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# The isolation and characterization of RNA coded by the *micF* gene in *Escherichia coli*

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## ABSTRACT

A new species of micF RNA, which contains 93 nucleotides (a 4.5S size), was isolated from Escherichia coli. The sequence of the 4.5S micF RNA corresponds to positions G82 through U174 of the micF gene. The 5' terminal end of this smaller micF RNA is triphosphorylated signifying that it is a primary transcript. Its promoter region, which is situated within the greater micF structural gene, has been identified and characterized by lacZ fusion analysis. A 6S micF RNA species, which has a base composition predicted for a transcript from the full length gene has also been detected; however, the 4.5S micF RNA is the predominant species. The work clearly shows by biochemical identification the presence of chromosomally encoded micF RNA.

## INTRODUCTION

The expression of the E. coli outer membrane proteins, OmpC and OmpF, is regulated such that the total amount of OmpC and OmpF in the membrane remains constant while the level of each varies in response to the osmolarity of the growth medium. Two cellular proteins, OmpR and EnvZ, are known to participate in this regulation at the level of transcription (1,2). In addition to these two proteins, Mizuno et al. (3) proposed the existence of an anti-sense RNA, micF RNA in E. coli, that inhibits the translation of the OmpF mRNA through complementary base pairing to the message. This was based on the finding that the expression of the OmpF protein was suppressed by placing the micF gene on a multicopy plasmid. Subsequently, Matsuyama and Mizushima (4) reported that the deletion of the micF gene from the E. coli chromosome had little apparent effect on OmpF expression. Recently, however, Mizuno, Mizushima and their coworkers have discovered in a more detailed analysis of this micF deletion strain that the response to changes in the osmolarity of the medium with respect to OmpF protein production is significantly slower than in the wild type (T. Mizuno, personal communication).

Here we report the isolation and characterization of ($^{32}$P) labeled micF RNA both from E. coli cells having only the chromosomal micF gene and from cells having the micF gene amplified on a multicopy plasmid. The major species is a primary transcript (4.5S in size) which originates from only a part of the full length micF gene. This

is the first isolation and characterization of an RNA coded by an E. coli chromosomal gene which is believed to function as a regulatory antisense RNA.

## MATERIALS AND METHODS

Plasmid construction. To construct pAM336 (Figure 1),the XbaI fragment approximately 300 base pairs in length containing micF was isolated from CX28 (3) and inserted into the XbaI site of pUC18 (5). A derivative was isolated which contains three micF genes and designated pAM336. The orientation and number of micF inserts were determined by digestion with restriction enzymes. $Amp^r$, lac I', po, and lac Z' designate the ampicillin resistance gene, part of the lacI gene, the lac promoter/operator, and part of the lacZ gene, respectively, which were derived from pUC18.

Growth of cells and labeling of RNA. In vivo labeled RNA was produced by the addition of 25 mCi of [$^{32}$ P] inorganic orthophosphate during growth of one liter of E. coli JA221 / F'$LacI^q$ (hsdR leuB6 lacY thi recA $\Delta$trpE5 / F'$lacI^q$ $lac^+$ proAB) (6) in low phosphate media (7) containing 0.3 M NaCl. Total RNA was isolated from E. coli by lysis of cells with hot Holmes-Bonner solution containing sodium dodecyl sulfate and 7M urea (3) followed by phenol extraction and chromatographed on a Sephadex G-100 column to segregate 4S to 10S size RNAs.

For the in vitro labeling of micF RNA ten liters of E. coli cells harboring the plasmid pAM336 were grown in LB medium with high salt (1% bactotryptone, 0.5% yeast extract, 0.3 M NaCl) to $A_{550}$= 0.9. Low molecular weight RNAs were prepared from these cells as described above. RNA was 5'-labeled in vitro by the treatment of low molecular weight RNAs (1 mg) with alkaline phosphatase (1mg), followed by phosphorylation of the RNAs with polynucleotide kinase (100 units) using 5 mCi ATP-$\gamma$ [$^{32}$ P].

Preparation of DNA and filters for hybridization. The preparation of the minus strand of the restriction fragment containing micF DNA and filters for hybridization of micF RNA was as follows. Three tandem repeats of the XbaI restriction fragments containing the micF gene (3) were inserted into double-stranded M13 mp18 DNA at the Eco R1 and Pst I sites that inactivate the $\beta$ -galactosidase gene (LacZ). The double-stranded M13 containing the micF genes was then used to transfect E. coli JM 103 competent cells. Colorless plaques were picked from agar plates containing IPTG and Xgal and the phage from these plagues were used to infect liquid cultures of E. coli JM 103. Double-stranded M13 DNA was isolated from each culture and double checked by gel analysis of DNA fragments produced from restriction enzyme digestions in order to assure the maintenance of the inserted genes. Single-stranded DNA bacteriophage was isolated from the

supernatant of the infected cultures by precipitation with polyethylene glycol. The samples were phenol extracted and ethanol precipitated. The purified DNA was adsorbed onto nitrocellulose filters and immobilized onto the filters by subsequent baking at 80° C. for two hours (8,9).

In vivo labeled low molecular weight RNAs (one billion counts) or in vitro 5' labeled low molecular RNAs were resuspended in 50% formamide, 0.6 M NaCl, 0.1 M PIPES pH 6.6 and incubated with the filters at 50° C. for 16 hours. The hybridization solution was removed from the filter and a series of high salt and low salt washes were performed before micF RNA was eluted from the filter. The bound RNA was eluted in 1 ml of deionized water by heating the filter at 80 ° C for 5 minutes (9). Subsequently the RNA in the eluant and low salt washes were separately precipitated in ethanol each with 25µg of tRNA carrier. These precipitates and less than 1 µl of the hybridization solution and high salt washes were electrophoresed on denaturing 6% polyacrylamide gels containing 7M urea and a buffer consisting of 50mM Tris-borate pH 8.3 and 1mM EDTA (TBE) along with RNA markers. The RNA bands were visualized by autoradiography of the gel (Figure 2). Using the autoradiogram as a template, the RNA bands were excised from the gel and the RNAs extracted by soaking the gel slice overnight at 37° C in a salt solution containing sodium dodecyl sulfate (10). The extracted RNA was subsequently precipitated in ethanol with 25 µg of tRNA carrier and dried in a vacuum.

In vivo uniformly labeled micF RNA samples extracted from polyacrylamide gels were digested to completion with RNase $T_2$ and nucleotides were separated by chromatography on polyethyleneimine thin layer plates (10). [$^{32}$P] containing spots were removed from the plates with the use of an autoradiogram as a template and counted with a liquid scintillation counter (Table I). Samples were counted to at least a total of 5,000 CPM. The same counting method was used to measure the radioactivity of undigested samples.

lacZ fusion analysis. lacZ was fused at different sites of the micF gene. For this purpose, three new restriction sites were created in the micF region by site-specific mutagenesis. Gene fusion was carried out with a promoter proving vector, pKM005 (11). Plasmids I and II were constructed from pAM331, on which new XbaI sites were created respectively; an upper XbaI site for plasmid I and a lower site for plasmid II. pAM331 was derived from pJDC406 (12) to which the CX28 fragment (3) was inserted at a unique SmaI site. In the case of plamid III, the promoter region for the 6S RNA was deleted using Bal31 from the newly created NruI site immediately upstream of the 6S RNA promoter. This treatment deleted the 6S RNA promoter up to a G residue 42 bases upstream of the gene. The lacZ gene was then fused at the MspI site which was converted to an XbaI site. E. coli SB4288

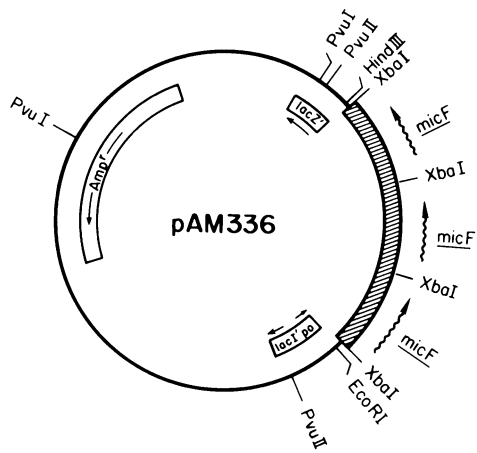


Figure. 1. Plasmid pAM336 showing three tandem repeats of micF. The thick shaded bar designates the three Xba I fragments containing the micF genes. The regions corresponding to the 6S micF gene shown in Fig. 6 are indicated by wavy arrows.

lacZ (3) was transformed with these plamids, and β-galactosidase activity was then measured for each plasmid.

## RESULTS

The strategy used to isolate micF RNAs in sufficient quantities for characterization was 1) to amplify the micF gene by constructing a plasmid containing multiple copies of the micF gene; 2) to purify low molecular weight RNA from E. coli cells harboring this plasmid and 3) to separate [$^{32}$ P] labeled micF RNA from low molecular weight RNAs by filter hybridization.

The multicopy plasmid, pAM336, was constructed by the insertion of three tandem copies of an XbaI fragment (approximately 300 base pairs) containing the micF gene (3), into the XbaI site of pUC18 (5) (Figure 1). Plasmid carrying E. coli strain JA221 (6) clones were selected by resistance to ampicillin. These clones were grown under steady state high osmolarity conditions.

To isolate micF RNA, in vivo [$^{32}$ P] labeled low molecular weight RNAs were incubated with nitrocellulose filters where M13 phage containing three tandem repeats of the coding strand of the micF gene had been immobilized. Gel electrophoresis of the eluant from the filter revealed isolates of more than one size, i.e., 6S, 4.5S and 4S fractions (Figure 2). The 6S micF RNA is detectable only in overexposed gels (Figure 2C). The sizes of the isolates agree with what is observed in the Northern hybridizations using the same gel system (Figure 3).

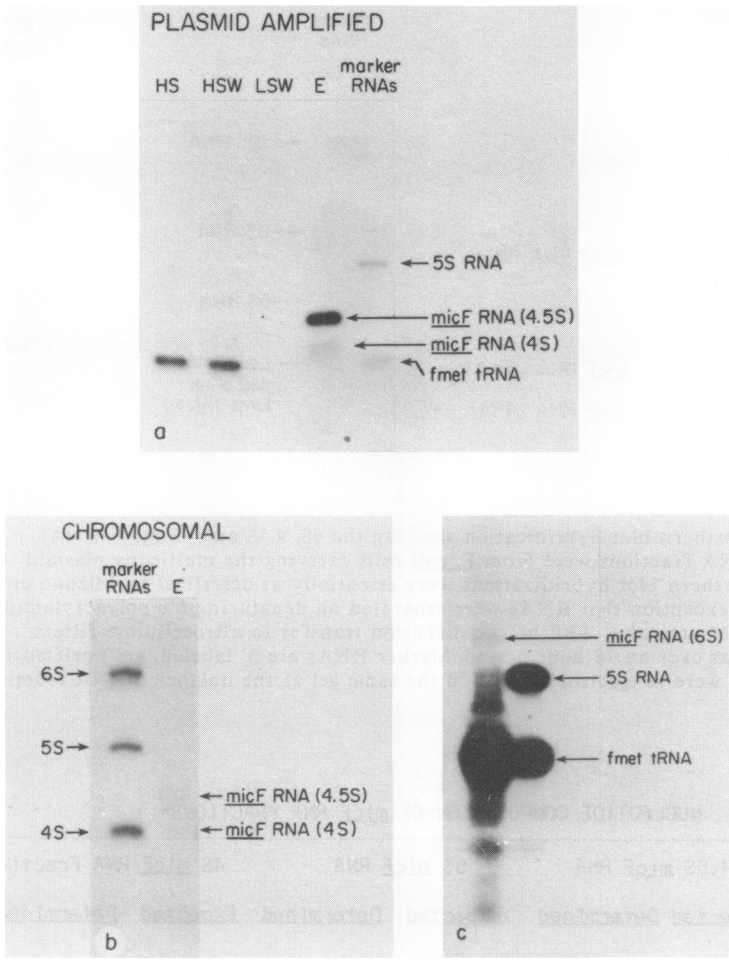


Figure. 2. Autoradiograms of 6% polyacrylamide-7M urea gels showing the electrophoresis patterns of [$^{32}$P] uniformly-labeled micF RNA fractions which were isolated by filter hybridization (8,9). (A), Autoradiogram showing in vivo labeled micF RNA fractions isolated from cells containing plasmid pAM336 (30 minute exposure). The mobilities of [$^{32}$P] labeled marker 5S ribosomal RNA and fmet tRNA are shown. HS, an aliquot of hybridization solution after incubation with the filter; HSW, an aliquot of the high salt (0.6 M NaCl) wash of the filter; LSW, low salt (0.06 M) wash of the filter; E, eluant containing micF RNA species (B). Autoradiogram showing in vivo labeled micF RNAs isolated from cells containing only chromosomally coded micF genes (18 hour exposure) (C). An overexposure of the gel shown in (A) revealing the 6S micF RNA band. (The 4.5S RNA band had been excised from the gel before the 16 hour exposure.) Marker 5S RNAs refer to 3' labeled E. coli 6S RNA (Brownlee, (7)), 5S ribosomal RNA, and fmet tRNA. E, eluant containing micF RNA species after high and low salt washes of the filter as described in (A).

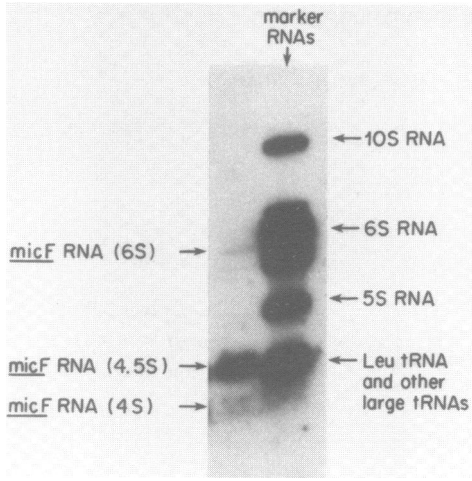


Figure. 3. Northern blot hybridization showing the 6S, 4.5S and 4S micF RNA fractions. RNA fractions were from E. coli cells carrying the multicopy plasmid pAM336. Northern blot hybridizations were essentially as described by Mizuno et al. (3) with the exception that RNAs were separated on denaturing 6% polyacrylamide gels containing 7M urea and TBE before diffusion transfer to nitrocellulose filters. Diffusion was over an 18 hour period. Marker RNAs are 3' labeled, gel purified RNA samples that were re-electrophoresed on the same gel as the unlabeled RNA fractions being tested.

TABLE 1

NUCLEOTIDE COMPOSITION OF micF RNA FRACTIONS*

| | 4.5S micF RNA | | 6S micF RNA | | 4S micF RNA Fraction | |
|---|---|---|---|---|---|---|
| | Expected | Determined | Expected | Determined | Expected | Determined |
| Gp | 13% | 11% | 11% | 42% | 15% | 28% |
| Ap | 19% | 19% | 29% | | 17% | |
| Cp | 23% | 22% | 23% | 19% | 23% | 21% |
| Up | 45% | 48% | 37% | 39% | 45% | 51% |

* In vivo uniformly labeled micF RNA fractions were isolated by filter hybridization and gel purified (see Figure 2). Details of digestions can be found in material and methods. The data represent the averages of two seperate determinations. For 6S and 4S micF fractions, nucleotides Ap and Gp separated poorly.

Base composition analyses of the in vivo labeled 6S RNA suggest that this isolate is a transcript of the full length gene (Table I). Base composition analyses of the 4.5S and 4S isolates suggest that they correspond in sequence to the 3' portion of the micF gene. These fractions of micF RNA are high in U content and low in G and A content. Fingerprint analyses using two-dimensional homochromatographic separation of RNase T1 digests of the 4.5S and 4S isolates show similar patterns except that the 4S RNA fingerprint has missing oligonucleotides that appear on the fingerprint of the 4.5S RNA (data not shown). Sufficient radioactive counts were not obtained for further analysis of the oligonucleotides. Initially the 4.5S and 4S isolates were considered to be break-down products from

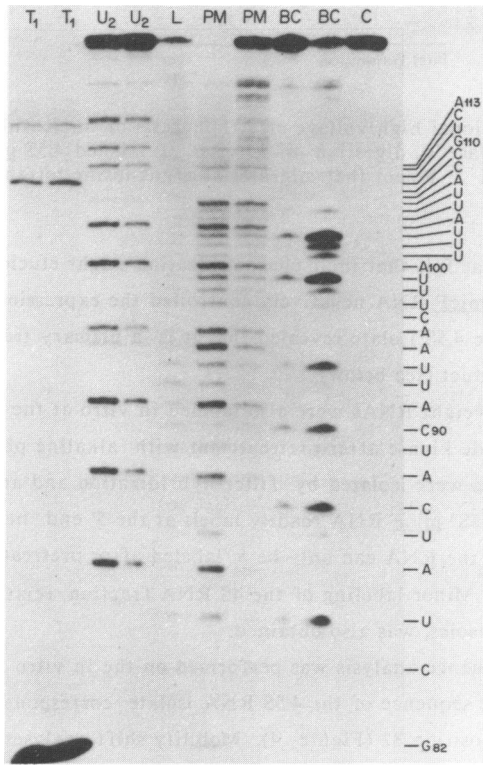


Figure 4. Autoradiogram of a 20% polyacrylamide sequencing gel showing the separation by electrophoresis of partial nuclease digests of 5' end-labeled 4.5S micF RNA (13,15). Lanes $T_1$, $U_2$, PM, and BC are partial digestions with RNases $T_1$, $U_2$, Phy M and B. cereus, respectively. Lane C is the undigested control. Lane L was the ladder generated by partial alkaline hydrolysis of labeled RNA.

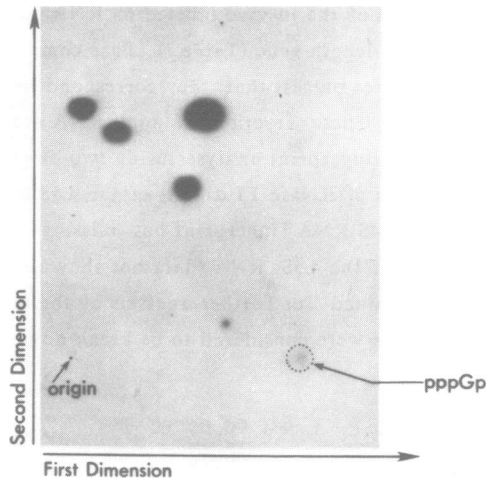


Figure. 5. Two dimensional high voltage electrophoresis of nucleotides resulting from the complete RNase $T_2$ digestion of in vivo $^{32}P$ labeled 4.5S micF RNA. The bent arrow points to a $^{32}P$ spot that migrates as a guanosine tetraphosphate (see text).

the 6S RNA and it was felt that their characterization might elucidate the mechanism by which micF RNA negatively controlled the expression of OmpF. Further characterization of the 4.5S isolate revealed that it is a primary transcript and not a break-down product (see below).

Low molecular weight RNAs were also labeled in vitro at the 5' end using ATP-$\gamma$ [$^{32}P$] and polynucleotide kinase after pretreatment with alkaline phosphatase (13). The micF RNA species were isolated by filter hybridization and analyzed by gel electrophoresis. The 4.5S micF RNA readily labels at the 5' end but the end is phosphorylated since the RNA can only be 5 'labeled after pretreatment with alkaline phosphatase. Minor labeling of the 4S RNA fraction, relative to the labeling of the 4.5S species, was also obtained.

Nucleotide sequence analysis was performed on the in vitro 5' end labeled micF 4.5S RNA (13,14). The sequence of the 4.5S RNA isolate corresponds to the micF gene sequence starting at position 82 (Figure 4). Mobility shift analyses (15,16) and 5' end nucleotide determinations (10) confirm that the 5' end begins at position G82 (data not shown). The in vitro labeling of the 4.5S micF RNA at the 3'-end with T4 RNA ligase and [32P] pCp revealed that the terminal nucleoside is uridine.

End-labeling of the 6S micF RNA at either the 5' or 3' ends was undetectable. This may be due to the low abundance of 6S micF RNA. The 4S RNA isolate labels at its 5' end to the same extent with and without pretreatment with alkaline

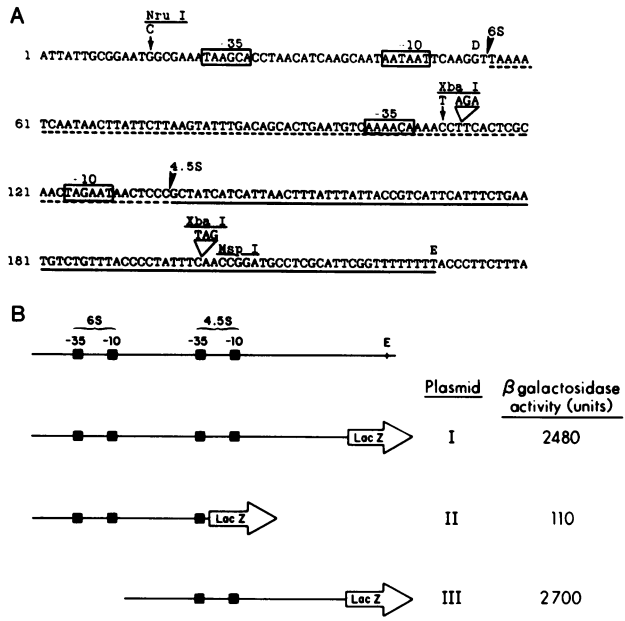


Figure 6. A. The DNA sequence of the micF gene. Boxes indicate -10 and -35 promoter regions; thick arrows, transcription initiation; E, transcription termination; thin arrows, base substitutions; triangles, positions of base insertions; and D, 3' border of the deletion for the NruI site. The 4.5S transcript is underlined by a continuous line and the 6S transcript by both broken and continuous lines. B. Schematic representation of lacZ gene fusion. Continuous lines correspond to the micF gene sequences and black boxes correspond to -10 and -35 promoter regions represented in panel A.

phosphatase. It is likely to be a breakdown product of 4.5S or 6S micF RNA and its 5' end is believed to start at A100 on the micF gene (data not shown).

In order to determine if the 5' end of the 4.5S RNA is triphosporylated, in vivo [$^{32}$P] labeled 4.5S RNA was completely digested with RNase T2 and the products of the digestion were analyzed by two-dimensional high voltage electrophoresis (17). These products were separated electrophoretically on cellulose acetate strips in the first dimension, transferred to DEAE- cellulose thin layer plates and, in the second dimension, separated by ionophoresis. In both dimensions, a [$^{32}$P] containing spot was found to have the mobility similar to that of guanosine tetraphosphate (17). In addition, transfers from cellulose acetate strips following high voltage electrophoresis were also made onto polyethyleneimine thin layer plates and samples were chromatogrammed in ammonium formate, pH 3.5 for the second dimension (Figure 5). Again, the [$^{32}$P] containing spot which migrates rapidly in the first dimension (approximately 1.7 times that of Up) remains at the origin of

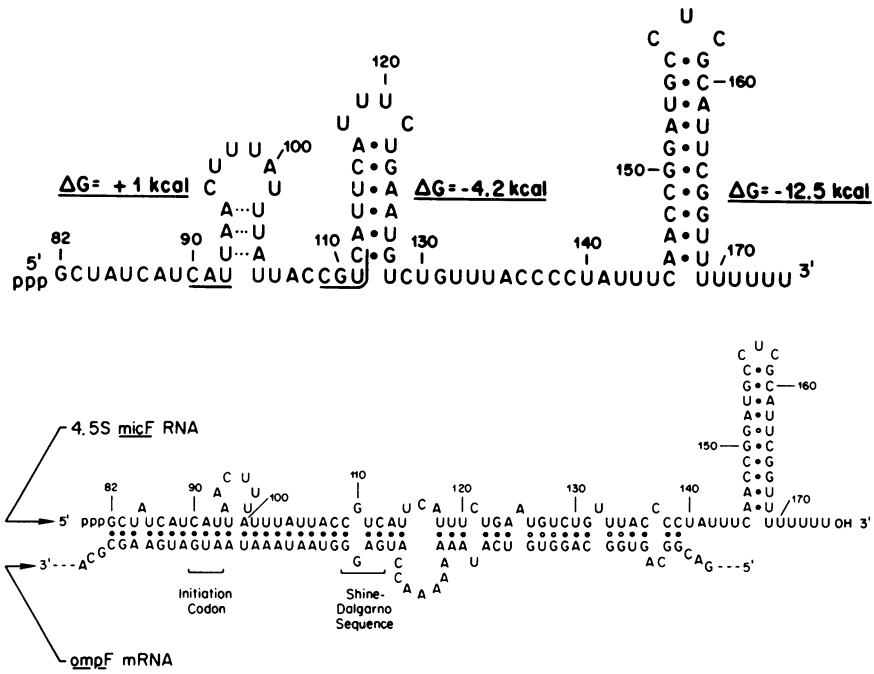


Figure 7. Top. Proposed secondary structural model for the 4.5S micF RNA showing that only two stable hairpins can be formed (Δ G's were calculated according to Tinoco et al (18). Underlined nucleotides are those that are complementary to the AUG condon and partially complementary to the Shine-Dalgarno sequence on the ompF mRNA. Bottom. Proposed complementary binding of the 4.5S micF RNA to the OmpF messenger RNA. Modified from Mizuno et al (3). G82 is the determined 5' end of 4.5S micF RNA. A100 is proposed as the 5' start of the 4S micF RNA fragment. The 6S micF RNA is believed to start at U1 according to previous work (3).

the second dimension. These data indicate that the 5' terminal end of the 4.5S micF RNA is triphosphorylated. We conclude that the 4.5S RNA is a primary transcript.

To characterize the micF promoter regions, lacZ was fused at different sites of the micF gene (Figure 6A and Material & Methods). Figure 6B shows the β -galactosidase levels found for cells harboring the three resulting plasmids. The basal level for the control vector, pKM005 was 25 units, which was substracted for each estimate. The results indicate that the 4.5S promoter (plasmid I and III) is about 25 times stronger than the 6S promoter (plasmid II). This agrees well with the relative amounts of 4.5S and 6S RNA found in the cell (see discussion).

The yield of 4.5S micF RNA was determined by comparisons of recovered [$^{32}$P] radioactivity in micF fractions from nitrocellulose filters relative to recovered

5S ribosomal RNA. Approximately 1-2 copies/cell of 4.5S micF RNA was obtained where micF is coded only by the chromosomal genes in cells grown under the above mentioned conditions. Rehybridization experiments using new filters containing bound micF genes showed that the filter capacity was not limiting since only 10-20% additional micF RNA was recovered after rehybridization.

## DISCUSSION

The work described here shows the isolation of a new transcript of micF RNA from E. coli, the 4.5S species. This RNA has nucleotide segments that are complementary to the regions surrounding the Shine- Dalgarno and initiation codon sequences on the ompF mRNA (Figure 7) and therefore has the potential to participate in the hypothesized mechanism of translational control proposed by Mizuno et al. (3). The 4.5S micF RNA species appears to have little possible secondary structure of its own (Figure 7) to interfer with its interaction with the ompF mRNA, especially in the region that has complementarity with the message.

Surprisingly, the ratio of the 6S to 4.5S micF RNA is approximately 0.01 under the growth conditions used in the present experiment. The significance of the low abundance of the 6S species relative to the 4.5S and the unusual feature of the apparent presence of two micF RNA transcripts is unknown but may be related to complex functions of the micF gene products. The 6S and 4.5S micF RNAs both have complementarity to a portion of the OmpF messenger RNA. However, the 5' end of the 6S micF RNA also shows homology to the OmpC messenger RNA (3).

Of interest are the promotor sequences upstream from position G82 at the -10 region (TAGAAT) and the -35 region (AAAACA) which can function as a transcriptional promotor for the 4.5S micF RNA (Figure 6A). lacZ fusion experiments confirm that a promotor exists in this region of the DNA even when the promotor for the 6S micF RNA is deleted. In addition, the 6S promoter was found to be approximately 30 fold weaker than the 4.5S promoter. These data are consistent with the results showing the presence of a triphosphate on the 5' terminal end of the 4.5S micF RNA and correlate well with the relative levels of 6S to 4.5S micF RNAs.

The function of the micF gene in osmoregulation has been investigated by gene deletion studies. Matsuyama and Mizushima (4) constructed a deletion mutant lacking the chromosomally coded micF and reported that under steady-state conditions, the expression of OmpF protein appeared similar to that in wild type cells. However, a large difference in the rate at which ompF is regulated was found between wild type cells containing the micF gene and the micF deletion mutant (T. Mizuno, personal communication). In another study, the deletion of the micF and ompC region of the chromosome resulted in the expression of ompF that was partially constitutive and the deletion mutant produced an ample amount of ompF protein under high osmolarity

(19). The present work substantiates the presence of cellular micF RNA transcripts in E. coli and thus reaffirms the potential function of the RNA in the regulation of OmpF.

In this study, the yield obtained for the 4.5S micF RNA is a few copies/cell in cells having only chromosomally coded micF and grown under steady state growth conditions. The levels of micF RNA, however, vary with cell growth conditions (unpublished data, J. Andersen, K. Zhao, M. Inouye, and N. Delihas). The levels of the OmpF protein are controlled not only by osmolarity but by other factors including temperature (20). We are now determining the cellular expression of the micF gene as a function of the temperature and osmolarity of the growth media.

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