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A unique mechanism regulating gene expression: Translational inhibition by a complementary RNA transcript (micRNA)

(Shine–Dalgarno sequence/initiation codon/outer membrane proteins/osmoregulation)

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ABSTRACT **The expression of the genes for the major outer membrane proteins OmpF and OmpC are osmoregulated. The *ompC* locus was found to be transcribed bidirectionally under conditions of high osmolarity and a 174-base transcript encoded upstream of *ompC* was found to inhibit the OmpF production and to substantially reduce the amount of the *ompF* mRNA. This RNA [mRNA-interfering complementary RNA (micRNA)] has a long sequence that is complementary to the 5′ end region of the *ompF* mRNA. We propose that the micRNA inhibits the translation of the *ompF* mRNA by hybridizing with it. This RNA interaction may cause premature termination of the transcription of the *ompF* gene or destabilization of the *ompF* mRNA or both.**

The major outer membrane proteins of *Escherichia coli* OmpF and OmpC are essential proteins that function as passive diffusion pores for small, hydrophilic molecules (1). These proteins are encoded by the structural genes *ompF* and *ompC*, which map at 21 and 47 min on the *E. coli* chromosome, respectively (2). The expression of these genes is regulated by the osmolarity of the culture medium (3, 4). As the osmolarity of the medium increases, the OmpF production decreases, while the OmpC production increases so that the total amount of the OmpF and OmpC proteins is constant (5). This osmoregulation is controlled by another unlinked locus, *ompB*, which maps at 74 min (6, 7). The *ompB* locus contains two genes, *ompR* and *envZ*. The DNA sequences of both genes have been determined and their gene products have been characterized (8, 9). The EnvZ protein is assumed to be a membrane receptor protein that serves as an osmosensor and transmits the signal from the culture medium to the OmpR protein. The OmpR protein then serves as a positive regulator for the *ompF* and *ompC* expression (4). Recently, the *ompF* (10) and *ompC* (11) genes were subjected to sequence analysis. During the course of the characterization of the *ompC* gene, we found evidence for a unique regulatory mechanism of gene expression mediated by a new species of RNA called mRNA-interfering complementary RNA (micRNA). The micRNA is produced from an independent transcriptional unit (the *micF* gene). This gene is located immediately upstream of the *ompC* gene but is transcribed in the opposite direction. The 174-base micRNA appears to block the translation of the *ompF* mRNA by hybridizing to it. Because the production of micRNA is assumed to be proportional to the production of *ompC* mRNA, this regulatory mechanism appears to be a very efficient way to maintain a constant total amount of OmpF and OmpC proteins.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* MC4100 (F^- *lacY169 araD139 rspL thiA tibB relA ompB*$^+$), MH1160 [*ompB101* (*ompR1*), mutant from MC4100], MH760 [*ompB427* (*ompR2*), mutant from MC4100], MH1461 [*tpoll* (*envZ*), mutant from MC4100] (5, 6), SB4288 (F^- *recA thi-7 relA mal24 spc12 supE-50* Δ *proB-lac*), and plasmids pMY111 (11), pKM004, and pKM005 (12) were used.

Other Materials and Methods. Cells (10-ml culture) were grown in a nutrient broth (Difco) to a late logarithmic phase at 37°C. Total membrane fractions were prepared as described (13). Outer membrane proteins were obtained by sodium lauryl sarcosinate treatment (14). $NaDodSO_4$/polyacrylamide gel electrophoresis was carried out as described (15). The DNA sequence was determined according to the method of Maxam and Gilbert (16). S1 nuclease mapping was carried out according to Berk and Sharp (17). RNA transfer blot hybridization was performed according to Rave *et al.* (18).

RESULTS AND DISCUSSION

A DNA Fragment Suppressing *ompF* Expression. While characterizing the *ompC* promoter, we found that a DNA fragment of ≈300 base pairs (bp), located upstream of the *ompC* promoter, inhibited the production of OmpF protein when $OmpF^+$ cells were transformed with a multicopy plasmid harboring this DNA fragment. For this experiment, plasmid pMY150 was constructed from the original *ompC* clone, pMY111 (11), by changing the *Hpa* I sites of pMY111 to *Xba* I sites followed by removal of the 1.1-kilobase (kb) *Sal* I fragment (Fig. 1*a*). The resulting plasmid pMY150 (Fig. 1*b*), contains the entire *ompC* coding region and ≈500 bp of upstream sequences, including the *ompC* promoter and the DNA encoding the 5′-end untranslated region of *ompC* mRNA. The *ompC* promoter fragments of different sizes were obtained as illustrated in Fig. 1*b*, which were subsequently transferred to a promoter-cloning vector, pKM005 (12). These experiments revealed that transcription of the *ompC* gene initiates at a site located between 390 and 440 bp downstream from the upstream *Xba* I site (originally *Hpa* I site). Surprisingly, *E. coli* transformed with these pKM005 derivatives, including the clone of the shortest *Xba* I fragment of only 300 bp, CX28, lost the ability to produce OmpF protein. The $NaDodSO_4$ gel pattern of the membrane proteins of the cells harboring the plasmid containing the CX28 fragment (plasmid III in Fig. 1*c*) is shown in Fig. 2. OmpF protein was clearly produced in the host cells (*ompB*$^+$ *ompF*$^+$ *ompC*$^+$) (Fig. 2, lane 1), while in the same cells carrying the clone of the CX28 fragment the production of OmpF protein was almost completely blocked (Fig. 2, lane 4). The same effect could be observed with cells harboring a clone of a longer fragment such as plasmid I in Fig. 1*c* (Fig. 2, lane 2). In this clone the *lacZ* gene was fused immediately after the initiation codon of the *ompC* gene, resulting in the $LacZ^+$ phenotype of the cells carrying this plasmid. However,

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Abbreviations: micRNA, mRNA-interfering complementary RNA; bp, base pair(s); kb, kilobase(s).

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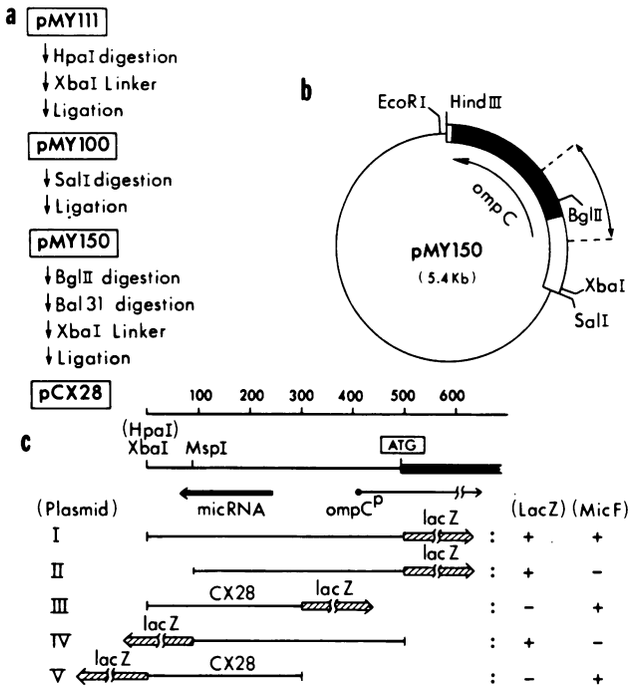

FIG. 1. Construction of the subclone of the *ompC* gene and various plasmids carrying the *ompC* promoter region. (*a*) Schematic presentation of the subcloning of the *ompC* gene. Plasmid pMY111 carrying a 2.7-kb *E. coli* chromosomal DNA in pBR322 was described previously (11). The plasmid (1 μg of DNA) was digested with *Hpa* I and religated in the presence of an *Xba* I linker (C-T-C-T-A-G-A-G, 150 pmol). Thus, the ≈400-bp *Hpa* I fragment was removed and a unique *Xba* I site was newly created (pMY100). Plasmid pMY100 (1 μg of DNA) was further digested with *Sal* I and religated to remove a 1.1-kb *Sal* I fragment (pMY150). To obtain *ompC* promoter fragments of different sizes, plasmid pMY150 was digested by BAL31 nuclease after cleavage of the unique *Bgl* II site (see *b*). Subsequently, the plasmid was religated in the presence of *Xba* I linkers. Plasmid pCX28, thus constructed, is one of the clones carrying an *Xba* I–*Xba* I fragment of ≈300 bp in length as shown in *b*. (*b*) Simplified restriction map of the plasmid pMY150 carrying the entire *ompC* gene. The 1.8-kb *Hin*dIII–*Sal* I fragment (boxed region) in pBR322 contains the entire *ompC* coding region as well as the 5′- and 3′-noncoding region. Transcription of the *ompC* gene proceeds in the direction shown by an arrow. A bidirectional arrow indicates the approximate deleted region (*ca.* 600 bp) for plasmid pCX28. (*c*) Various β-galactosidase (*lacZ*) gene fusions to the DNA fragments derived from the *ompC* promoter and its upstream region. Plasmid I, a 507-bp *Xba* I–*Rsa* I fragment, was isolated from pMY150 (an *Rsa* I site is present just downstream of the ATG codon) and inserted between *Xba* I–*Sma* I sites of plasmid pICIII, which is derived from plasmid pINIII carrying the *lacZ* gene (12). During the ligation, a *Hin*dIII linker was inserted between the *Rsa* I and *Sma* I ligation site. The *Xba* I–*Hin*dIII fragment was then reinserted into plasmid pKM005 (12) to create a *lacZ* gene fusion in the right reading frame. Plasmids II and IV carry the ≈430-bp *Msp* I–*Bam*HI fragment that was isolated from clone I (a *Bam*HI exists downstream of the ATG codon for the β-galactosidase coding sequence in plasmid I) and treated with S1 nuclease to crease blunt ends. After adding *Xba* I linkers at both ends, the fragment was inserted into plasmid pKM005 at its *Xba* I site in the possible two orientations. To construct plasmids III and V, an ≈300-bp *Xba* I–*Xba* I fragment was isolated from plasmid pCX28 (*a*) inserted into plasmid pKM005 at its *Xba* I site in the two possible orientations. These plasmids (I–V) were transformed into a *lacZ* deletion strain SB4288, and those β-galactosidase activities were tested on MacConkey plates (Difco). Results are shown as $LacZ^+$ or $LacZ^-$. Ability of these clones to inhibit the expression of OmpF protein are also shown as $MicF^+$ or $MicF^-$ on the basis of results from Fig. 2.

when the *Xba* I–*Msp* I fragment of 87 bp was removed from plasmid I, the cells carrying the resulting plasmid (plasmid II) were able to produce OmpF protein (Fig. 2, lane 3). It should be mentioned that a similar DNA fragment of 430 bp

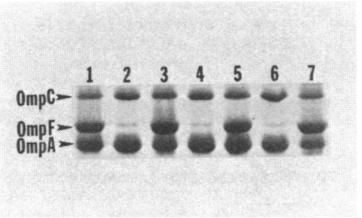

FIG. 2. $NaDodSO_4$/polyacrylamide gel electrophoresis of outer membrane proteins from cells carrying various plasmids. Strain SB4288 is carrying the following plasmids: lane 1, no plasmid; lane 2, plasmid I in Fig. 1*c*; lane 3, plasmid II in Fig. 1*c*; lane 4, plasmid III in Fig. 1*c*; lane 5, plasmid IV in Fig. 1*c*; lane 6, plasmid V in Fig. 1*c*; and lane 7, plasmid $pOmpF^P$-A1 (unpublished data), which contains the ≈430-bp promoter region of the *ompF* gene (including the first three amino acid residues of pro-OmpF protein) fused to the *lacZ* gene. Outer membrane proteins were solubilized with a 1% $NaDodSO_4$ solution. Samples (15 μg of protein) were subjected to urea/$NaDodSO_4$/polyacrylamide gel electrophoresis (7 M urea/8% acrylamide; ref. 15). Gels were stained by 0.1% Coomassie brilliant blue in 10% acetic acid/25% isopropyl alcohol.

in length containing the upstream region of the *ompF* gene did not block the production of either OmpF or OmpC protein (Fig. 2, lane 7).

DNA Sequence Homology Between CX28 and the *ompF* Gene. The results described above demonstrate that the stretch of DNA ≈300 bp long, located upstream of the *ompC* promoter, is able to block *ompF* expression. To elucidate the function of this DNA fragment (CX28), the DNA sequence of this region was determined. Fig. 3 shows the DNA sequence of 500 bp from the *Xba* I site (originally *Hpa* I) to the initiation codon, ATG, of the *ompC* gene. It was found that the sequence from residues 99 to 180 (Fig. 3) has 70% homology with the 5′ end region of the *ompF* mRNA (10), which includes the Shine–Dalgarno sequence, the initiation codon, and the codons for the first nine amino acid residues of pro-OmpF protein (bases marked by + are homologous to the *ompF* sequence). A plausible model to explain the above result is that the 300-bp CX28 fragment contains a transcription unit that is directed towards the region upstream of the *ompC* gene so that the RNA transcript from this region has a sequence complementary to the *ompF* mRNA. Presumably, the hybridization between the two RNAs thus blocks the production of OmpF protein.

Existence of a New Transcription Unit. To determine whether the CX28 fragment contains an independent transcription unit oriented in a direction opposite from the *ompC* gene, the *lacZ* gene was fused as two different sites within the CX28 fragment. In plasmid V, the CX28 fragment was inserted in the opposite orientation with respect to plasmid III (Fig. 1*c*). This clone was still fully active in suppressing the production of OmpF protein (Fig. 2, lane 6), although it did not produce β-galactosidase ($LacZ^-$) (see Fig. 1*c*). When the fusion junction was shifted to the *Msp* I site at nucleotide 88 (Fig. 3; also see Fig. 1*c*), the newly constructed clone (plasmid IV) was capable of producing β-galactosidase but could not suppress the production of OmpF protein (Fig. 2, lane 5). On the other hand, plasmid V was fully active in the suppression of OmpF protein production. These results demonstrate that there is a transcription unit in the CX28 fragment that is independent from the *ompC* gene promoter and that the CX28 fragment and the *ompC* gene are transcribed in divergent directions. The fact that plasmid IV can produce β-galactosidase and plasmid V cannot indicates that the CX28 transcription unit terminates between residues 1 and 88 (Fig. 1*c*). In fact, a very stable stem-and-loop structure can form between nucleotides 70 and 92 (arrows with letter a in Fig. 3), which is followed by oligo(T). This structure is characteristic of ρ-factor-independent transcription

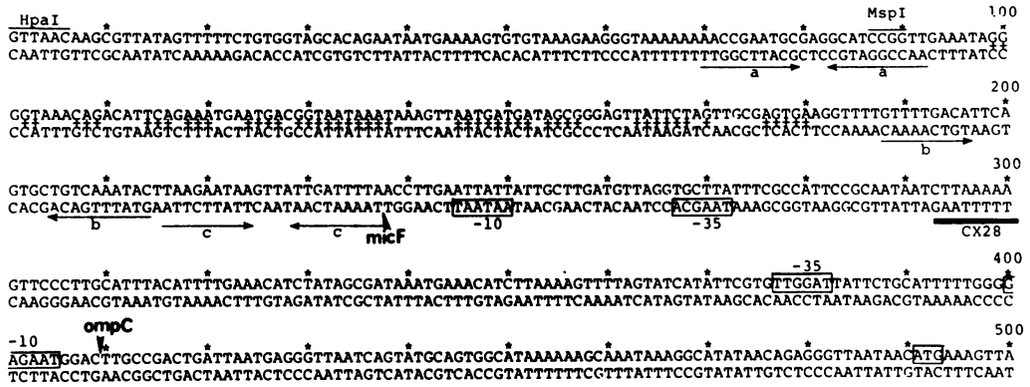

FIG. 3. Nucleotide sequence of the promoter region and upstream of the *ompC* gene. Restriction DNA fragments prepared from pMY111 or pMY150 were labeled at their 3′ end by the method of Sakano *et al.* (19). The DNA sequence was determined by the method of Maxam and Gilbert (16). The RNA polymerase recognition site (−35 region) and the Pribnow box (−10 region) for the *ompC* and *micF* promoter as well as the initiation codon of the *ompC* gene are boxed. The transcriptional initiation sites determined by S1 nuclease mapping for the *ompC* and *micF* genes (see Fig. 4) are indicated by a large arrow. Arrows with small letters (a–c) represent three inverted repeat sequences. Plus signs (+) between the two strands (nucleotides 99–180) indicate sequences homologous to the nucleotide sesquence of the *ompF* gene (10). The region where an *Xba* I linker was inserted in pCX28 (see Fig. 1*a*) is indicated by a thick bar with CX28. The exact location of the linker was not determined.

termination sites in prokaryotes (20). The ΔG value for this structure was calculated to be −12.5 kcal according to Salser (21).

The initiation site for the CX28 transcript was positioned at nucleotide 237 (Fig. 3) by S1 nuclease mapping, as shown in Fig. 4*a*, indicating that the CX28 transcript consists of 174 nucleotides. This was further proven by RNA transfer blot hybridization, as shown in Fig. 4*b*. In the RNA preparation extracted from cells carrying plasmid III (Fig. 1*c*), an RNA species, which migrated approximately as a 6S RNA, hybridized with the CX28 fragment (Fig. 4*b*, lane 2). In the control cells, no such RNA was detected (Fig. 4*b*, lane 1). When the same filter was hybridized with a probe specific for the *ompF* mRNA, the amount of the *ompF* mRNA was substan-

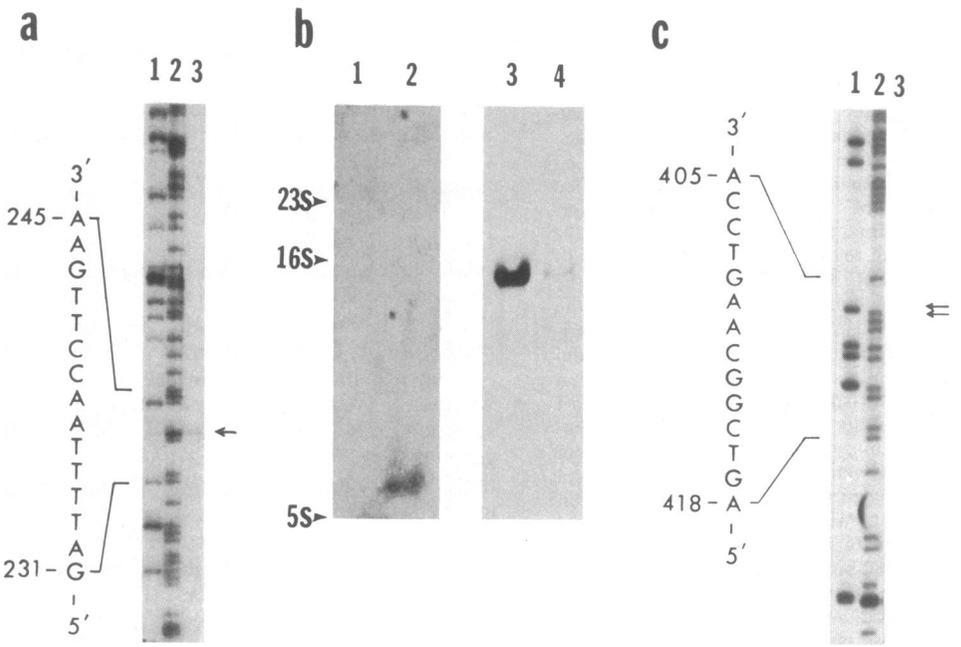

FIG. 4. S1 nuclease mapping of *micF* RNA and *ompC* mRNA and RNA transfer blot hybridization of *micF* RNA. (*a*) S1 nuclease mapping of *micF* RNA. Total cellular RNA was prepared (22) from *E. coli* MH1461 grown in M9 medium (23) supplemented with 0.2% glucose/0.1% Casamino acids. The *Msp* I–*Bam*HI fragment, ≈430 bp in length, from plasmid II (see Fig. 1) was ^{32}P-labeled at the 5′ end of the *Msp* I site and used to map the transcripts. The *Msp* I site between nucleotides 87 and 90 is shown in Fig. 3. The results were analyzed on a 10% acrylamide/7 M urea gel. An arrow indicates the major protected band in lane 3. Lane 1, the G sequencing reaction (16); lane 2, the A+G sequencing reaction. The nucleotide sequence of the *micF* gene around the protected region is shown to the left. The nucleotide numbers correspond to those in Fig. 3. (*b*) RNA transfer blot hybridization of *micF* RNA and *ompF* mRNA. The 300-bp *Xba* I–*Xba* I CX28 fragment from plasmid III (see Fig. 1) and the 513-bp *ompF* gene fragment (from residues 42–554 of the *ompF* gene; see ref. 10) that encompasses the 5′-end noncoding region of the *ompF* and the coding region for the first 33 amino acid residues were nick-translated with α-^{32}P-labeled dNTP and used as the probes. RNA from *E. coli* SB4288 (lane 1) RNA from *E. coli* SB4288 carrying plasmid III (lane 2) were hybridized with the ^{32}P-labeled CX28 fragment. The same filter was used after 3 months for hybridization with the ^{32}P-labeled *ompF*-specific probe. Lanes 3 and 4 correspond to lanes 1 and 2, respectively. Arrows indicate the hybridized bands. The positions of 23S, 16S, and 5S RNA were determined by staining the gel with ethidium bromide. (*c*) S1 nuclease mapping of *ompC* mRNA. The experiment was performed as described for *a*, except that the 510-bp *Xba* I–*Bam*HI fragment from plasmid I (see Fig. 1), ^{32}P-labeled at the 5′ end of the *Bam*HI site, was used to map the transcripts. The same RNA preparation was used for both *a* and *c*.

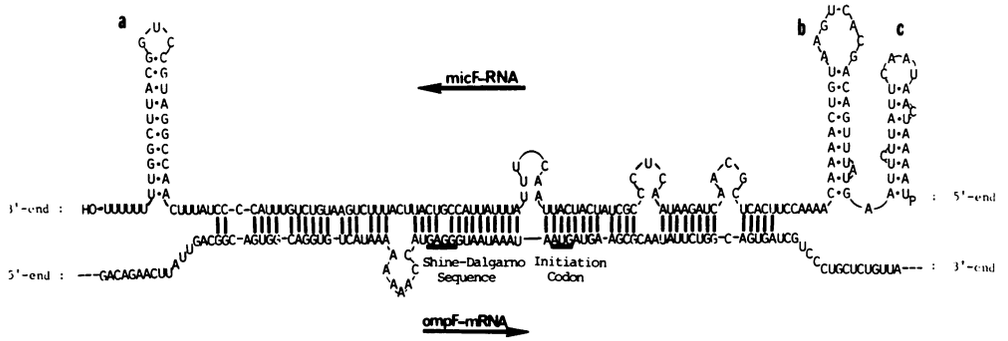

FIG. 5. Hybrid formation between *micF* RNA and *ompF* mRNA. The sequence of *micF* RNA corresponds to the sequence from residues 237 to 64 in Fig. 3. The *ompF* mRNA sequence was cited from Inokuchi *et al.* (10). The ΔG values for the secondary structures a, b, and c were calculated to be −12.5, −4.5, and +2.9 kcal, respectively (21).

tially reduced in the cells carrying the CX28 fragment (Fig. 4*b*, lane 4).

Function of the CX28 RNA. The CX28 RNA is unlikely to produce a protein, because it has no open reading frame preceded by an initiation codon and a Shine–Dalgarno sequence. On the other hand, because the CX28 DNA fragment has extensive homologies with a portion of the *ompF* gene, the CX28 RNA can form a very stable hybrid with the *ompF* mRNA, as shown in Fig. 5. Forty-four bases of the 5′-end untranslated region of the *ompF* mRNA, including the Shine–Dalgarno sequence and 28 bases from the coding region, are involved in the hybrid formation. This hybrid structure is sandwiched by the two stable stem-and-loop structures of the CX28 RNA: the ρ-independent transcription termination signal of the 3′ end (loop a) and the other at the 5′ end (loop b). The ΔG values for loops a and b were calculated to be −12.5 and −4.5 kcal, respectively (21). Loop c is unlikely to be formed because of its ΔG value (+2.9 kcal). We propose that the formation of the hybrid primarily blocks the translation of *ompF* mRNA, which may also result in reduction of the amount of the *ompF* mRNA in the cells because of coupling between translation and transcription and/or because of destabilization of the mRNA due to the formation of the hybrid. This would explain why clones carrying the CX28 DNA fragment suppress the production of OmpF protein (Fig. 2) as well as reducing the amount of the *ompF* mRNA (Fig. 4*b*). Thus, CX28 RNA is designated as the micRNA for *ompF* and the gene is designated *micF*. It should be noted that when loop a was eliminated by fusing the *micF* gene with the *lacZ* gene, the MicF function was abolished (plasmid IV, Fig. 1*c*). This may be due to the instability of the *micF* RNA or, alternatively, due to the requirement of loop a for the MicF function.

To examine whether the *micF* gene is under the control of the *ompB* locus, various *lacZ* clones were therefore put into three different *ompB* mutants. The *lacZ* gene under *micF* promoter control (plasmid IV) produces β-galactosidase in the same manner as the *lacZ* gene under *ompC* promoter control (plasmid I): high β-galactosidase activity was found in both the wild-type and *envZ*$^-$ (phenotypically OmpF$^-$ OmpC$^+$) strains, but very low activity ($\approx$1/15th of the wild-type activity) was observed in *ompR1*$^-$ (OmpF$^+$ OmpC$^-$) and *OmpR2*$^-$ (OmpF$^+$ OmpC$^-$) mutants (data not shown). On the other hand, the *lacZ* gene under the control of the *ompF* promoter was not expressed in the *ompR1*$^-$ cells. In addition, the *lacZ* gene under the control of the lipoprotein promoter, used as a control, was expressed in all strains (data not shown). These results indicate that the *micF* gene is regulated by the *ompB* locus in the same fashion as the *ompC* gene. It is interesting to note that the *lacZ* gene under the control of the *ompF* promoter was constitutively expressed at a somewhat lower level in the *envZ*$^-$ (OmpC$^+$ OmpF$^-$) strain (data not shown). This suggests that the *OmpF*$^-$ phenotype of this *envZ*$^-$ strain is due to the inhibition of translation of the *ompF* mRNA by micRNA and that the complementary hybridization between the *micF* RNA and the mRNA for the *ompF* and *lacZ* fusion is not stable enough to block the translation of the mRNA.

Promoters of the *micF* and *ompC* genes. Because both the *micF* and *ompC* genes appear to be regulated by the *ompB* locus, the promoters of these genes should have sequence homologies. As shown in Fig. 4*c*, major transcription initiation was found to take place at the T residues at positions 410 and 411 (Fig. 3; also see Fig. 6). Thus, −10 regions for the *micF* and *ompC* genes are assigned as A-A-T-A-A-T (nucleotides 250–245) and G-A-G-A-A-T (nucleotides 400–405 in Fig. 3), respectively (Fig. 6), both of which show good homology to the consensus sequence, T-A-T-A-A-T (24). RNA polymerase recognition sites (−35 regions) for the *micF* and *ompC* genes are also assigned as T-A-A-G-C-A and T-T-G-G-A-T, respectively (Fig. 6), both of which show 50% homology to the consensus sequence, T-T-G-A-C-A (24). However, no significant sequence homologies are found between the *micF* promoter of 63 bp (nucleotides 300–238) and the *ompC* promoter (nucleotides 301–409 in Fig. 3). On the other hand, homologous sequences are found in the 5′ end regions of both of the transcripts as shown in Fig. 6. Twenty-eight of 44 bases are homologous (64% homology), and these regions are probably the sites recognized by the OmpR protein, a positive factor (5).

CONCLUSION

Regulation of gene expression in *E. coli* is generally controlled at the level of transcription. It has been well established that expression of some genes is suppressed by their specific repressors or activated by their specific inducers (20, 21, 24, 25). Positive protein factors such as cAMP receptor protein (26) and OmpR protein (5) are also known to reg-

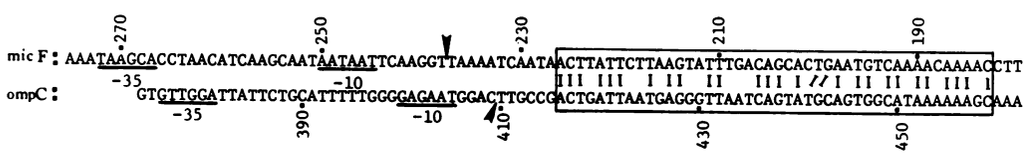

FIG. 6. Homologous sequences between the *micF* and the *ompC* genes. Nucleotide numbers correspond to those in Fig. 3. The boxed sequences show the homologous sequences between the two genes. Bars between the two sequences indicate the identical bases. The arrows indicate the transcription initiation sites determined in Fig. 4.

ulate gene expression at the level of transcription. Another transcriptional regulatory mechanism is attenuation, which plays an important role in controlling expression of operons involved in the biosynthesis of various amino acids of other compounds (27). In addition, some proteins have been shown to regulate gene expression at the level of translation (28). The present results demonstrate regulation of bacterial gene expression at the level of translation by means of an RNA factor complementary to the translational start region. The possibility that micRNA directly blocks the expression of the *ompF* gene at the level of transcription has not been ruled out. However, this is highly unlikely because the *lacZ* gene fused with the *ompF* promoter (*lacZ* was fused at the 4th amino acid residue of pro-OmpF proteins) was expressed in the *envZ*$^-$ cells. The substantial reduction in the amount of the *ompF* mRNA observed in the cells producing the *micF* RNA (Fig. 4*b*) may, therefore, occur because (*i*) the block of *ompF* mRNA translation due to the hybrid formation causes premature termination of *ompF* transcription or (*ii*) the hybrid formation destabilizes the *ompF* mRNA or both.

Regulation by micRNA appears to be an extremely efficient way to block production of a specific protein without hampering other protein production. In particular, the *micF* system is considered to be playing an important role in maintaining the total amount of OmpC and OmpF proteins constant. Recently, we have constructed a mic system for the *E. coli lpp* gene under the *lac* regulation. When the production of the micRNA complementary to *lpp* mRNA was induced by a *lac* inducer, the lipoprotein production as well as the amount of the *lpp* mRNA was substantially reduced (unpublished data). This result indicates that the mic system is not specific for the *ompF* gene and that an artificial mic system can be a very effective method for blocking the formation of a specific protein. Furthermore, it is a unique and powerful method to study the functions of unknown or essential genes (or both) in various organisms.

It seems reasonable that the micRNA regulatory system may be a general regulatory phenomenon in *E. coli* and in other organisms, including eukaryotes. In this regard, it is interesting to note that a similar mechanism has been proposed to explain a translational block in a mutant of bacteriophage (T7 (29). RNA species may have additional roles in the regulation of various cellular activities. In fact, small RNA species have been shown to be involved in the regulation of DNA replication of some plasmids (30, 31).

A preliminary report on the present results has been published elsewhere (32). After this research was completed, Simons and Kleckner (33) reported that translation of the "transposase" mRNA of transposon Tn*10* is inhibited by direct pairing with a small RNA of 180 bases in length. In this case, however, the two RNA species are transcribed in opposite directions, from the two overlapping genes such that the first 36 bases of the two RNAs are completely complementary to each other.

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