hfq is important in both capacities for OmrA and OmrB in vivo action.

Role of the 5’ leader of cirA mRNA for control by OmrA/B

Many targets of sRNAs have relatively long 5’ untranslated regions (UTRs) that participate in the regulation, for instance by adopting a structure that can promote the interaction with the regulatory sRNA (34). At first sight, cirA could fall into the category of these targets, since it carries a long UTR. Indeed, two promoters of cirA have been identified: p1, leading to transcription of a 173-nt untranslated leader, and p2, leading to transcription of a 160-nt untranslated leader (35). Both are present in the cirA-lacZ translational fusion used in this study. Two deletions were constructed to examine the role of this UTR in regulation by OmrA and OmrB. Deletion Δ1 extends from nts –149 to –54, deleting 96 nt; deletion Δ2 extends from nts –149 to –21, deleting 129 nt (Figure 4A). Activity of the cirA-lacZ fusion was similar to the full-length fusion, and was subject to OmrA and OmrB-dependent repression (Figure 4B). Although the degree of repression was less than that observed with the full-length leader, these results show that OmrA and OmrB do not require cirA sequences beyond those from –54 to +30 for regulation.

The Δ2 deletion increased the basal activity (in the absence of OmrA and OmrB) by 1.75-fold, and control by OmrA and OmrB was almost fully abolished (Figure 4B, Table S1), suggesting that at least some of the region between nts –54 to –21 of the cirA leader is crucial for OmrA/B action.

Several mutations were then introduced into the cirA-lacZ fusion to further examine whether pairing with the TIR of cirA is required for OmrA/B action. In the predicted pairing (Figure 4A), the longest stretch of continuous pairing, arbitrarily referred to as pairing region I, is an 8- or 9-nt region that extends from nts –35 to –27 of cirA, pairing with a region that is not completely conserved between OmrA and OmrB.
The second stretch of pairing involves 12 nt of OmrA and B, starting very close to the 5′ C of these sRNAs, and three regions of cirA, of 3 nt, 6 nt and 3 nt respectively, separated in each case by a single unpaired base (Figure 4A). These pairing regions were arbitrarily referred to as IIA, IIB and IIc, respectively. Changes in region I or IIA of cirA that are expected to disrupt pairing in these regions (mutI and mutIIa respectively, Figure 4A) did not disrupt regulation by OmrA/B (Figure 4B, Table S1). In addition, combining these mutations (mutI+IIa) gave regulation very similar to that seen in the wt case (Figure 4B).

Therefore, pairing in regions I and IIA is not required for control by OmrA/B in our experimental conditions, which leaves only the pairing of 9 nt in regions IIB and IIc. Consistent with a crucial role for these regions, a mutation expected to disrupt the three last base-pairs of region IIB (mutIIB), almost fully abolished control by OmrA or OmrB (Figure 4B).

**OmrA and OmrB base-pair with cirA mRNA in vivo**

To directly test whether the predicted cirA-OmrA/B pairing in regions IIB and IIc was critical for regulation, effects of mutations and compensatory mutations in the sRNAs and the cirA-lacZ fusion were tested in vivo. Mutations in cirA-lacZ that changed blocks of 3–5 nt were made as shown in Figure 5; the compensating mutations, indicated with an asterisk (*), were constructed in both OmrA and OmrB and gave wt levels of RNA (Figure 5C).

In the cirA-lacZ mutant referred to as mut2, the nine predicted base-pairs in regions IIB and IIc are disrupted by a 3-nt change (Figure 5A). Probably because the first G of the cirA Shine–Dalgarno (SD) sequence is changed into a C with this mutation, the activity of the cirAmut2-lacZ fusion was extremely low (<2 Miller units when transformed with the empty vector, data not shown). This rendered it virtually impossible to measure the effect of OmrA/B on the expression of this fusion. Therefore, we took advantage of an up-mutant that we constructed while in the process of characterizing the cirA untranslated region. This mutant, referred to as ‘up’, consists in a change of 4 out of the 6 nt between the SD sequence and the AUG start codon (Figure 5A). The activity of the resulting cirAup-lacZ fusion is more than 8-fold higher than the activity of the wt fusion (Figure 5B, compare scale on two panels). In addition, consistent with the fact that the ‘up’ change affects nucleotides that are not predicted to base-pair with the sRNAs, OmrA and OmrB were found to efficiently repress the activity of the cirAup-lacZ fusion (Figure 5B, Table S1). Although the molecular mechanism underlying the increase in expression with the ‘up’ mutation is still unknown, we combined this change with other changes in the pairing region in order to study the effects of compensatory mutations in OmrA/B.
Two sets of mutations in the 5' end of OmrA or OmrB were tested. Mutating positions 3–6 of the 5' end of OmrA/B to create mut2/C3 (Figure 5A) drastically reduced the ability of the Omr mutant RNAs to regulate the cirAup-lacZ fusion (Figure 5B, right panel), consistent with required pairing of the 5' end of OmrA and OmrB.

Mutating the 5 nt between positions 6 and 10 (Figure 5A, mut3/C3) also greatly reduced regulation of the 'up' fusion. Both sets of mutations also reduced regulation of the wt fusion (Figure 5B, left panel), consistent with the 'up' mutation not changing the basic-pairing properties of the cirA-lacZ fusion. Complementary mutations in the pairing regions of cirA were tested, in the context of the 'up' version of the cirA-lacZ fusion. While the up/2 double mutant had relatively low expression in the absence of the sRNAs, there was very little effect of expression of wt OmrA or OmrB (1.5- and 1.3-fold decrease, respectively). In contrast, OmrAmut2/C3 and OmrBmut2/C3 repressed the expression of the cirAup/2-lacZ fusion significantly (repression factors are 3.1 and 2.7 for OmrAmut2/C3 and OmrBmut2/C3, respectively), supporting the predicted base pairing (Figure 5B, Table S1).

All together, data presented in Figures 4 and 5 clearly show that the 5' terminal region of OmrA/B interacts in vivo with the TIR of cirA mRNA and that a relatively short pairing region of no more than 9 nt is sufficient for the regulation, at least when OmrA/B are overexpressed. It is worth noting that none of these mutations fully abolished regulation under these growth conditions, suggesting that the remaining even shorter pairing can provide some modest degree of regulation.

OmrA and OmrB base-pair with ompT mRNA in vivo

As for cirA, the importance of RNase E and Hfq in the control of ompT expression (see above) suggested a base-pairing with OmrA/B and such a base-pairing can be predicted in the vicinity of the ompT TIR (Figure 6A). Interestingly, the region of OmrA/B that base-pairs with cirA (i.e. nts 2 to 10 of the sRNAs) is also predicted to be involved in the interaction with ompT, since nts 1 to 33 of OmrA or 1 to 32 of OmrB are expected to form an imperfect duplex with nts -15/-14 to +20 of ompT mRNA. Surprisingly, most of the region of ompT mRNA involved in this base-pairing is part of the coding sequence rather than of the 5' UTR.
In order to determine whether this interaction occurs in vivo, the effect on the control of \( \text{ompT} \) expression of mutations in the longest stretch of predicted base pairs (the 9 bp between nts 1 and 9 of OmrA/B and nts 12 to 20 of \( \text{ompT} \) mRNA) was analyzed. Mutants in OmrA/B used in this set of experiments are OmrA/Bmut2/C3, also used previously, while the mutant in \( \text{ompT} \) mRNA, referred to as \( \text{ompTmut2} \), consists of the compensatory change (Figure 6A).

Results obtained using these compensatory mutations and the previously described \( \text{ompT-lacZ} \) translational fusion as a reporter for \( \text{ompT} \) expression support the pairing between the 5’ end of OmrA/B and the early coding region of \( \text{ompT} \) mRNA (Table S1). However, given the very modest effect seen with the \( \text{ompT-lacZ} \) reporter, a number of other approaches were used to examine the importance of the predicted base-pairing. In both of the approaches discussed below, a full-length \( \text{ompT} \) gene is present and therefore, if sequences downstream of the \( \text{ompT-lacZ} \) fusion joint are critical for regulation, these should be present.

An \( \text{ompT-Flag} \) allele was used to further confirm this direct interaction in vivo. In this OmpT-Flag construct, an eight-amino-acid Flag tag (sequence DYKDDDDK) was introduced between amino acids S110 and N111 of OmpT (the numbering refers to the full-length protein, i.e. including the signal sequence). Based on the structure of OmpT (36), the Flag tag is expected to be in the second extracellular loop. As shown by western analysis using an anti-Flag antibody, the amount of OmpT-Flag clearly decreases in the presence of the plasmids overproducing OmrA or OmrB compared to the empty vector, but the mut2* forms of OmrA/B are less effective (Figure 6B, lanes 1–5). In this regard, this OmpT-Flag is a better reporter than the OmpT-\( \beta \)-galactosidase fusion for the action of OmrA/B, consistent with the idea that regions outside of those present in the fusion may contribute to regulation. Consistent with the predicted pairing, OmrAmut2/C3 and OmrBmut2/C3, but not the wt OmrA and OmrB, decreased the cellular level of the OmpTmut2-Flag protein (Figure 6B, lanes 6–10) made from an \( \text{ompTmut2-Flag} \) allele where the compensatory mutation was introduced. This clearly shows that OmrA/B base-pair with \( \text{ompT} \) mRNA in vivo in the beginning of the coding region. Therefore, the first 10 nt of both OmrA and OmrB mediate their interaction with at least two direct targets, \( \text{ompT} \) and \( \text{cirA} \).

Finally, the ability of OmrA/B or OmrA/Bmut2* to induce the degradation of \( \text{ompT} \) or \( \text{ompTmut2} \) mRNAs, as well as their flagged derivatives, was investigated. As previously shown, wt OmrA and OmrB decreased the cellular level of the \( \text{ompT} \) mRNA as soon as 10 min after induction. Surprisingly however, the level of \( \text{ompT-Flag} \) or \( \text{ompTmut2-Flag} \) mRNAs were not affected by the induction of wt or mut2* OmrA/B, respectively (Figure 6C), even though the synthesis of the corresponding proteins are controlled by these sRNAs (Figure 6B). This strongly suggests that mRNA degradation is not required for
translational control of opmT by Omr sRNAs. Finally, opmTmut2 message was not degraded after a 10-min induction of mut2/C3 forms of OmrA/B (Figure 6C) and similar results were obtained after a 30-min induction of the sRNAs (data not shown). A 4-nt change in the opmT mRNA is thus sufficient to change its sensitivity to Omr-promoted degradation.

The 5' end of OmrA/B is also involved in the regulation of other targets of these sRNAs

Results presented so far show the crucial role of the conserved 5' end of OmrA/B in the base-pairing with opmT and cirA targets. Consistent with these results, mutants in the 5' end of these sRNAs, OmrAmut2/C3 or OmrBmut2/C3, failed to induce the decay of opmT and cirA mRNAs seen with the wt sRNAs (Figures 6C and 7B). We have previously shown that OmrA and OmrB have other targets, including two additional genes for outer membrane proteins, fecA and fepA. The effect of the mut2* mutation in OmrA/B on the mRNA level of these other targets was investigated as well. Figure 7B clearly shows that, as for opmT and cirA, mRNA levels of fecA and fepA are decreased after induction of the wt forms of OmrA/B (see also reference 20), but not after induction of their mut2* counterparts. Therefore, the 5' end of OmrA/B plays a major role in the regulation of at least four targets. In the case of opmT and cirA, this is due to a direct base-pairing between the target-mRNA and OmrA/B 5' end. The same may be true for fecA and fepA as well, but we cannot exclude indirect regulation of these targets by OmrA/B, in which case the mut2* mutation could affect the level of an intermediate regulator instead.

Both OmrA and OmrB directly regulate the expression of their transcriptional activator, ompR

Given these results, we used the program TargetRNA (37) to search for potential pairing between the first 10 nt of OmrA/B and the TIR of E. coli mRNAs, with the idea that additional potential targets might be found. The search was done using the following criteria: focus around nts –30 to +20 of mRNA relative to the start codon, a hybridization seed of 6 bp allowing G:U base-pairs, and a P-value threshold of 0.3. With these fairly permissive criteria, opmT was ranked 5 (P-value = 0.033828) and cirA was ranked 48 (P-value = 0.137068), whereas fecA and fepA were not present among the first 100 predicted targets. Interestingly, the first predicted target is yjcH (P-value = 0.00800168), and mRNA levels for both genes of the yjcH-yjcG operon
were strongly decreased after induction of OmrA or OmrB in microarrays experiments (20). Similarly, ranked at the eighth position with a \( P \)-value of 0.0338288 is \( \text{ompR} \), encoding the transcriptional regulator of several genes including \( \text{omrA} \) and \( \text{omrB} \). \( \text{ompR} \) is the first gene of the \( \text{ompR-envZ} \) bicistronic operon and both \( \text{ompR} \) and \( \text{envZ} \) mRNA levels were found to be reproducibly decreased by 2-fold after OmrA or OmrB induction in \( E. \ coli \) (20). The same was true for \( \text{ompR} \) in \( S. \ aureus \) (Papenfort, K. and Vogel, J., personal communication). Altogether, these results suggest that OmrA/B could base-pair with the leader of \( \text{yjcH} \) or \( \text{ompR} \) and thereby regulate the expression of the corresponding operons. Besides \( \text{ompT} \), \( \text{yjcH} \) and \( \text{ompR} \), the other genes among the 10 first predicted targets were either poorly expressed or their expression was unchanged in the presence or absence of OmrA or B as assayed by microarray experiments (20).

The possibility that \( \text{ompR} \) could be a direct target of OmrA/B was further reinforced when we investigated the effect of ectopically overproducing wt and mut2\(^*\) OmrA/B on \( \text{ompR} \) mRNA levels by northern blot. When we used a 42-nt probe complementary to nts 98 to 137 of \( \text{ompR} \) mRNA, two distinct mRNA species were detected (Figure 7B). The larger band migrates between 2.0 and 2.5 kb (data not shown), which is consistent with the size of the \( \text{ompR-envZ} \) bicistronic mRNA. The smaller band migrates above 1.0 kb and could correspond to an alternative transcription product, a processing product or could be due to a nonspecific hybridization, but this was not investigated further. As shown in Figure 7B, induction of wt OmrA or OmrB, but not of OmrAmut2\(^*\) or OmrBmut2\(^*\), decreased the level of \( \text{ompR-envZ} \) mRNA, while the amount of the smaller transcript was not affected. This result shows that OmrA/B negatively controls the expression of the \( \text{ompR-envZ} \) operon, and that, as for other targets of these sRNAs, the 5' end of OmrA/B is involved in this control.

Given these results, it seems likely that the predicted pairing mentioned above between the \( \text{ompR} \) 5' UTR and OmrA/B (shown in Figure 7A) exists \textit{in vivo}. In order to test this, a translational \( \text{ompR-lacZ} \) fusion was constructed. Transcription of this fusion is driven by the PBAD promoter and the transcription start site is expected to be the same as that for the \( \text{ompR} \) promoter p1 (38). The 5' UTR should therefore be 123-nt long. \( \text{lacZ} \) is fused in frame after the 30th nucleotide of the \( \text{ompR} \) coding region (i.e. after the 10th amino acid). The activity of the resulting fusion was decreased by 3- or 2.5-fold, respectively when OmrA or OmrB was ectopically overexpressed.

![Figure 7](https://academic.oup.com/nar/article-abstract/36/21/6781/2410296)
Nevertheless, members of the family of outer membrane proteases defined by OmpT, the Omptin family, are encoded by Yersinia (pla gene) or Salmonella (pgtE gene), but the OmrA/B-ompT pairing is not conserved with these genes (Figure S1). Therefore, the synthesis of Yersinia Pla and Salmonella PgtE may not be directly controlled by OmrA/B, even though we cannot exclude an unidentifiable alternative pairing in these cases. Pairing with the same 5' region of OmrA and OmrB is also required for regulation of envZ/ompR; in this case, pairing is again upstream of the AUG and partially overlapping the RBS. As for cirA, this pairing is conserved in other enterobacteria (Figure S1). Finally, the same 4-nt mutation (mut2*) that disrupts pairing with ompR, ompT, and cirA also abolishes regulation of fepA and fecA, suggesting that all OmrA and OmrB targets depend on this 5' region.

Pairing of the same region of an sRNA to multiple targets may be a general rule; for instance, a conserved region of GcvB sRNA is involved in its interaction with as many as seven different targets (41). In the case of OmrA/B, the participation of the 5' end of the sRNA may provide increased flexibility or allow easier accessibility to mRNAs. Pairing to multiple targets through the 5' end of a sRNA has also been observed with Salmonella RybB sRNA, that interacts with several OMP-encoding mRNAs (Mika,F. et al., submitted). Interestingly, MicC and MicF, that respectively inhibit synthesis of OmpC and OmpF porins, also paired with the corresponding target-mRNAs through their 5' end (42,43). This is reminiscent of the 5' end seed used for pairing by eukaryotic miRNAs (44).

The conservation of this 5' end region between OmrA and OmrB is consistent with the fact that both sRNAs can regulate all targets identified so far. Such redundancy in regulation of shared targets is not unusual for duplicated sRNAs (45,46), but does raise the question of why these small RNAs are duplicated in some species, but not others such as Yersinia pestis. It is also worth noting that, in addition to conservation of the 5' end (which extends well beyond the pairing region, to nt 21), the last 35 nt of OmrA/B are conserved as well. Although some of these 3' end nts are part of the transcriptional terminator, this conservation pattern may suggest that additional targets of OmrA/B remain to be identified that pair with the 3' conserved regions, or that this region plays other roles in sRNA function.

**Other components of cirA regulation**

cirA mRNA has a rather long leader [173- or 160-nt long if transcription is driven by p1 or p2, respectively (31)]. Even though pairing of OmrA/B with nts –10 to –19 of cirA leader is sufficient for the control in our experimental conditions, we found that deleting nts –20 to –149 (i.e. outside of the sufficient pairing region) almost fully abolished this regulation (Figure 4, deletion Δ2). Some of the leader may therefore ensure an optimal conformation for the pairing region to interact with the sRNAs.

On the other hand, it is clear that most of this leader is not necessary for control by OmrA/B, given that a deletion of nts –54 to –149 of the leader still gives good pairing.
regulation (Figure 4, deletion Δ1). It is therefore conceivable that, in addition to the effects of OmrA/B, other post-transcriptional controls regulate the expression of cirA via this leader region.

Yet another level of control is suggested by the surprising effect of the ‘up’ mutation (Figure 5): changing 4 out of the 6 nt between the SD sequence and the start codon of cirA increased the activity of the cirA-lacZ fusion by more than 8-fold. Preliminary data indicate that changing either the G or A just upstream of the AUG into an U is sufficient to increase the expression of cirA-lacZ by about 3-fold (our unpublished data). It seems therefore that the increase in expression could be due to the disruption of the GGAA sequence between nts –3 and +1, which resembles an SD sequence and may be used to initiate translation from an alternative start codon. Interestingly, this GGAA sequence is conserved in Salmonella and Klebsiella, and, in Yersinia, there is an AGAG sequence instead, that could also be considered as a potential SD sequence (Figure S1). Whether this model is true and whether it is a process regulating, rather than simply decreasing, cirA expression remains to be investigated.

Complexity of ompT regulation and mRNA turnover

ompT regulation by OmrA and OmrB is easily demonstrated at the mRNA level for the wt ompT message; mRNA disappears rapidly upon induction of the sRNA (Figures 1, 3 and 6). However, in experiments done with an ompT-lacZ fusion carrying the 5′ UTR and the first 30 nt of ompT, regulation by OmrA/B overproduction is poor (Figure 3A). Since this suggested the possibility that elements beyond nt +30 were required, control was analyzed using the chromosomal copy of ompT, either in its wt or Flag-tagged form. In contrast to the ompT-lacZ fusion, ompT mRNA downstream of nt +30 is present in these constructs, but may be modified by the insertion of the 24-nt Flag sequence. The accumulation of the Flagged proteins is clearly controlled by OmrA/B, both in the wt situation as well as when compensatory changes were introduced in ompT-flag mRNA and in OmrA/B (Figure 6B). However, compensatory changes did not restore the ability of OmrA/B to induce the degradation of ompTmut2 mRNA or ompTmut2-Flag mRNA (Figure 6C). In general, degradation of the ompT mRNA in response to the Omr RNAs was perturbed by every change we made. For instance, the ompT-Flag mRNA (not carrying a mutation) is poorly sensitive to degradation by wt sRNAs (Figure 6C).

The mut2 mutation, in which the fifth and sixth codons of ompT are changed from CUUCUG (Leu-Leu) to CUGUUC (Leu-Phe), also decreases the level of OmpT-Flag protein (Figure 6B, compare the two vector lanes). This is independent of OmrA/B, since the experiment was done in a ΔomrAB strain. This sequence is part of the signal sequence and it is possible that the mutation affects the efficiency of export of the resulting protein to the outer membrane. Thus, it seems likely that mRNA structure, stability, and translation efficiency are perturbed by the addition of a Flag tag and by the mut2 change, hinting at further complexity in the regulation of ompT.

It was shown previously that the control of the IICB\textsuperscript{Glc} inner membrane protein synthesis by SgrS sRNA required the membrane localization of the target-mRNA, presumably because this decreased ribosome loading on the mRNA, allowing the sRNA to successfully compete with the ribosome (47). Even though the localization process is somewhat different for outer membrane proteins such as OmpT, one can hypothesize that, in a similar way, the translation of ompT may be affected by sequences within the signal sequence in ways that perturb OmrA/B action. This could explain, at least in part, why OmrA/B only weakly decreases the expression of the ompT-lacZ fusion.

Autoregulation by OmrA and OmrB

The expression of several sRNAs is subject to feedback control. For instance, RybB sRNA, whose transcription is regulated by the alternative sigma factor sigmaE, indirectly downregulates the expression of sigmaE by decreasing the synthesis of several OMPs (48,49). Similarly, production of the Fur-regulated RybB sRNA represses the synthesis of iron-binding proteins, thereby increasing the cellular level of free iron, which will in turn repress the expression of the Fur regulon, which includes RybB itself (50). Another example comes from Vibrio cholerae, where the four redundant Qrr sRNAs inhibit expression of the HapR regulator, which indirectly represses synthesis of the Qrr (51). In our case, OmrA/B sRNAs negatively autoregulate their transcription by directly repressing the ompR-envZ operon that encodes their transcriptional activators. This feedback loop should limit the production of OmrA/B after induction of the EnvZ-OmpR two-component system. However, it was found that there was almost no change in the transcription of two members of the EnvZ-OmpR regulon, ompC and ompF, over a wide range of EnvZ and OmpR levels (52), and the physiological relevance of OmrA/B autoregulation remains therefore to be investigated.

Length of pairing regions

Our results show that relatively short regions of complementarity between OmrA/B and their targets are sufficient for translational control by these sRNAs in our experimental conditions. It is especially striking for cirA, where mutagenesis of the leader indicates that two stretches of 6 and 3 bp with the sRNA are sufficient for control. sRNA–mRNA interactions relying on very short regions of complementarity are not limited to the Omr sRNAs, since Aiba and co-workers (33) showed that, when mutagenizing the duplex between SgrS sRNA and ptsG mRNA, only 6 bp affected control when point mutants were introduced. One drawback of both our and Aiba’s group results is that the effect of mutations in either the sRNA or the target-mRNA was analyzed under conditions where the sRNA was overproduced, which could compensate for weaker interactions. Therefore, we cannot exclude that additional base-pairs contribute to the interaction with the targets when sRNAs are expressed at lower levels from the chromosome. In the future, it might be worthwhile to undertake the same kind of study after inducing the sRNA synthesis.
under conditions that would be closer to the physiological levels. It would also be interesting to determine whether having the pairing region at the very 5’ end of OmrA/B allows control through shorter base-pairing interactions.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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