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**Discovery and characterization of the first non-coding RNA that regulates gene expression, *micF* RNA: A historical perspective**

Delihas N. History of discovery of regulatory RNA

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**Abstract**

The first evidence that RNA can function as a regulator of gene expression came from experiments with prokaryotes in the 1980s. It was shown that *E. coli micF* is an independent gene, has its own promoter, and encodes a small non-coding RNA that base pairs with and inhibits translation of a target messenger RNA in response to environmental stress conditions*.* The *micF* RNA was isolated, sequenced and shown to be a primary transcript. *In vitro* experiments showed binding to the target *ompF* mRNA. Secondary structure probing revealed an imperfect *micF* RNA/*ompF* RNA duplex interaction and the presence of a non-canonical base pair. Several transcription factors, including OmpR, regulate *micF* transcription in response to environmental factors. *micF* has also been found in other bacterial species, however, recently Gerhart Wagner and Jörg Vogel showed pleiotropic effects and found *micF* inhibits expression of multiple target mRNAs; importantly, one is the global regulatory gene *lrp*. In addition, *micF* RNA was found to interact with its targets in different ways; it either inhibits ribosome binding or induces degradation of the message. Thus the concept and initial experimental evidence that RNA can regulate gene expression was born with prokaryotes.

**Key words:** Non-coding RNAs; Regulation of gene expression; *micF* RNA; RNA/RNA interaction; Trans-acting RNA gene

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**Core tip:** The original discovery and characterization of the first non-coding RNA gene and its transcript was with prokaryotes in the 1980s. At that time the *Escherichia coli* *micF* RNA gene was characterized in terms of properties, its promoter region, and activation by environmental stress conditions; and the *micF* RNA transcript structure as well as the *micF* RNA/target messenger RNA duplex interaction were elucidated. This occurred over 5 years before the discovery of the first eukaryotic regulatory miRNA, which is not generally recognized. Prokaryotic and eukaryotic non-coding RNAs greatly differ in terms of RNA processing, but the basic principle of an RNA gene locus encoding a regulatory RNA that targets gene expression *in trans* *via* RNA/target RNA duplex formation is similar. Thus the concept and discovery of regulatory non-coding RNAs and their functions in messenger RNA inhibition originated with prokaryotes.

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**INTRODUCTION**

It is now recognized that regulation of gene expression by non-coding RNAs *via* RNA/mRNA interactions is a vastly widespread phenomena that occurs in all biological domains, including viruses, and it has become a basic principle in biology. Current research on non-coding RNAs has essentially “exploded”[1-7]. Yet before the 1980s, RNAs were thought of as macromolecules that primarily supported the protein synthesis machinery, were considered inert, and the concept of RNA as a regulator was unheard of.

In this review we focus on early history of the experimental work that showed that RNA is a regulator of gene expression, and this occurred approximately 30 years ago in the 1980s. *micF* RNA was the first regulatory RNA discovered[8-11]. This preceded the discovery of regulation of gene expression by RNAs in eukaryotes, which was in the 1990s[12]. Prokaryotic non-coding RNAs that regulate gene expression are termed sRNAs; the eukaryotic RNAs are termed microRNAs (miRNAs).

The second bacterial regulatory RNA discovered was DsrA.This RNA transcript was found to mediate *rpoS* expression in 1995[13] but its mechanism of action *via* RNA/RNA binding was not determined until 1998[14,15].

***E. COLI* 6S RNA, SPOT 42, EUKARYOTIC 7SK RNA AND CRISPRS**

The discovery that RNA can serve a regulatory role was in the 1980s, however, three regulatory RNAs were found before that but were uncharacterized: *E. coli* 6S RNA, Spot 42 and the eukaryotic 7SK RNA. These RNAs were discovered and sequenced about 40 years ago but their functions were not elucidated until decades later. The 6S RNA was first detected in 1967[16] and was sequenced by George Brownlee in 1971[17]. Its function was determined approximately 30 years later in 2000 by Wassarman and Storz[18] who showed that 6S RNA binds the RNA polymerase-sigma70 complex and inhibits sigma factor-dependent gene transcription.

The second non-coding RNA found and also uncharacterized until decades later was the transcript termed Spot 42 encoded by the *E.coli* *spf* gene. It was first found in 1973 as a transcript on two-dimensional gel electrophoresis by Dahlberg *et al*[19], but its function as a regulatory non-coding RNA was not determined until 2002 when Valentine-Hansen’s lab showed that Spot 42 binds the *galK* mRNA and inhibits the galactose operon[20]; however, more recently it has been shown to also target fourteen other operons[21].

As to eukaryotic RNAs, in 1976 Gary Zieve and Sheldon Penman found several small RNAs in HeLa cells, one was the 7SK RNA, but its function and the function of the other RNAs were unknown at the time[22]. Almost 30 years later it was shown that 7SK RNA binds to a HEXIM1 protein complex, which then binds to the transcription elongation factor P-TEFb and inhibits transcription[23,24].

There are parallels between the prokaryotic 6S RNA and the eukaryotic 7SK RNA – both were found decades before any functional roles were determined and both bind proteins resulting in inhibit of transcription. The majority of small non-coding RNAs regulate post-transcriptionally by binding target mRNAs.

Clustered regularly interspaced short palindromic repeats (CRISPRs) were discovered in *E.coli* in 1987 by Ishino *et al*[25]. CRISPRs are an array of genomic repeat sequences, which are separated by spacer sequences that originate from viral or plasmid DNA. They represent an acquired bacterial immune system. Similar to the RNAs discussed above, functions and mechanism of regulation of the CRISPR system as small RNA/target DNA inhibitors were not elucidated until about two to three decades later[26-29] (references shown represent only a partial list of contributors).

The bacterial CRISPR-specific RNA processing and targeting mechanism has been compared to the eukaryotic RNAi mechanism and they display striking similarities, although they are not homologous[30]. The CRISPR system currently represents one of the fastest moving fields in molecular biology, primarily because of its potential to alter gene structure and induce chromosomal rearrangements. On the other hand, Morange points out that some earlier pioneer work with the CRISPR system went underappreciated[31].

**COLE1 RNAI**

In 1981 two labs showed that antisense RNAs can inhibit plasmid replication in *E. coli*[32,33].These are milestone experiments as they were the first to demonstrate a regulatory function for RNA. In addition, Tomizawa *et al*[34], working with ColE1 plasmid replication, demonstrated that antisense RNA I binds to and inhibits the primer RNA II that is involved in initiating DNA replication.

Subsequently, Tomizawa *et al*[34] presented a model of antisense RNA/primer RNA recognition by the so-called “kissing interaction”. This involves a two-step process, whereby initially there is a loop-loop interaction *via* Watson-Crick base-pairing between the short stem loops of RNA I and RNA II, followed by pairing of unstructured single-stranded segments of the two RNAs and eventual melting of the stem loops and formation of an antisense/sense RNA/RNA duplex (Figure 1).

The kissing interaction was another pioneer proposal by Tomizawa *et al*[34] whereby we now know there is board significance to the initial recognition of two RNAs *via* stem loop interactions[35-39]. The loop-loop interaction has also been predicted to occur in both the prokaryotic and eukaryotic non-coding RNA/target RNA recognition process[40-42].

There were also other significant plasmid-related experiments at that time. In 1981 Rosen *et al*[43] found three RNA transcripts encoded by plasmids R100 and R1, one a counter transcript, but RNA functions were not fully elucidated. Additionally, Simons *et al*[44] and Kleckner *et al*[45] showed that an antisense counter transcript from the insertion sequence of transposon IS10 inhibits transposase expression *via* RNA/RNA interactions and predicting an initial a loop interaction.

***MICF* RNA**

In 1984, Mizuno *et al*[8] were the first to present the concept of a regulatory RNA gene that controls expression of another gene *via* RNA/RNA base-pairing. This is the *E. coli micF* gene whose transcript inhibits the expression of the target gene *ompF*. This was based on multicopy plasmid repression of OmpF protein from a sequence upstream of the *ompC* gene. This sequence has complementarity *ompF* mRNA[8]. OmpF is an outer membrane protein, a porin protein that allows small molecules to diffuse passively through the cell. Thus it is an important protein to regulate in terms of protecting the cell from deleterious molecules.

At that time it was a rather bold and innovative proposal that an RNA can regulate gene expression, There were no role models for such a function, and RNA was for the most part still considered a passive macromolecule that participates in the protein synthesis process and formed the scaffolding for ribosomal proteins, notwithstanding the previous work on antisense RNA repression of plasmid replication[32,33].

Dr. Inouye should be credited for his creativity in first proposing the existence of a regulatory RNA gene. But his lab had only multicopy plasmid effects and had not isolated the RNA transcript. An RNA molecular biologist was needed and I was asked to contribute to the work. Subsequently my lab isolated and sequenced the chromosomal-encoded *micF* RNA, found that *micF* is an independent gene with its own promoter, determined the regulatory RNA/target RNA base-pairing by structure probing, and determined its function in terms of regulation of target gene in response to environmental stress conditions[9,10].

By using *in vivo* P32 labeling, isolating low molecular weight RNAs and separation on gels, we found a 4.5S RNA transcript. Its sequence showed that it was *micF* RNA and that it was 93 nt long with a rho independent termination site[9].To characterize the micF promoter regions, lacZ was fused at different sites upstream of the *micF* gene and *micF* was found to have a strong promoter based on beta galactosidase activity.

***MICF* RESPONSE TO STRESS CONDITIONS**

A search for function was initiated by looking at the response of *micF* expression to various environmental factors. A major effect was found with temperature change and other factors. Levels of *micF* RNA increased dramatically in response to cell growth at high temperature[10]. The levels of *ompF* mRNA and the OmpF protein were also measured and found to decrease under these conditions. Figure 2 shows a marked decrease in *ompF* mRNA levels with temperature increase in the wild type strain, but no significant change in the *micF* deletion strain (SM3001). The findings revealed that *micF* RNA plays a major role in the thermal regulation of *ompF* mRNA and OmpF protein. However, the mechanism of thermal regulation of *micF* expression is still an open question, but temperature has been shown to regulate DNA supercoiling that is associated with the induction of stress related genes[46-49]. *micF* expression was also found to respond to other environmental stress conditions such as osmolarity change and exposure to ethanol[10,50].

The upstream *micF* regulatory region is complex in that it contains binding sites for a number of transcription factors; these regulate *micF* expression in response to different environmental stress factors[51]. Five transcription factors are known to bind in the upstream regulatory region (Figure 3)*.* With respect to osmolarity change, there are three binding sites (C1-C3) for transcription factor OmpR that activates expression of *micF* in response to osmolarity increase[50]. MarA responds to weak acids[52], SoxS to oxidative stress[53], Rob to peptide antibiotics[54,55] and Lrp to nutritionally poor media[56]. These four transcription factors share the same binding site on the *micF* promoter region (Figure 3). MarA, SoxS and Rob are related proteins that activate *micF*. Rowena Matthews’ lab showed that Lrp represses *micF* transcription[56]; Lrp is part of the nucleoid-associated protein goup[57]. The crystal structure of transcription factor Rob bound to the *micF* promoter region has been elucidated (by Kwon*et al*[58]).The model shows one protein helix-turn-helix motif bound to the major groove of *micF* DNA. Additional biochemical studies showed a high affinity of binding[58].

*micF* is also regulated at the post-translational level when cells are grown in a nutrient poor medium[59]. Dorman and co-workers hypothesized that Stp induces a conformational change in *micF* RNA that makes it is susceptible to degradation by nucleases[59]. Stp is also a nucleoid-associated protein and a paralogue of H-NS[60,61]. Thus *micF* gene expression is regulated at both the transcriptional and post-transcriptional levels in response to different environmental conditions.

***MICF* RNA/TARGET *OMPF* MRNA DUPLEX**

It was demonstrated that an RNA/RNA duplex could be formed *in vitro* between *micF* RNA and the target *ompF* mRNA[62]. Full length *micF* RNA and a 150 nt 5’ end fragment of *ompF* mRNA were synthesized *in vitro* and the RNA/RNA duplex was formed by annealing at 55 oC and 37 oC. Subsequently, in vitro formed duplexes were used for secondary structure probing[63]. Single and double stranded specific RNases and chemical modification with a NiCR complex that is single strand G-specific[64] were used for probing. As nuclease probes can be sensitive to steric hindrance, chemical modification was performed using the NiCR reagent to try to gain additional information[63-65]. A schematic of the deduced *micF* RNA/*ompF* mRNA duplex base-pairing is in Figure 4.

The RNA/RNA interaction shows an imperfect duplex, and a non-canonical G99-G29 base-pair that was determined from the absence of RNase T1 cleavage and NiCR reaction, but also the presence of double stranded specific RNase V1 cleavages[63,65].The Shine-Dalgarno (S-D) ribosome binding site is blocked, which implies that *micF* RNA inhibits ribosome binding. *In vivo* experiments show that *ompF* mRNA levels decrease with temperature increase in an *E. coli* strain carrying *micF* (Figure 2); however, *micF* RNA has not been shown to directly participate in degradation of the message, but to block ribosome binding.

Long-range pairing based on the Maximum Weighted Matching computational program of Tabaska *et al*[66] was predicted to also contribute to the RNA/RNA interaction (Figure 4)**.** The pairing is supported by phylogenetic comparisons and for the most part, by structure probing. Long-range pairings should contribute to the three-dimensional structure of the interacting RNAs.

***MICF* RNA HAS MULTIPLE TARGETS AND PARTICIPATES IN GLOBAL GENE REGULATION**

Recently it has been shown that the functions of *micF* RNA are multifaceted and extend far beyond regulation of *ompF* expression. Gerhart Wagner and his lab showed that *micF* is part of a global regulatory network involving the leucine responsive protein (Lrp)[67], and Jörg Vogel and co-workers at the University of Würzburg in Germany showed that *micF* targets different mRNAs and base-pairs with these RNAs in different ways[68].

Approximately 10% of all genes in *Escherichia coli* are controlled by Lrp, a transcription factor which responds to nutrient availability. Wagner’s group[67] showed participation of *micF* RNA in a circular regulatory pathway, *i.e*., Lrp regulates transcription of *micF* RNA and *micF* RNA regulate *lrp* expression post-transcriptionally, depending on the nutritional content of the cell growth media (Figure 5). Both Lrp and *micF* RNA function as repressors. *micF* RNA participates in this global regulatory network by regulating *lrp* mRNA; thus this greatly expands its role in regulation of gene expression and cell metabolism.

On the other hand, using *Salmonella* as a model, Vogel and his lab demonstrated that *micF* RNA targets multiple mRNAs in addition to *ompF* mRNA and *lrp* mRNA (Figure 5). Interestingly, its mode of binding to different mRNAs is multifaceted. On the one hand, *micF* RNA can induce destabilization of the *yahO* mRNA by unfolding a stem-loop structure located at the 5′-terminus, and thus make the mRNA accessible to exonuclease action. With *lpxR* mRNA, *micF* RNA binds at two sites. One site is within the coding region of the message, and when *micF* RNA binds at this site, it induces a conformational change exposing an AU region that can be targeted by the endonuclease RNase E, which then degrades the message[68]. *yahO* encodes a periplasmic protein and *lpxR* encodes an outer membrane lipid A-modifying enzyme.

**HFQ PROTEIN**

*In vitro* studies showed that *micF* RNA binds to a protein based on mobility shifts of protein-RNA complexes on non-denaturing gels[62]. UV-cross-crosslinking experiments suggested that the protein was 80-kDa, however the protein was not further characterized. It is well known that many sRNAs bind the Hfq protein and that Hfq facilitates the binding of sRNAs to their target mRNAs, including *micF* RNA[70]. The molecular weight of Hfq is 11.2-KDa[71], but the 80-kDa protein found to bind *micF* RNA[62] is inconsistent with this and there is a need for further investigation.

Hfq is a central component of the pairing of bacterial non-coding RNAs with their target messenger RNAs. The multifaceted ways that Hfq, together with sRNAs can participate in the inhibition of translation and/or mRNA degradation has been outlined[72]. Hfq also interacts with another protein, catalase HPII. The crystal structure of Hfq bound to catalase HPII has been solved[71], but the crystal structure of an Hfq-sRNA complex has not been determined,

**OTHER NON-CODING RNA FUNCTIONS**

In this paper we focused on initial findings involving the regulation of gene expression by RNA, however, there is a wealth of discoveries with other functions of non-coding RNAs that also occurred during the 1980s, and it is important to mention this. Table 1 lists several initial findings that include enzymatic RNA, catalytic RNA and RNA functions in protein translocation. The work on regulation of gene expression by RNA and the studies outlined in Table 1 provide a breakthrough on understanding functions of RNAs whereby before the 1980s, RNAs such largely considered inert with no dynamic functions.

**CONCLUSION**

The initial discovery that RNA can function as a regulatory molecule occurred in the 1980s. Table 2 summarizes the chronology. Bacterial plasmid RNAs were first shown by two labs to regulate DNA replication *via* sense/antisense RNA/RNA base-pairing[32,33] and the *E. coli* *micF* gene was the first found to regulate expression of another gene *in trans* *via* imperfect RNA/RNA base-pairing, also by two labs[8-10]. These finding were major breakthroughs that came approximately 5-10 years before the discovery of eukaryotic microRNAs and their functions. Subsequently, numerous labs added to the molecular genetics of *micF* and to functional properties of the transcript that greatly advanced our knowledge of the role of this gene in cell survival. So it was essentially work with prokaryotes that first opened the door to a new form of regulation of gene expression by RNA that is now found in all biological kingdoms, including viruses. However, there are many different types of regulatory RNAs that are found only in eukaryotes (*e.g.*, piRNA, siRNA, lncRNA), and the prospects of finding new and unique functions of RNAs may lie primarily with eukaryotic RNAs. Looking towards the future, there are tens of thousands of lncRNAs that have been detected, and for the most part are uncharacterized. But the few that have been characterized display very diverse and intricate functions[78-82].

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**Table 1 Discovery of other non-coding RNA functions**

|  |  |  |  |
| --- | --- | --- | --- |
| **Non-coding RNA**  | **Date discovered** | **Function**  | **Ref.** |
| RNase P RNA | 1978, 1983  | Catalytic RNA | [73,74] |
| Tetrahymena intron | 1982  | RNA self-splicing | [75] |
| snRNA | 1982  | Involved in RNA splicing | [76] |
| 7SL RNA | 1982 | Participates in protein translocation | [77] |

**Table 2 Discovery of regulatory non-coding RNA functions**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Date determined to be a regulator** | **Function**  | **Ref.** |
| **Non-coding RNA (prokaryotic**) |  |  |  |
| ColE I RNA I  | 1981  | Inhibits DNA replication  | [31,32] |
| *micF* RNA | 1984/1987 | Inhibits *ompF* mRNA+ other mRNAs | [8-10] |
| 6S RNA | 2000  | Binds RNA polymerase (transcription inhibitor) | [18] |
| Spot 42 | 2002  | Inhibits *galK* mRNA + other mRNAs | [20] |
| **Non-coding RNA (eukaryotic**) |  |  |  |
| lin 4  | 1993  | Inhibits *lin14*  | [12] |
| 7SK RNA  | 2004  | Binds pTEFb (transcription inhibitor) | [23] |



**Figure 1 Model of two step interaction between antisense RNA I and sense RNA II-initial stem loop recognition followed by antisense/sense RNA/RNA duplex formation.** Reproduced from ref. [34] with permission**.**



**Figure 2 *ompF* mRNA levels after temperature shift from 24 oC to 42 oC.** Lanes marked a-k correspond to time 0’-120’. Left, RNA from strain MC4100 that has the *micF* gene; right, strain SM3001 that is a *micF* deletion strain. Data from Andersen *et al*[10]. Reproduced with permission.



**Figure 3 Schematic of *micF* gene, upstream P-10, P-35 promoter region and transcription factor binding sites.** Diagram modified from Delihas and Forst[51].



**Figure 4 Model of micF RNA/*ompF* mRNA interaction.** Duplex based on secondary structure probing; the long range pairing of five bases at the 5’ end of *ompF* mRNA with positions 45-49 of *micF* RNA was based on computational analysis[66]. Reproduced from Delihas and Forst[51].



**Figure 5 Multifaceted functions of *micF* RNA showing a “circular” regulatory process involving Lrp and *micF* RNA.** Also shown are multiple mRNAs targeted by *micF* RNA. Based on work of Holmqvist *et al*[64] and Corcoran *et al*[65]. Drawing reproduced from Delihas[69].