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micF RNA Binds to the 5′ End of *ompF* mRNA and to a Protein from *Escherichia coli*†

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ABSTRACT: *micF* RNA regulates the levels of outer membrane protein F (OmpF) in *Escherichia coli* in response to temperature increase and other stress conditions by decreasing the levels of *ompF* mRNA (Andersen et al., 1989). A 93-nucleotide *micF* RNA was synthesized in vitro directly from polymerase chain reaction generated DNA which was designed to contain a functional T7 RNA polymerase promoter upstream of the *micF* RNA gene and an appropriate restriction site for transcription termination. A transcript (150 nucleotides) containing the ribosomal binding domain of *ompF* mRNA messenger was synthesized in vitro from the *ompF* gene cloned into a T7 expression vector. A stable duplex was formed between *micF* RNA and the 150-nucleotide 5′ transcript of *ompF* mRNA after incubation at 37 °C in a physiological buffer. The melting curve of the duplex formed by *micF* RNA and 150-nucleotide transcript revealed a T_m of 56 °C and a ΔT_m that spans about 20 °C; both are consistent with the proposed structure for the *micF*/*ompF* duplex. In addition, as determined by competition studies and UV cross-linking/label-transfer analyses, an *E. coli* protein was found to bind specifically to *micF* RNA. The protein also bound weakly to the 150-nucleotide *ompF* transcript. The data are the first to demonstrate the complex between *micF* RNA and the 5′ end of *ompF* mRNA and suggest that in vivo a *micF* ribonucleoprotein (RNP) particle may participate in the destabilization *ompF* mRNA during thermoregulation of OmpF porin.

The outer membrane protein OmpF, a major porin protein of *Escherichia coli*, is regulated in response to changes in temperature, osmolarity, and other stress conditions during growth [Lugtenberg et al., 1976; Hall & Silhavy, 1981; Andersen et al., 1989; see Forst and Inouye (1988) for a review]. It has been shown that chromosomally derived 4.5S *micF* RNA plays an essential role in the thermal regulation of OmpF (Andersen et al., 1989). The 93-nucleotide *micF* RNA represses OmpF synthesis by decreasing the levels of *ompF* mRNA (1.1 kb) in response to temperature increase as well as to the other stress-related factors (Andersen et al., 1989). While *micF* RNA is necessary for the observed decreased levels of *ompF* MRNA, it is not sufficient; another factor, possibly a cognate protein, was deduced to participate with *micF* RNA in the regulation of the messenger's levels (Andersen et al., 1989).

Although the *micF* RNA gene is at 48′ on the *E. coli* chromosome and distal from the *ompF* gene at 21′ (Inokuchi et al., 1982; Mizuno et al., 1983; Bachmann, 1987), *micF* RNA is believed to function as a natural antisense RNA against the mRNA for OmpF. Consistent with its hypothesized role as an antisense RNA, the suppression of OmpF via *micF* RNA has been shown to occur at a posttranscriptional level (Misra & Reeves, 1987; Cohen et al., 1988), and *micF* RNA can significantly inhibit translation of *ompF* mRNA if overexpressed (Mizuno et al., 1983, 1984; Andersen et al., 1989). A model of *micF* RNA/*ompF* mRNA has been proposed (see Figure 1). In this model, the primary sequence of *micF* RNA shows extensive complementarity with the 5′ end of *ompF* mRNA in and around its ribosome binding domain; however, the complementarity is imperfect since there are several looped-out positions and non-Watson–Crick base pairing in the proposed duplex (Mizuno et al., 1984; Andersen et al., 1987).

Since *micF* RNA is found in low levels in the cell (Andersen et al., 1987), it is difficult to obtain in sufficient quantities for in vitro studies. In a novel approach, polymerase chain reaction (PCR) was used to generate DNA from which bona fide *micF* RNA could be synthesized by T7 RNA polymerase. Using T7-synthesized RNAs, we show that *micF* RNA hybridizes to the 5′ end of *ompF* mRNA and forms a stable duplex. We also show the *micF* RNA binds specifically to a protein from *E. coli*, suggesting that a *micF* RNP particle may regulate the stability of *ompF* mRNA.

Experimental Procedures

Polymerase Chain Reaction Generated DNA. The PCR technique (Mullis & Faloona, 1987) was used to introduce a T7 RNA polymerase promoter upstream of the *micF* RNA gene and to create a restriction site for run-off transcription at the end of the gene (Figure 2A). Due to paucity of restriction sites around the *micF* RNA gene, this novel method was chosen in lieu of conventional cloning to position the

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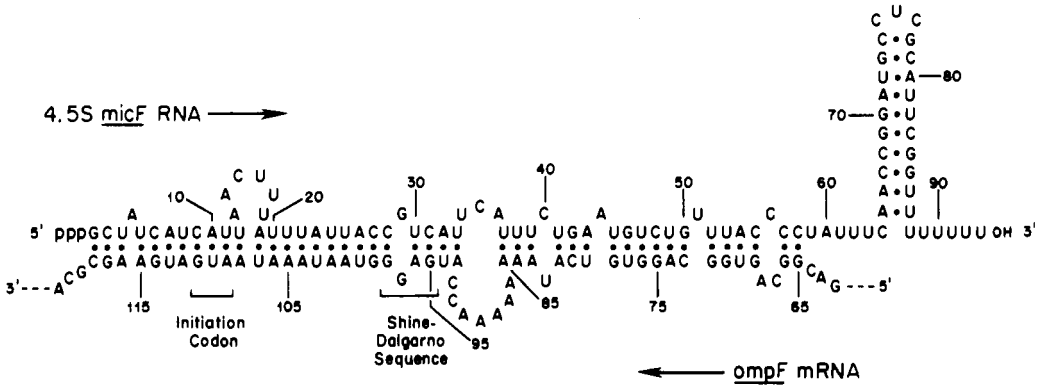

FIGURE 1: Proposed secondary structural model of the duplex formed by *micF* RNA and *ompF* mRNA around the region of the initiation codon and Shine–Dalgarno sequence on the messenger (Mizuno et al., 1984; Andersen et al., 1989). The top strand shows the complete sequence of *micF* RNA, and the bottom strand shows the sequence of the 5′ end of *ompF* mRNA from positions 62 to 121.

promoter upstream of the gene and create a site for the run-off transcription so that bona fide *micF* RNA could be synthesized by T7 RNA polymerase in vitro. A similar PCR strategy was employed to obtain sufficient transcripts for reverse transcription reactions in a technique called genomic amplification with transcript sequencing (GAWTS) (Stoflet et al., 1988). As in GAWTS, it was not necessary to clone the resulting PCR DNA in order to transcribe its *micF* RNA gene with T7 RNA polymerase.

A 300 bp restriction fragment carrying the *micF* RNA gene (the CX28 fragment; Mizuno et al., 1984) was cut from plasmid pAM336 (Andersen et al., 1987) with *Xba*I restriction endonuclease, and the DNA fragment was gel-purified on low melting point (LMP) agarose. The DNA was extracted from the agarose using Elutip-D columns according to the direction from the manufacturer (Schleicher & Schuell).

Two oligonucleotides were designed to be used in the PCR reaction along with the gel-purified 300 bp restriction fragment carrying the *micF* RNA gene. Two restriction sites (*Eco*RI and *Hin*dIII) were built into the DNA sequence as potential cloning sites. Also, at the 5′ ends of each oligonucleotide, two extra nucleotides were added beyond the restriction site sequence in an effort to avoid interference with restriction cutting of the PCR DNA which might result from "breathing" at the ends of the double-stranded DNA.

The sequence of the 5′ oligonucleotide (51-mer) is 5′-GCgaattcCGAAATTAATACGACTCACTATAG**GCTATCATCATTAACTTTA**-3′. It contains (5′ to 3′) an *Eco*RI sequence (lower case), the consensus T7 RNA polymerase promoter sequence (underlined) plus 6 upstream nucleotides (Ikeda & Richardson, 1986), an extra G residue to assure accurate transcription initiation, and the sequence of the first 19 nucleotides from the noncoding strand [(+) strand] of *micF* gene sequence (boldface letters).

The sequence of the 3′ oligonucleotide (36-mer) is 5′-ATaagctt**aaaAAAAACCGAATGCGAGGCATCCGG**-3′. It contains (5′ to 3′) overlapping *Hin*dIII and *Dra*I sequences in antisense orientation (lower case) followed by 17 nucleotides complementary to the noncoding strand [(+) strand] at the 3′ end of the *micF* gene (boldface letters). The *Dra*I sequence also overlaps A residues that appear in the *micF* sequence. Cutting the PCR-generated DNA with *Dra*I, a blunt-end cutter, results in run-off transcription at the last A/T base pair of the *micF* gene. Since cloning of the PCR product is not necessary in order to use the template DNA in in vitro transcription reactions, the sequence of the 3′ oligonucleotide may be abbreviated to only those sequences complementary to the noncoding strand. PCR T7 template has been made that synthesizes correct-size U6 snRNA using an abbreviated 3′ oligonucleotide (Andersen and Zieve, unpublished results).

The 5′ and 3′ oligonucleotides were made on an Applied Biosystems DNA synthesizer, Model 380B, processed according to the manufacturer's directions and gel-purified on a thin sequencing-type polyacrylamide gel containing 50 mM Tris–borate, pH 8.3, 1 mM EDTA (TBE), and 7 M urea. Purified oligomer concentration was determined from A_{260} readings using an estimated extinction coefficient of 25 μg/mL for $A_{260} = 1$.

The PCR reaction was done according to the instructions from the Perkin Elmer Cetus GeneAmp DNA amplification kit on a PCR machine from the same company, and all reagents used in the reaction, other than the restriction fragment and the oligonucleotides, came from the purchased kit. The appropriate annealing temperature was estimated from the number of G/C (4 °C per bp) and A/T (2 °C per bp) base pairs formed between the oligomers and the CX28 restriction fragment, and a temperature about 5 °C less than the estimated lowest value was chosen as the annealing temperature for the reactions, in this case, 43 °C. Default parameters of Perkin-Elmer Cetus program 3 were used for the chain reaction except the program was modified to deliver an annealing temperature of 43 °C and, due to the small size of the expected product (~130 bp), to allow polymerization for only 30 s. One nanogram of the CX28 restriction fragment was sufficient to obtain about 4–5 μg of finished product under these conditions.

ompF Gene Cloned into T7 Expression Vector. The *ompF* gene was cloned into a T7 expression vector by conventional cloning techniques (Maniatis, 1982) as outlined in Figure 2B. Plasmid pGR201 (Ramakrishnan et al., 1985) was first cut with *Eco*RI and *Ava*I restriction endonucleases. After phenol/chloroform extraction and ethanol precipitation, the cut DNA was subjected to Klenow treatment so that *Bam*HI linkers (8-mers) could be ligated to the resulting blunt ends. The ligated linkers were subsequently restricted with *Bam*HI endonuclease and kinased with T4 polynucleotide kinase and ATP before the DNA was purified by electrophoresis on LMP agarose. A 2.6-kb restriction fragment was extracted from the gel by using Elutip-D columns and subsequently ligated into the prepared T7 expression vector, pET7 (Rosenberg et al., 1987). Plasmid pET7 had been prepared by restriction with *Bam*HI, treatment with bacterial alkaline phosphatase, and gel purification. Ligation occurred overnight at 14 °C using T4 DNA ligase.

The resulting plasmid, pJAN010, was subsequently cut with *Stu*I and *Xba*I blunt-end restriction endonucleases, and gel-purified. Religation of the large restriction fragment resulted in a second plasmid, pJAN011, which has a functional T7 promoter and two initiating G residues (from the *Stu*I cut)

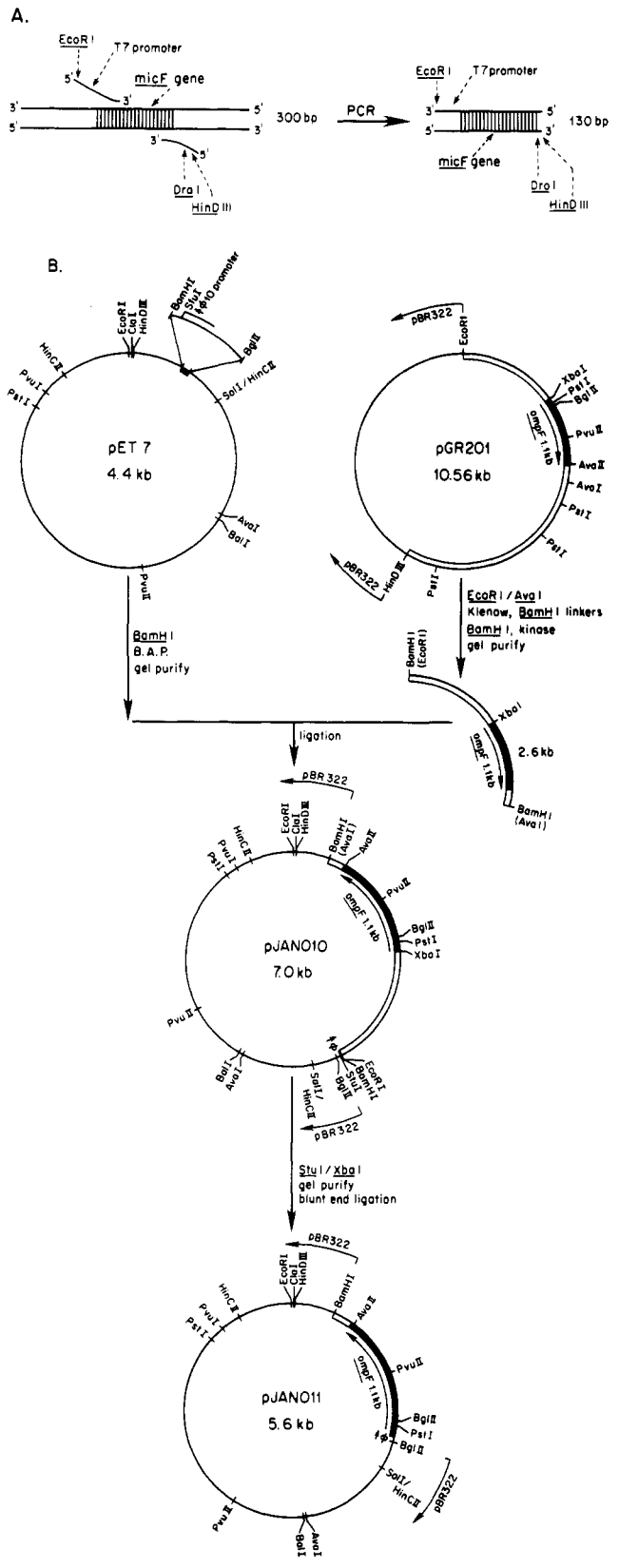

FIGURE 2: Schematics of strategies employed to create T7 expression systems for *micF* RNA and *ompF* mRNA. (Panel A) PCR strategy which created DNA having a functional T7 promoter upstream of the *micF* RNA gene and a usable restriction site, *Dra*I, for run-off transcription termination so that bona fide *micF* RNA could be made in vitro. (Panel B) Strategy for cloning the *ompF* gene into the T7 expression vector pET7 (see Experimental Procedures for details).

directly upstream of the structural *ompF* gene (see Figure 2B). The sequence at the border of the T7 promoter (underlined) and the *ompF* gene is 5′-TAATACGACTCACTATAG-GAGA-3′.

In Vitro Transcription with T7 RNA Polymerase. The PCR-generated DNA was digested with *Dra*I restriction endonuclease, phenol/chloroform-extracted, and ethanol-precipitated. About 0.5 μg of labeled *micF* RNA resulted when 40 ng of the cut DNA was incubated at 37 °C for 60 min in a transcription buffer (Krupp & Söll, 1987) containing 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 500 μM each of ATP, GTP, and CTP, 40 μM UTP, 10 μCi of [α-^{32}P]UTP (at 3000 Ci/mmol), and 0.08 μg/μL T7 RNA polymerase, which had been prepared as previously described (Van Der Werf et al., 1986). To obtain unlabeled transcripts, 500 μM UTP was substituted for the ^{32}P-labeled UTP.

"Full length" *ompF* mRNA was synthesized by T7 RNA polymerase from plasmid pJAN011 (Figure 2B) which had been cut with *Ava*II. *Ava*II cuts 15 base pairs before the end of the structural *ompF* gene, and so a potential terminal hairpin in the natural mRNA is incomplete in the T7-synthesized product. The "full length" *ompF* mRNA transcript was determined to be unstable under the experimental conditions used. To prepare for transcription of the 150-nucleotide 5′-end *ompF* transcript, plasmid pJAN011 was linearized with restriction enzyme *Bgl*II, phenol/chloroform-extracted, and ethanol-precipitated. The transcription reactions were performed as described above using 100 ng of the prepared plasmid in each case.

A 70-nucleotide transcript containing the 5′ end of poliovirus (type 1, Mahoney strain) was synthesized from plasmid pT7PV1-5 (Van der Werf et al., 1986) linearized with restriction endonuclease *Kpn*I. *Xenopus borealis* somatic (Xbs) 5S RNA was also made by the T7 expression system using pT75S (gift from Drs. M. Sands and D. Bogenhagen) which is similar to the plasmid constructed by Romaniuk et al. (1987). The transcription reactions were as described above for pJAN011.

Transcription was terminated by the addition of 0.2 M KOAc, pH 6.5, and 2 volumes of ethanol. The precipitated RNA was denatured by heating at 50 °C for 5 min in a buffer containing 5 mM Tris–borate, pH 8.3, 1 mM EDTA, and 7 M urea and purified by gel electrophoresis on a 6% polyacrylamide gel containing TBE and 7 M urea. The RNA bands were excised from the gel using the autoradiogram as a template, and the RNA was eluted from the gel by vigorous shaking at 37 °C overnight in an SDS buffer (Donis-Keller et al., 1977) containing 7.5 μg of wheat germ tRNA. The eluted RNAs were ethanol-precipitated and redissolved in sterile double-deionized water.

Formation of micF/ompF Duplex. The duplex of *micF* RNA and *ompF* mRNA or the 150-nucleotide 5′ transcript was formed by incubating the transcripts in a physiological buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, and 25 mM KCl (TMK). Since the transcripts were labeled with [α-^{32}P]UTP and the 150-nucleotide transcript contained only one more uridine residue than the *micF* RNA transcript, molar ratios were estimated from counts per minute (cpm) of gel-purified transcripts. Initial incubations were done on ice (4 °C) for 1 h, at 37 °C for 1 h, or at 55 °C for 5 min and then slow cooled at 1 °C/min until 24 °C. Subsequent incubation were done only at 37 °C for 1 h. The resulting duplexes were separated from the unhybridized transcripts by nondenaturing gel electrophoresis at 4 °C on a 4% polyacrylamide gel containing 50 mM Tris–borate, pH 7.5, and 1 mM $MgCl_2$ (TBM), and discerned by autoradiography (Figure 4A). From the image on the autoradiograph, bands

were excised from the gel, and the RNAs were eluted and denatured as described above before being electrophoresed on a 6% polyacrylamide gel containing TBE and 7 M urea (Figure 4B), or the bands were dropped into scintillation cocktail and cpm determined (see below).

Melting Curve. *micF* RNA and the 150-nucleotide *ompF* transcript were incubated in TMK buffer at 37 °C for 1 h and then placed on ice. The incubation mixture was divided into aliquots. Each aliquot was heated for 3 min at one of several temperatures at 5 °C intervals between 35 and 65 °C and then placed back on ice. The heat-treated incubation mixtures were electrophoresed on a nondenaturing gel at 4 °C (Figure 5A). After autoradiography, duplex bands were excised from the gel, and the cpm of the bands was measured by scintillation counting for greater than 5×10^3 counts.

Identification of the micF Binding Protein. A 500-mL culture of *E. coli* SM3001, a *micF* deletion strain (Matsuyama & Mizushima, 1985), was grown at 37 °C to mid-log phase in L broth (Maniatis et al., 1982). This strain was used so as to eliminate competition for a putative *micF* RNA cognate protein by endogenous *micF* RNA itself. Pelleted cells were resuspended in 20 mM sodium phosphate, pH 7.1 (phosphate buffer), and cell integrity was disrupted by sonication on ice. Cell membranes were pelleted by centrifugation as described previously (Forst et al., 1989). The resulting supernatant was passed over a 1-mL DEAE column equilibrated with phosphate buffer. The column was washed with phosphate buffer, and proteins were eluted from the column in phosphate buffer containing 0.35 or 0.5 M NaCl. Protein concentrations of the flow-through and 0.5-mL fractions were determined by the method of Lowry et al. (1951). The *micF* RNA binding protein eluted in the first fractions after addition of phosphate buffer containing 0.35 M NaCl.

Approximately 25 ng of ^{32}P-labeled *micF* RNA and 375 ng of wheat germ tRNA (used in precipitation of labeled RNA) were incubated on ice in TMK buffer with 600 ng of protein from each column fraction that contained the most protein. The incubated mixtures were electrophoresed at 4 °C on a nondenaturing polyacrylamide containing TBE and viewed by autoradiography.

In addition, the fraction that contained the *micF* RNA binding protein was incubated with labeled *micF* RNA as described above, and the mixture was subjected to ultraviolet (UV) radiation for increasing times with the short-wave (254 nm) light on a hand-held UVP 254 mineral lamp (Model UVGL-25). The RNA/protein incubations were irradiated in microtiter dishes on ice at a distance of 1 cm for 30 s, and 1, 5, 10, and 15 min initially. Only the 15-min irradiation time was used in successive experiments. The irradiated samples were mixed with 1 mg/mL ribonuclease A, incubated at 37 °C for 30–45 min, and then boiled in standard Laemmli protein solubilizing buffer (Laemmli, 1970; Wilusz et al., 1988; Smith, 1976). Prestained proteins used as molecular weight markers were purchased from Sigma Chemical Co. Label transfer to the protein was analyzed by gel electrophoresis on 10% polyacrylamide–SDS Laemmli gels (SDS–PAGE) followed by autoradiography.

Results

In Vitro Transcription of micF and ompF Genes. *micF* RNA was synthesized from a PCR-generated piece of DNA containing a T7 RNA polymerase promoter, the *micF* RNA gene, and a *Dra*I restriction site for transcription termination (Figure 2A). Transcription of the PCR DNA cut with restriction enzyme *Dra*I resulted primarily in a 93-nucleotide RNA (Figure 3, lane 2) while transcription of uncut DNA

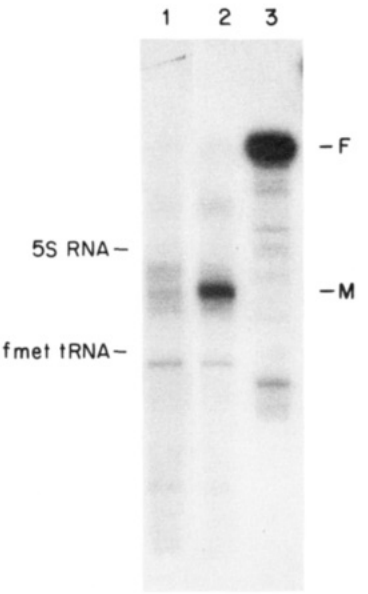

FIGURE 3: Gel purification of ^{32}P-labeled RNAs made from T7 expression systems of Figure 2. Transcripts made from (lane 1) uncut *micF* PCR DNA, (lane 2) *micF* PCR DNA which had been cut with *Dra*I, and (lane 3) pJAN011 containing *ompF* cut with *Bgl*II. Lines mark the mobility of bona fide *micF* RNA (M) in lane 2 and the 150-nucleotide 5′ transcript of *ompF* mRNA (F) in lane 3. As determined by UV shadowing, the mobilities of unlabeled marker RNAs, 5S RNA, and $tRNA^{Met/f}$ are also shown.

resulted in multiple RNA products, including a small amount of the 93-nucleotide RNA (Figure 3, lane 1). In addition, about 50% of the labeled transcripts from the uncut PCR template were retained at the origin (not shown in Figure 3). The synthesis of the 93-nucleotide RNA from the uncut DNA suggests that T7 RNA polymerase can recognize, albeit at low efficiency, the endogenous transcription termination signal (hairpin followed by U's) of the *micF* RNA gene. The RNA product from transcription was initially confirmed to be *micF* RNA by gel mobility in relationship to 5S RNA and $tRNA^{Met/f}$ and by Northern hybridization with a ^{32}P-labeled oligodeoxynucleotide complementary to 54 nucleotides in the central portion of *micF* RNA (Andersen et al., 1989). In addition, the 93-nucleotide RNA product was subsequently 3′ labeled (England & Uhlenbeck, 1978) and its sequence confirmed to be that of *micF* RNA by the RNA sequencing methods of Donis-Keller et al. (1977) (data not shown).

A 150-nucleotide transcript containing the 5′ end of *ompF* mRNA was synthesized from pJAN011 cut with *Bgl*II (Figure 3). The *ompF* transcript includes positions 65–118 which show the striking complementarity with *micF* RNA (see Figure 1). RNAs were gel-purified before being used in subsequent reactions (see Figure 3, lane 3).

Hybridization of micF RNA with the 5′ End of ompF mRNA. *micF* RNA and the 150-nucleotide transcript of *ompF* mRNA which contains the ribosome binding domain were labeled with [α-^{32}P]UTP and incubated in equimolar ratios in TMK buffer, a physiological buffer commonly used in the isolation of ribosomes. Several incubation parameters were used: on ice for 1 h, at 37 °C for 1 h, or at 55 °C for 5 min and then slow-cooled to 24 °C at about 1 °C/min. Electrophoresis of the incubation mixtures on a nondenaturing polyacrylamide gel showed a slower moving component in the incubations at 37 and at 55 °C/slow-cooled (Figure 4A, lanes 4 and 5) which was not present in the incubation mixture left on ice (Figure 4A, lane 3). When the slower moving components from each line were excised from the gel, and the RNAs were eluted and reelectrophoresed on a denaturing gel

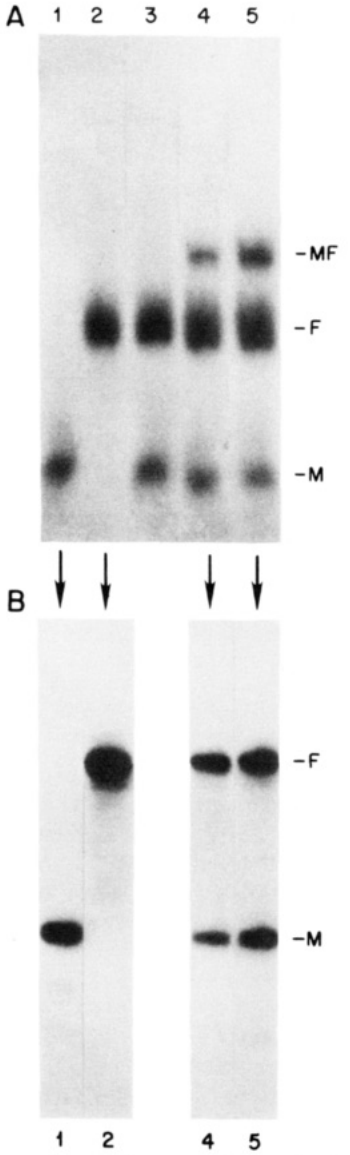

FIGURE 4: Formation of the *micF*/*ompF* duplex. (Panel A) Nondenaturing 4% polyacrylamide gel showing the duplex (MF) formed between *micF* RNA (M) and the *ompF* 5′-end transcript (F). Shown are *micF* RNA alone (lane 1), *ompF* 5′-end transcript alone (lane 2), *micF* RNA and the *ompF* 5′-end transcript after incubation on ice (lane 3), at 37 °C for 1 h (lane 4) or for 5 min at 55 °C and then slow-cooled to 24 °C (lane 5). (Panel B) Denaturing 6% polyacrylamide gel showing the RNA composition of the duplex band cut from the gel of panel A. Shown are *micF* RNA alone (from lane 1), *ompF* 5′-end transcript alone (from lane 2), and duplex bands cut from lanes 4 and 5 of the gel in panel A and denatured before rerunning on the gel in panel B.

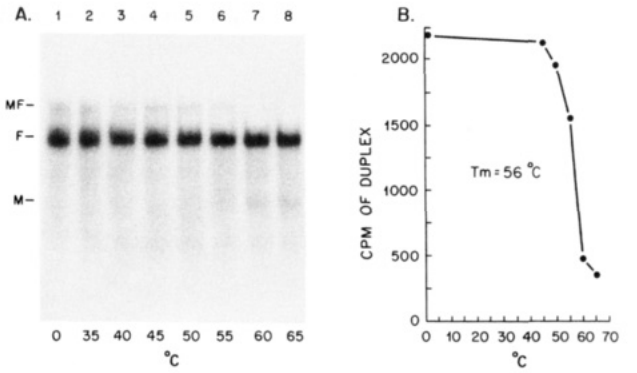

FIGURE 5: Melting curve of the *micF*/*ompF* duplex. (Panel A) Nondenaturing 4% polyacrylamide gel showing the melting of the duplex band (MF) during 3-min incubations at each of the designated temperatures from 35 to 65 °C. The sample in lane 1 was kept on ice. The duplex bands were excised from the gel of panel A and subjected to scintillation counting. The cpm of each duplex was graphed against incubation temperature to obtain the melting curve shown in panel B.

containing 7 M urea (Figure 4B), RNAs with the mobility of *micF* RNA and the 150-nucleotide transcript of *ompF* mRNA appeared. We concluded that the slower moving component in the nondenaturing gel was the duplex formed between *micF* RNA and the 150-nucleotide transcript containing the 5′ end of *ompF* mRNA. Failure to see the duplex form at 0 °C may be due to interference from higher order structures present in the transcripts at that temperature.

Unbound *micF* RNA and the 150-nucleotide transcript were present in samples incubated at 37 or at 55 °C followed by slow cooling (see Figure 4A). Attempts to drive the formation of the duplex to completion were more successful in the presence of excess *ompF* transcript. Therefore, subsequent hybridizations were routinely done using 5–10-fold molar excess of the 150-nucleotide *ompF* transcript or of the "full length" mRNA. Before each experiment, the molar ratios of *micF* RNA and *ompF* mRNA were determined by cpm of the gel-purified in vitro transcription products; however, for unknown reasons, the autoradiographs generally show a loss of *micF* RNA upon reelectrophoresis (see Figures 4 and 5).

Run-off transcription from the *Ava*II cut of the *ompF* gene creates a transcript which is 15 nucleotides shorter than the natural mRNA and is missing a potential 3′-terminal hairpin. In order to visualize the binding of *micF* RNA (93 nucleotides) to this "full-length *ompF* mRNA (~1.1 kb), labeled *micF* RNA was incubated at 37 °C with an unlabeled "full-length" transcript from the *ompF* gene, and the incubation mixture was analyzed by nondenaturing polyacrylamide gel electrophoresis. A band having a mobility slightly slower than labeled "full length" *ompF* mRNA was detected on the autoradiograph (data not shown). The "full-length" *ompF* transcript is particularly labile in the TMK buffer (with or without *micF* RNA) even though *micF* RNA and the 150-nucleotide transcript appear to be stabile. Therefore, the interaction of *micF* RNA with the 5′ end of *ompF* mRNA was analyzed by using the 150-nucleotide transcript containing the 5′ end of *ompF* mRNA.

Melting Curve of the micF/ompF Duplex. *micF* RNA and the 150-nucleotide *ompF* transcript (molar ratio 1:6) were incubated in TMK buffer at 37 °C for 1 h to form the duplex. Aliquots of incubation mixture were each subjected to one of several temperatures for 3 min (see Experimental Procedures). After gel electrophoresis on a nondenaturing gel at 4 °C (Figure 5A) and autoradiography, duplex bands were excised from the gel, and the radioactivity of the bands was measured (Figure 5B). This analysis discloses the T_m of the imperfect RNA/RNA duplex to be 56 °C and the ΔT_m spans about 20 °C (Figure 5B).

micF RNA Binds to a Cognate Protein. Gel mobility shift analyses initially suggested that a cell extract taken from sonicated *E. coli* SM3001 cells, a *micF* deletion strain, contained a *micF* RNA binding protein. The cell extract was fractionated on a DEAE column to partially purify the protein. *micF* RNA exhibits slower mobility during gel electrophoresis after incubation with the protein fraction that elutes at 0.35 M salt from the column than when it is incubated in buffer alone (Figure 6, compare lanes 1 and 2). It is unlikely that the shift in mobility is due to *micF* RNA binding to a piece of *ompF* mRNA since the incubation was done at 4 °C and *micF* RNA did not hybridize to the purified 5′ end of *ompF*

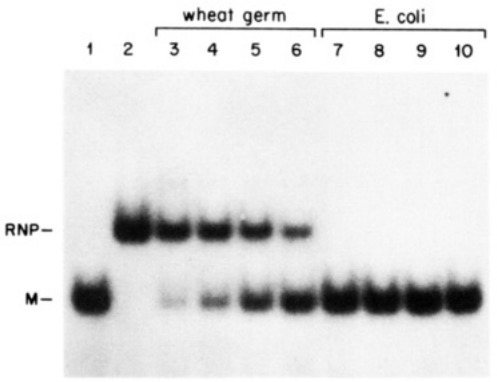

FIGURE 6: Nondenaturing 6% polyacrylamide gel showing the RNP band formed by *micF* RNA and an *E. coli* protein. The mobility of *micF* RNA alone is shown in lane 1. The mobility of *micF* RNA (25 ng) shifts when it is incubated on ice with 600 ng of protein extract partially purified on a column (lane 2). Two types of competitive RNAs, wheat germ tRNA (lanes 3–6) and *E. coli* low molecular weight RNAs enriched in *micF* RNA (lanes 7–8), were preincubated in molar excess with the protein extract, before incubation of labeled *micF* RNA: lanes 3 and 7, 2.5 μg (100-fold excess); lanes 4 and 8, 4.5 μg (300-fold excess); lanes 5 and 9, 15 μg (600-fold excess); lanes 5 and 10, 45 μg (1800-fold excess).

mRNA at that temperature (see Figure 4A, lane 3). In addition, treatment of the incubation mixture with proteinase K eliminates the shift in *micF* RNA's mobility (C. De Loughery and N. Delihas, personal communication), implying that the mobility shift is caused by *micF* RNA binding to a protein.

To test the specificity of *micF* RNA binding to the protein, competitive low molecular weight RNAs from wheat germ or from *E. coli* were preincubated in molar excess (compared to *micF* RNA) for 1 h with the protein fraction before labeled *micF* RNA was added for incubation. The results show that the 100- and 300-fold molar excess, wheat germ tRNA fails to significantly compete for protein binding (Figure 6, lanes 3 and 4). When wheat germ tRNA is in 600- or 1800-fold molar excess, some competition for protein binding is seen (Figure 6, lanes 5 and 6); however, this may be the result of nonspecific RNA interference with *micF* RNA binding to the protein due to the high concentration of RNA in the mix. In contrast, *E. coli* low molecular weight RNAs containing plasmid-amplified quantities of *micF* RNA show distinct competition for the protein when in 100-fold excess (Figure 6, lane 7). Only at 10-fold molar excess (i.e., 250 ng) did the *micF*-enriched RNA sample begin to loose the ability to compete significantly with labeled *micF* RNA for the protein (data not shown). In addition, Xbs 5S RNA does not show a gel mobility shift when incubated with the protein extract (data not shown). It appears from these data that *micF* RNA complexes specifically with a protein from *E. coli*.

Label Transfer from micF RNA to Its Binding Protein Using UV Light. To further characterize the *micF* binding protein, the RNA/protein complex formed using [α-^{32}P]-UTP-labeled *micF* RNA and unlabeled protein was irradiated with UV light (254 nm) for various times, digested with RNase A, and boiled in SDS protein sample buffer, and the results, were analyzed by SDS–PAGE. One distinct radiolabeled protein appeared with increased time of irradiation (Figure 7A). This protein migrated at an apparent molecular weight of 80 000.

When a ^{32}P-labeled 70-nucleotide transcript from the 5′ end of poliovirus was incubated with the protein fraction and the mixture processed for label-transfer, two *E. coli* proteins which migrate faster than the *micF* RNA binding protein receive

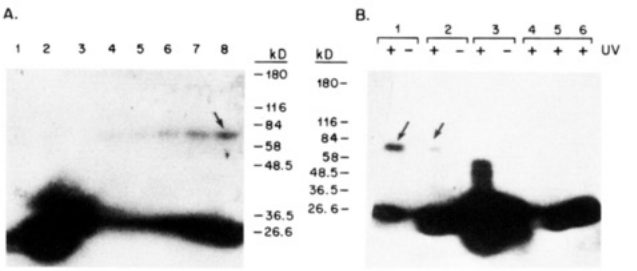

FIGURE 7: UV cross-linking of *micF* RNA to its binding protein. (Panel A) ^{32}P-Labeled *micF* RNA was complexed with its binding protein and exposed to 254-nm UV light for increasing times, digested extensively with RNase A, and subjected to SDS–PAGE. The arrow marks the label-transferred protein band that emerges with time of irradiation. The mobilities of prestained protein markers are shown next to their molecular weights. Lanes 1 and 2, *micF* RNA alone with and without digestion, respectively. Lanes 3–8, RNP mixture with increased irradiation times: 0, 30 s, and 1, 5, 10, and 15 min, respectively. (Panel B) The protein fraction was incubated with labeled *micF* RNA (lane 1), with the 5′-end *ompF* transcript (lane 2), or with a 70-nucleotide transcript from poliovirus (lane 3), subjected to UV light for 15 min (+) or not (–), digested extensively with RNase A, and run on SDS–PAGE. The *micF* RNA binding protein, as determined by its characteristic mobility, is shown by the arrow in lanes 1 (+) and 2 (+). Lanes 4–6 are UV-irradiated and RNase A-digested *micF* RNA, *ompF* 5′ transcript, and 70-nucleotide poliovirus transcript, respectively. The mobilities of prestained protein markers are again shown next to their molecular weights.

transferred label from this transcript (Figure 7B, compare lanes 1+ and 3+). Although potentially intriguing, the specificity of this binding has not been determined. A radiolabeled protein with the mobility of the *micF* RNA binding protein is not detected after UV cross-linking with the poliovirus transcript (i.e., the 80-kDa protein does not bind the poliovirus transcript), giving added evidence that the interaction of *micF* RNA with the protein is specific.

When the 150-nucleotide *ompF* 5′-end transcript, however, is incubated with the protein fraction (in the absence of any *micF* RNA) and the mixture processed for label-transfer, a very faint ^{32}P-labeled protein band with the same mobility as the *micF* binding protein band appears on the autoradiograph (Figure 7B, lane 2+, see arrow; band may be too faint to be seen in photo). The moles of *ompF* transcript used in this sample was 10 times that of *micF* RNA in lane 2 (and therefore 10 times the cpm), and yet only a small amount of label transferred to the 80-kDa protein. The data suggest that while the *micF* binding protein can bind to the 150-nucleotide *ompF* transcript (in the absence of *micF* RNA), it has a significantly weaker association constant for this transcript than it does for *micF* RNA.

To further examine the interaction of the *micF* binding protein with the 5′ end of *ompF* mRNA, the 150-nucleotide *ompF* transcript was incubated with the protein fraction on ice for 1 h, and the results were analyzed by electrophoresis on a nondenaturing gel. Incubation of the 150-nucleotide *ompF* transcript with the protein fraction on ice results in a smear of radioactivity with mobility slower than the transcript itself (data not shown). This "smeary" band is possibly an unstable complex between the *ompF* transcript and the *micF* RNA binding protein; however, further investigations have to be done in this regard.

Discussion

This present study shows for the first time that *micF* RNA forms a stable duplex with the 5′ end of *ompF* mRNA, supporting the notion that *micF* RNA functions as a natural antisense RNA in vivo (Mizuno et al., 1984). The melting curve of the duplex formed by *micF* RNA and the 150-nu-

cleotide *ompF* transcript is in accord with what one might expect for the proposed duplex depicted in Figure 1. For example, the T_m of the perfect RNA/RNA duplex formed by 108-nucleotide RNA I and the primer RNA for Col E1 DNA replication is 93 °C (Tamm & Polisky, 1985), over 35 °C higher than the T_m for the duplex which is believed to be formed by *micF* RNA and *ompF* mRNA. The looped-out positions and non-Watson–Crick base pairs of the *micF*/*ompF* duplex contribute to the lower *micF*/*ompF* T_m which is in the same temperature range as intramolecular melting temperatures (Kochetkov & Budovskii, 1971). On the other hand, the ΔT_m of the *micF*/*ompF* duplex spans about 20 °C while the ΔT_m of the melting curve of a tRNA spans about 30 °C in buffers of similar salt content. This is in contrast to the ΔT_m of a complete DNA/DNA duplex or RNA/RNA duplex which spans about 5 °C or less (Kochetkov & Budovskii, 1971; Tamm & Polisky, 1985). As might be expected from the proposed secondary structural model, the duplex formed between *micF* RNA and the 5′ end of *ompF* mRNA appears to have an intermediate ΔT_m between that of intramolecular RNA structure and nucleic acid perfect duplex.

In vivo, chromosomally encoded *micF* RNA has been shown to be a necessary factor in the negative regulation of OmpF porin in response to temperature increase and appears to be involved in the destabilization of *ompF* mRNA. However, *micF* RNA was not sufficient for the observed decrease in *ompF* mRNA levels, and thus another factor (e.g., a cognate protein) was inferred to also be necessary for the destabilization of the mRNA (Andersen et al., 1989).

In the present study, gel mobility shift analyses, RNA competition experiments, and label-transfer methods show that *micF* RNA complexes with a protein found in a partially purified extract from *E. coli* and that this protein appears to have some affinity for the 5′ end of *ompF* mRNA. In addition, although *micF* RNA alone does not appear to degrade the 5′-end *ompF* transcript, at least under the conditions described here, the 5′-end *ompF* transcript is specifically cleaved by some factor present in the partially purified extract. When the *ompF* transcript is incubated with the protein extract at 37 °C for 10 min, a discrete breakdown product of the *ompF* transcript, appears which is absent if the transcript is incubated alone in TMK buffer at 37 °C (unpublished observation). Cleavage of the *ompF* transcript by a component in the protein extract appears to be specific for the *ompF* transcript since neither *micF* RNA nor Xbs 5S RNA degrades when incubated with the protein extract under the same conditions. It is possible that the *micF* RNA binding protein alters the conformation of the *ompF* transcript or that the RNA transcript forms an alternate conformation at 37 °C, thus making it is susceptible to a ribonuclease also present in the extract. Another possibility is that the *micF* RNA binding protein itself is causing the cleavage of the 5′-end *ompF* transcript. We do not know the relationship of this cleavage to the in vivo destabilization of *ompF* mRNA seen during thermoregulation. Further identification and isolation of the *micF* RNA binding protein are necessary before experiments to determine whether this protein or another component in the extract produces the cleavage of the 150-nucleotide *ompF* transcript. In addition, further experimentation is necessary to confirm in vivo the RNA/RNA and RNA/protein interactions deduced from this present study.

RNase III, a processing enzyme in *E. coli*, has been implicated in the destabilization of the λ cII mRNA after it complexes with the small, phage-derived OOP RNA (Krinke & Wulff, 1987). We found that RNase III$^-$ strains of *E. coli* (BL107, BL321) exhibit the same thermal regulation of OmpF when compared to their respective isogenic parental strains; however, the parental strains (BL15, BL322, A19) do not exhibit the same thermal regualation of OmpF porin as seen in the strains of *E. coli* (JA221, MC4100) used in the original thermal regulation study (Andersen et al., 1989). Therefore, the in vivo results are inconclusive. However, the susceptibility of the *micF*/*ompF* duplex to RNase III cleavage was tested in vitro, and the protein-free duplex did not appear to be a substrate for RNase III cleavage.

The data presented in this study and in the previous study (Andersen et al., 1989) suggest that a *micF* RNP particle participates in the destabilization of *ompF* mRNA and that both the RNA and protein components are necessary for the decrease in *ompF* mRNA levels after an increase in temperature. While the *micF* RNA binding protein appears to have some affinity for the 5′ end of *ompF* mRNA in vitro, it may be that in vivo the protein does not have sufficient affinity to bind to the full-length mRNA and needs to be directed to the 5′ end of *ompF* mRNA through hybridization of *micF* RNA. If the 150-nucleotide *ompF* transcript is incubated at 37 °C with the protein extract in the presence of *micF* RNA, two distinct bands with mobilities slower than the *ompF* transcript appear concomitantly with the disappearance of the *micF* RNP band (unpublished results). These slow-moving bands may be a complex of the *micF* RNP with the 150-nucleotide *ompF* transcript and with a discrete breakdown product of the *ompF* transcript. Current work is directed at purifying the *micF* RNA binding protein in order to look directly at the interaction of the *micF* RNP with the 5′ end of *ompF* mRNA and its role in the destabilization of the messenger. The concept of an RNP particle regulating the stability of a messenger RNA is intriguing.

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In Vitro Reassembly of Active Large Ribosomal Subunits of the Halophilic Archaebacterium *Haloferax mediterranei*†

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ABSTRACT: The large ribosomal subunits of the halophilic archaebacterium *Haloferax mediterranei* have been reconstituted in vitro from the dissociated RNA and protein components. Efficient reassembly of particles fully active in poly(U)-directed polyphenylalanine synthesis requires a 2-h incubation at 42 °C in the presence of no less than 2.5 M concentrations of monovalent cations and of 60 mM magnesium. K^+ and NH_4^+ ions are equally effective in promoting subunit reconstitution; however, maximal efficiency is attained when they are combined in a 1:2 molar ratio. The reassembly process requires no heat activation step, as under the appropriate ionic conditions it takes place spontaneously within the temperature range optimal for growth of *H. mediterranei* cells (40-45 °C).

Extremely halophilic archaebacteria thrive in hypersaline environments. Unlike other kinds of halotolerant microorganisms, they counterbalance the strong external osmotic pressure by raising their internal concentration of potassium ions up to near saturation. This creates an intracellular milieu of uniquely high ionic strength, to which all cellular components are obliged to adapt.

Halobacterial ribosomes have long since attracted the biologists' attention as a suitable object for studying how macromolecules and macromolecular assemblies become modified in order to function at salt concentrations that would normally be destructive. However, apart from the well-established facts that halobacterial ribosomes are unstable in low-salt buffers (Bayley & Kushner, 1964; Bayley, 1966a; Visentin et al., 1972) and that they contain mostly acidic, instead of basic, proteins (Bayley, 1966b; Strom & Visentin, 1973), there is a considerable dearth of information on the mechanism of RNA-protein interaction and ribosome folding in hypersaline conditions.

To gain insight into these latter issues, we have developed an in vitro system that allows the spontaneous reassembly, from the separate RNA and protein moieties, of synthetically active

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