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Engineering of the mRNA-interfering complementary RNA immune system against viral infection

(anti-sense RNA/RNA phages/Shine–Dalgarno sequence/*Escherichia coli*)

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ABSTRACT **Creation of an artificial mRNA-interfering complementary RNA (micRNA) immune system, utilizing anti-sense RNAs to inhibit viral gene expression, has been shown to be an effective way to prevent viral infection. In the RNA coliphage SP, the gene for the maturation protein was found to be the best target for this type of immune system; mRNA-interfering complementary RNAs specific to the genes for coat protein and replicase were less effective in preventing infection. The greatest inhibitory effect was observed with a 240-base sequence encompassing the 24-base noncoding region of the maturation gene plus the 216-base coding sequence. Significantly, even a 19-base sequence covering only the Shine–Dalgarno sequence (ribosome-binding region) without the coding region exerted a strong inhibitory effect on phage proliferation. In contrast to the highly specific action against phage SP exhibited by the longer mRNA-interfering complementary RNA, the specificity with the shorter mRNA-interfering complementary RNA was broadened to phages Qβ and GA as well as SP, all of which are classified in the different groups of RNA coliphages. Therefore, this type of anti-viral reagent may be designed to have a particular breadth of specificity, thus increasing its value in various research and possibly clinical applications.**

An anti-sense RNA to a specific mRNA has been shown to function as a repressor for gene expression in *Escherichia coli* (1, 2). The *micF* gene maps at 21 min on the *E. coli* chromosome and encodes a small RNA, micF RNA, which is complementary to the mRNA for the major outer membrane protein, OmpF. Production of the micF RNA has been shown to inhibit OmpF production.

Since the artificial production of an anti-sense RNA against a specific gene can be easily achieved in both prokaryotes and eukaryotes, several attempts have been made to artificially regulate specific genes in a manner analogous to the *micF* system (refs. 3–12; for review see also ref. 13). We have termed these regulators micRNAs for mRNA-interfering complementary RNA.

In addition to regulating cellular genes by artificial micRNA, we have designed a specific *E. coli* immune system against viral infection (micRNA immune system) using micRNAs against viral genes (14). *E. coli* cells containing a plasmid carrying an inducible gene for micRNAs against coat protein and replicase of the positive, single-stranded RNA coliphage SP became resistant or immune to the phage upon induction of the micRNA (14).

In the present paper we explore the design of a construct that will more effectively confer immunity against phage SP and discuss how to broaden the specificity of the micRNA immune system to include other related RNA phages.

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MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* K12 strain JA221 (*lacY hsdR trpE5 leuB6 recA*/F′ *lacI*^{q} *lac*$^+$ *pro*$^+$) (15) and *E. coli* A/λ (F$^+$, *Su*$^+$, *pro*$^-$) were used. Cell culture and phage infection were carried out as described (14). Plasmid pJDC406 (14) was used for construction of various micRNA immune systems.

DNA Manipulation. DNA manipulation was carried out according to Maniatis *et al.* (16).

Phage Titers. Phage titers in Fig. 3 were estimated using *E. coli* A/λ as indicator. *E. coli* cells carrying pMIC-D1 and/or pJDC406 were grown at 37°C in L broth containing 5 mM $CaCl_2$, and isopropyl β-D-thiogalactoside (IPTG) was added at a final concentration of 2 mM at a Klett–Summerson reading of 10 to induce the micRNA gene. At a Klett unit of 55, phage SP was added at a multiplicity of 1. After 6 min, 0.1 ml of the culture was mixed with 0.9 ml of L broth containing an excess amount of anti-SP serum (K = 10) and 2 mM IPTG. The mixture was incubated at 37°C to remove unabsorbed phage particles. At 12 min after infection, the mixture was further diluted 1:10,000. At each point for the first 29 min, an aliquot, after an appropriate dilution, was immediately mixed with a large excess of stationary culture of *E. coli* A/λ and plated onto L-broth agar plates. After 35 min of infection, samples were treated with chloroform before an appropriate dilution. Numbers of plaques were measured after incubation at 37°C overnight.

RESULTS

Construction of micRNAs Against Maturation Protein. Of the three genes of phage SP, we have constructed the micRNA immune system against the second (coat protein) and the third (replicase) genes. The basic design of all constructions places the plasmid-carried genes under the control of the *lpp* plus *lac* promoters such that expression can be induced with the *lac* inducer IPTG. These mic immunity plasmids are as follows: pMIC-A, a plasmid carrying a gene that produces a 247-base-pair (bp) micRNA complementary to the translation initiation region of the mRNA for coat protein; pMIC-B, a plasmid inducibly producing a 159-bp micRNA complementary to the translation initiation region of the mRNA for replicase; and pMIC-C, a plasmid that produces a micRNA complementary to the 518-bp 3′ end of the phage RNA including the 411-bp sequence from the C-terminal coding region of the replicase gene and the 107-bp sequence from the 3′-noncoding region (see Fig. 1*a*).

These clones exerted inhibitory effects against phage SP proliferation upon induction with IPTG: 69% inhibition for pMIC-A, 42% for pMIC-B, and 40% for pMIC-C (ref. 14; see also Table 1). The first gene of phage SP, encoding the maturation protein, has been cloned, and the gene product,

Abbreviations: micRNA, mRNA-interfering complementary RNA; IPTG, isopropyl β-D-thiogalactoside; bp, base pair(s).

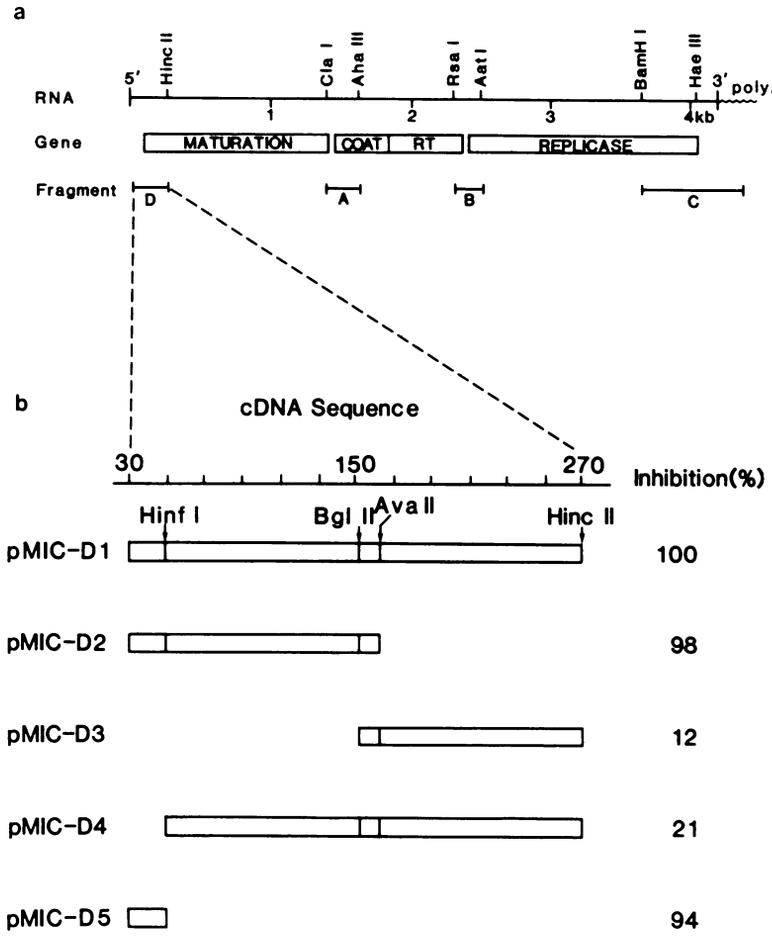

FIG. 1. (*a*) Diagrammatic representation of bacteriophage SP. Numbers refer to the distance (in kilobases) from the 5'-end terminus. Only the relevant restriction sites are shown. RT, coat readthrough protein. Fragments A, B, C, and D were used to construct the micRNA plasmids pMIC-A, -B, -C, and -D, respectively. These fragments were inserted into the unique *Xba* I site of pJDC406, a micRNA cloning vector as described (14). (*b*) Construction of various micRNAs from the D region. pMIC-D2 and pMIC-D5 were constructed in the same way as pMIC-D1 except that the cDNA clone was digested with *Mst* I plus *Ava* II for pMIC-D2 and with *Mst* I plus *Hin*fI for pMIC-D5 followed by Klenow fragment of polymerase I treatment to create blunt end fragments. In the cases of pMIC-D3 and pMIC-D4, the 123-bp *Bgl* II–*Hin*cII fragment and 221-bp *Hin*fI–*Hin*cII fragment from the cDNA clone were inserted into pJDC406, respectively. Nucleotide numbers represent those from the 5' end of phage SP. The exact nucleotide sequence is shown in Fig. 2. Inhibition of phage production with these micRNAs was examined using *E. coli* JA221/F'*lacI*^{q} pJDC406 as control in the same manner as described (14). The results were expressed as % inhibition of the control titer. All the experiments were carried out in the presence of 2 mM IPTG.

while known to be a very minor component of phage particles (one molecule per phage), is an essential factor for the binding of phage to its host cell (17). Therefore, it is now possible to construct micRNA immune systems using this region and to examine whether micRNAs against genes for minor viral proteins are more effective than those against major species such as coat protein and replicase.

The cDNA clone available at present covers the entire phage downstream of the nucleotide at position 31 (see Fig. 2). The upstream region of position 31 in this cDNA clone was replaced with a poly(A) sequence approximately 60 bases long. This poly(A) was derived from a poly(A) sequence created at the unique *Pst* I site of pBR322 for cDNA cloning. To isolate a DNA fragment encompassing the gene for

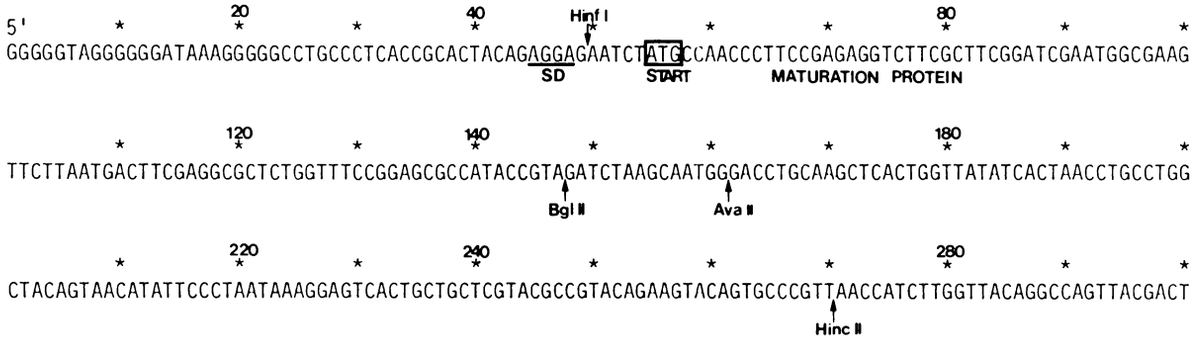

FIG. 2. The nucleotide sequence of the cDNA corresponding to the 5'-terminal region of bacteriophage SP. SD, Shine–Dalgarno sequence. ATG in a box represents the initiation codon for the maturation protein.

maturation protein, the cDNA clone was digested at the *Mst* I site of pBR322 located 21 bp downstream of the *Pst* I site as well as the *Hin*cII site at position 270 in the maturation gene (see Fig. 2). The resultant approximately 320-bp fragment is blunt at both the 5′ and the 3′ ends and consists of the 21-bp *Mst* I–*Pst* I fragment from pBR322, approximately 60 bp of poly(A) (at the 5′ end of the plus strand) and the 240-bp fragment from position 31 to position 270 of phage SP (see Fig. 2). This fragment was cloned into the unique *Xba* I site of a micRNA cloning vector pJDC406 (14), which was converted into blunt ends after *Xba* I digestion followed by DNA polymerase I treatment. One clone carrying a single copy of the DNA fragment inserted into pJDC406 in antisense orientation to the promoter of pJDC406 was designated pMIC-D1 (see also Fig. 1*b*). *E. coli* cells transformed with pMIC-D1 are thus able to produce the mic-D1 RNA (complementary to the region of phage SP from positions 31 to 270) upon the addition of *lac* inducer. The mic-D1 RNA covers the 24-base 5′-noncoding region including the Shine–Dalgarno sequence for ribosome binding and 216 bases of the 5′ end of the coding region for maturation protein including the initiation codon AUG. Furthermore, it contains approximately 60 bases of poly(A) plus a 21-base RNA derived from the pBR322 sequence at the 3′ end.

As shown in Table 1, the immunity to phage SP conferred by pMIC-D1 was much more effective than not only pMIC-A, pMIC-B, and pMIC-C but also pMIC-A:B:C. When the D1 fragment was inserted in the sense orientation [pMIC-D1 (sense)], there was no immune activity (Table 1). Fig. 3 shows the phage titer of the cells carrying pMIC-D1 or pJDC406 at various times after phage infection in the presence of IPTG. In the case of pMIC-D1, the number of infective centers was approximately five times lower than that in the case of pJDC406. In addition, the final phage production at 90 min in the case of pMIC-D1 was ≈2% of that in the case of pJDC406 (Fig. 3). Absorption efficiencies of phages were identical for both control cells and cells carrying pMIC-D1 (data not shown). Therefore, the lower numbers for infective centers in the case of cells carrying pMIC-D1 (Fig. 3) are likely due to an inhibitory effect of the micRNA on phage production. The micRNA immune activity by pMIC-D1 was much lower in the absence of IPTG as in the cases of other systems, although significantly higher backgrounds of these systems were observed (see Table 1). These high backgrounds are due to leaky expression of the immune systems in L broth.

Effective Regions for the micRNA Immune System. To demonstrate which part of the pMIC-D1 RNA is essential for the immune system, we next utilized three restriction sites to examine the immune activity of various regions of the mic-D1 RNA (see Figs. 1*b* and 2). The DNA fragments tested are

Table 1. Effects of various micRNA genes on plaque formation of bacteriophage SP

Plasmid	Gene	% inhibition +	% inhibition −
pMIC-A	Coat protein	69	32
pMIC-B	Replicase	42	22
pMIC-C	3′-Terminal region	40	4
pMIC-D1	Maturation protein	98	54
pMIC-D1 (sense)	Maturation protein in the sense orientation	2	0
pMIC-A:B:C	Coat protein/replicase/3′-terminal region	91	44

Phage titers were measured with use of *E. coli* JA221/F′ *lacI*^{q} harboring various micRNA clones in the presence (+) or absence (−) of 2 mM IPTG as described (14). % inhibition was calculated on the basis of the titer using the cells carrying pJDC406, a micRNA cloning vector.

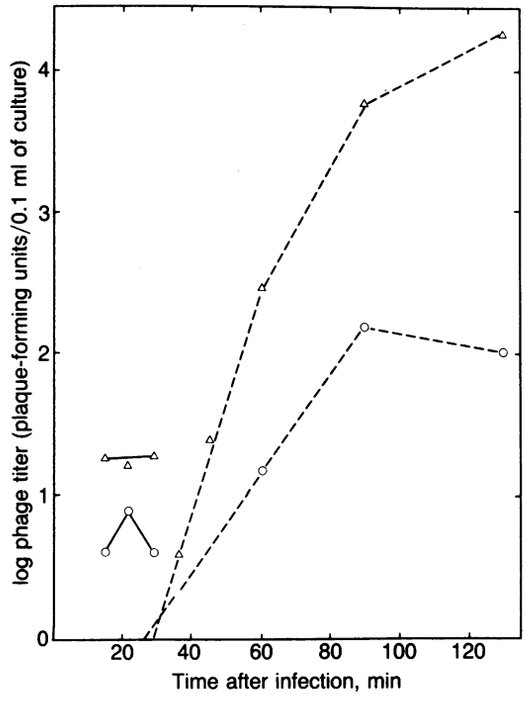

FIG. 3. Phage titers of the cell cultures at various times after the addition of bacteriophage SP. *E. coli* JA221/F′*lacI*^{q} harboring pJDC406 (△—△). Titers were measured without adding chloroform. The same cells but titers were measured after the addition of chloroform (△---△). *E. coli* JA221/F′*lacI*^{q} harboring pMIC-D1 (○—○). Titers were measured without the treatment of chloroform. The same cells but titers were measured after the addition of chloroform (○---○).

shown in Fig. 1*b*: pMIC-D2 and pMIC-D5 were constructed in the same way as pMIC-D1 except that the cDNA clone was digested with *Mst* I plus *Ava* II for pMIC-D2 and with *Mst* I plus *Hin*fI for pMIC-D5, instead of *Mst* I plus *Hin*cII. Therefore, pMIC-D2 lost the 109-bp DNA fragment from position 162 (*Ava* II site) to position 270 (*Hin*cII site) in the coding region of the maturation gene in comparison with pMIC-D1 (see Figs. 1*b* and 2). Similarly, pMIC-D5 lost the 251-bp fragment from position 50 (*Hin*fI site) to position 270 (*Hin*cII site), leaving only the 19-bp fragment from position 31 to position 49 from phage SP (see Figs. 1*b* and 2). In the cases of pMIC-D3 and pMIC-D4, the 123-bp *Bgl* II–*Hin*cII fragment and the 221-bp *Hin*fI–*Hin*cII fragment from the cDNA clone were cloned into pJDC406, respectively (see Figs. 1*b* and 2). Therefore, in contrast to pMIC-D1, -D2, and -D5, these two plasmids contain neither the 60-bp poly(T) sequence nor the 21-bp sequence derived from pBR322 at the 3′ ends of their transcripts.

The micRNA immune effects of these plasmids on phage SP production are shown in Fig. 1*b*. As is evident in the case of pMIC-D2, the micRNA immunity was hardly affected by removing approximately half of the coding region from pMIC-D1. On the other hand, the removal of the 5′-end half of the pMIC-D1 sequence resulted in significant decrease of the micRNA immune effect as can be seen with pMIC-D3 (from 100 to 12%; see Fig. 1*b*). The complete recovery of the coding region of pMIC-D1 by adding the 98-bp *Hin*fI–*Bgl* II fragment to pMIC-D3 (which includes the initiation codon AUG) improved the immune effect only to a small extent, from 12 to 21% (Fig. 1*b*). These results suggest that the 19-bp sequence from position 31 to position 49 (*Hin*fI site) encompassing the Shine–Dalgarno sequence plays an essential role in this micRNA immune system. Strong evidence for this conclusion was obtained with pMIC-D5, which contains only the 19-bp fragment from phage SP. This plasmid is able to

Table 2. Effects of pMIC-D1, pMIC-D2, and pMIC-D5 on plaque formation of phages SP, Qβ, and GA

Plasmid	% inhibition		
	SP	Qβ	GA
pMIC-D1	95	9	−6
pMIC-D2	94	38	53
pMIC-D5	94	78	98

E. coli JA221/F'*lacI*^{q} harboring one of the clones was used as indicator cells in the presence of IPTG. % inhibition was calculated as described in Table 1. The data presented are the averages of three independent experiments.

inhibit phage production almost as well as pMIC-D1 (Fig. 1*b*). The low immune activities by pMIC-D3 and -D4 are consistent with the results obtained for a micRNA regulatory system against the gene for the outer membrane lipoprotein (3). When micRNAs were complementary only to the coding region of the *lpp* mRNA, lipoprotein synthesis was inhibited by a factor of 2 in contrast to inhibition by a factor of 16 with a micRNA covering the ribosome-binding site. Therefore, the low immune activities by pMIC-D3 and -D4 are unlikely to be due to the lack of poly(dT) and a fragment derived from pBR322 that exists at the 3′ ends of micRNAs for pMIC-D1, -D2, and -D5.

We have also examined copy numbers of plasmids in all cases and found that there were no significant differences in plasmid copy numbers in the cells carrying pJDC406, pMIC-D1, -D2, -D3, -D4, and -D5 (data not shown). Therefore, different efficiencies by different mic systems are not due to gene dosage effect.

Specificity of the micRNA Immune System. It has been shown that there are other positive, single-stranded RNA coliphages such as Qβ and GA, which are in the different groups of RNA phages from phage SP (18). The nucleotide sequences of all these phages have been determined and show a similar gene arrangement (refs. 19 and 20; Y.I. and A.H., unpublished results; M. A. Billeter, personal communication).§ Therefore, we examined whether the present micRNA immune system against phage SP also exerts immune effects against phages Qβ and GA.

As shown in Table 2, the immune system of cells harboring pMIC-D1 was highly specific for phage SP, showing little effect on phage Qβ and no effect on phage GA. However, as a part of the anti-sense RNA covering the coding region for maturation protein was removed, the immune effect on phage Qβ as well as GA increased dramatically without decreasing the immune effect on phage SP (Table 2). The cells harboring pMIC-D2 became approximately 40% immune to phage Qβ and approximately 50% immune to phage GA. It was particularly surprising that pMIC-D5, the 19-base micRNA encompassing the Shine–Dalgarno sequence, was no longer specific for phage SP and exerted potent immune effects on both phages Qβ and GA (Table 2).

DISCUSSION

The present data demonstrate how to construct an effective micRNA immune system against viral infection and suggest a way to regulate specificity against various viral species. The results suggest the following conclusions: (*i*) A micRNA immune system against a very minor viral protein is able to confer very effective protection against viral infection. (*ii*) A micRNA encompassing the region required for ribosome binding (Shine–Dalgarno sequence) plus a longer coding region exerts strong immune activity. (*iii*) A micRNA that

§Ref. 19 amended in nine single nucleotide positions.

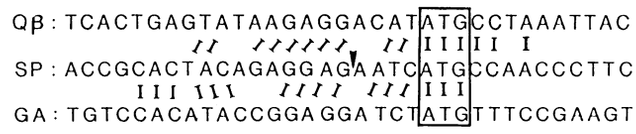

FIG. 4. Similarity in the nucleotide sequences of cDNAs of the region upstream of the initiation codon of the maturation protein among bacteriophages Qβ (21), SP (Y.I. and A.H., unpublished results), and GA (20). The initiation codons are boxed, and bars between the sequences indicate the identical nucleotides. An arrowhead indicates the site cleaved by *Hin*fI.

consists of only 19 bases and encompasses the region of the Shine–Dalgarno sequence without the initiation codon is able to block virus production very effectively. (*iv*) As the region complementary to the coding sequence is shortened, the specificity of the immune system against RNA phages is broadened.

It has been pointed out that micRNA function is most effective when it covers the sequence translation initiation region of the target mRNA (3, 11). This was again confirmed by the present work using mic-D1 clones. However, it is not well understood at present why the specificity of the micRNA immune system is broadened to related RNA phages when the sequence of the micRNA complementary to the coding region is shortened; inclusion of the coding region apparently prevents micRNA immune activity from inhibiting the related RNA phages. It is also surprising that the micRNA of only 19 bases (mic-D5 RNA), which is complementary to the region of the Shine–Dalgarno sequence of the maturation gene of phage SP, is able to exert micRNA immune activity against both phage Qβ and phage GA. The sequence similarities between the phages at these regions are shown in Fig. 4 (mic-D5 RNA covers the sequence upstream of the site indicated by a small arrow). However, the similarities at these regions between phage SP and phage Qβ and between phage SP and phage GA are only approximately 50%. Since we observed a phenomenon that a mic clone that carries a 27-bp fragment from the 5′ end of the SP genome also showed strong inhibition of SP phage infection as well as phages Qβ and GA (data not shown), micRNA immune activity of mic-D5 RNA against phages Qβ and GA may be due to another element within the RNA, which is able to block the replication of phage RNA rather than to block the binding of ribosomes to the Shine–Dalgarno regions of the gene for the maturation protein.

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