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Antisense RNA associated with biological regulation of a restriction–modification system

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ABSTRACT

Restriction–modification systems consist of a modification enzyme that methylates a specific DNA sequence and a restriction endonuclease that cleaves DNA lacking this epigenetic signature. Their gene expression should be finely regulated because their potential to attack the host bacterial genome needs to be controlled. In the EcoRI system, where the restriction gene is located upstream of the modification gene in the same orientation, we previously identified intragenic reverse promoters affecting gene expression. In the present work, we identified a small (88 nt) antisense RNA (Rna0) transcribed from a reverse promoter (P_{REV0}) at the 3′ end of the restriction gene. Its antisense transcription, as measured by transcriptional gene fusion, appeared to be terminated by the $P_{M1,M2}$ promoter. $P_{M1,M2}$ promoter-initiated transcription, in turn, appeared to be inhibited by P_{REV0}. Mutational inactivation of P_{REV0} increased expression of the restriction gene. The biological significance of this antisense transcription is 2-fold. First, a mutation in P_{REV0} increased restriction of incoming DNA. Second, the presence of the antisense RNA gene (*ecoRIA*) *in trans* alleviated cell killing after loss of the EcoRI plasmid (post-segregational killing). Taken together, these results strongly suggested the involvement of an antisense RNA in the biological regulation of this restriction–modification system.

INTRODUCTION

Restriction–modification (R–M) systems are found in many prokaryotes (1). The great abundance of R–M systems in prokaryotes reflects their mobility via transformation, transduction or conjugation (2,3). Many R–M systems are present on plasmids and other mobile elements and can spread from one bacterial host to another, sometimes crossing species boundaries and impacting genome evolution on a global scale (4–6). R–M systems consist of a modification enzyme that methylates a specific DNA sequence in a genome and a restriction endonuclease that cleaves DNA lacking this methylation. They are recognized as bacterial defense systems against invading DNAs, but their biological significance extends far beyond this (7).

R–M systems may represent one form of life, just as other mobile elements (8). Under some conditions, they may switch on bacterial death programs (8,9). Several type II R–M systems can program the death of a cell lineage once it has lost the R–M system (10,11). This is similar to the classical post-segregational host cell killing systems on plasmids (12–14). This post-segregational killing or genetic addiction helps in the maintenance and spread of the R–M system (6,11,15). This suggests that some R–M systems behave as selfish mobile elements, similar to viral genomes and transposons. R–M systems have also been linked with various genome rearrangements (6). Recent studies revealed that modification enzymes methylate many copies of a recognition sequence along the genome and define the epigenetic status of that genome in a combinatorial fashion, which may promote adaptation (16). R–M systems mediate DNA selection during horizontal gene transfer, serving as gene flow 'gatekeepers', using epigenetic signs as identification.

R–M systems are thought to possess mechanisms to tightly regulate their gene expression to suppress potentially lethal attacks on their host bacteria. When they enter a new host bacterial cell with a genome lacking proper methylation, they avoid cell killing by expressing the modification enzyme first (17). The restriction endonuclease and modification enzyme activities must be carefully

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balanced not only at the stage of R–M system establishment in a new host, but also during maintenance of this system. When R–M genes are lost from the cell, the restriction enzyme will attack the chromosome in an act of post-segregational killing.

Their regulatory machinery is expected to be host-independent to bypass differences in host factors affecting their establishment, maintenance and host attack. To date, these regulatory mechanisms have mostly been studied at the transcriptional level. Three main modes of regulation have been distinguished. One employs C-proteins (18), which specifically bind a DNA operator sequence through a helix–turn–helix (HTH) motif to temporally control the expression of the restriction enzyme, modification enzyme or both (19,20). This appears to be a tight, finely tuned regulation mechanism, operating via transcriptional feedback circuits (21). Moreover, C proteins can efficiently delay expression of the restriction enzyme during establishment in a new host cell (17,22). In the second type of regulation, the modification enzyme represses transcription of its own gene and sometimes stimulates expression of a restriction gene through DNA binding via its HTH domain (23–25). In the third mode, the coordinated expression of R–M systems depends on the methylation status of the cognate recognition site(s) within their promoter region (26,27).

In the EcoRI R–M system, where two genes form a linearly oriented operon, the restriction endonuclease gene (*ecoRIR*) precedes its modification enzyme gene (*ecoRIM*) (Figure 1A). We previously identified functional promoters for restriction and modification genes (P_R, $P_{M1,M2}$) (Figure 1A and B) (28,29). We also identified a composite reverse promoter, $P_{REV1,REV2}$ within the restriction gene (Figure 1A and B), associated with the negative regulation of the *ecoRIR* gene, and likely the *ecoRIM* gene (28,29).

One of the possible mechanisms underlying the action of the reverse promoter is the transcription of a small antisense RNA. In prokaryotes, small noncoding RNAs are involved in numerous cellular responses to changing environments. The largest group of RNAs studied act by base-pairing with a target RNA to modulate

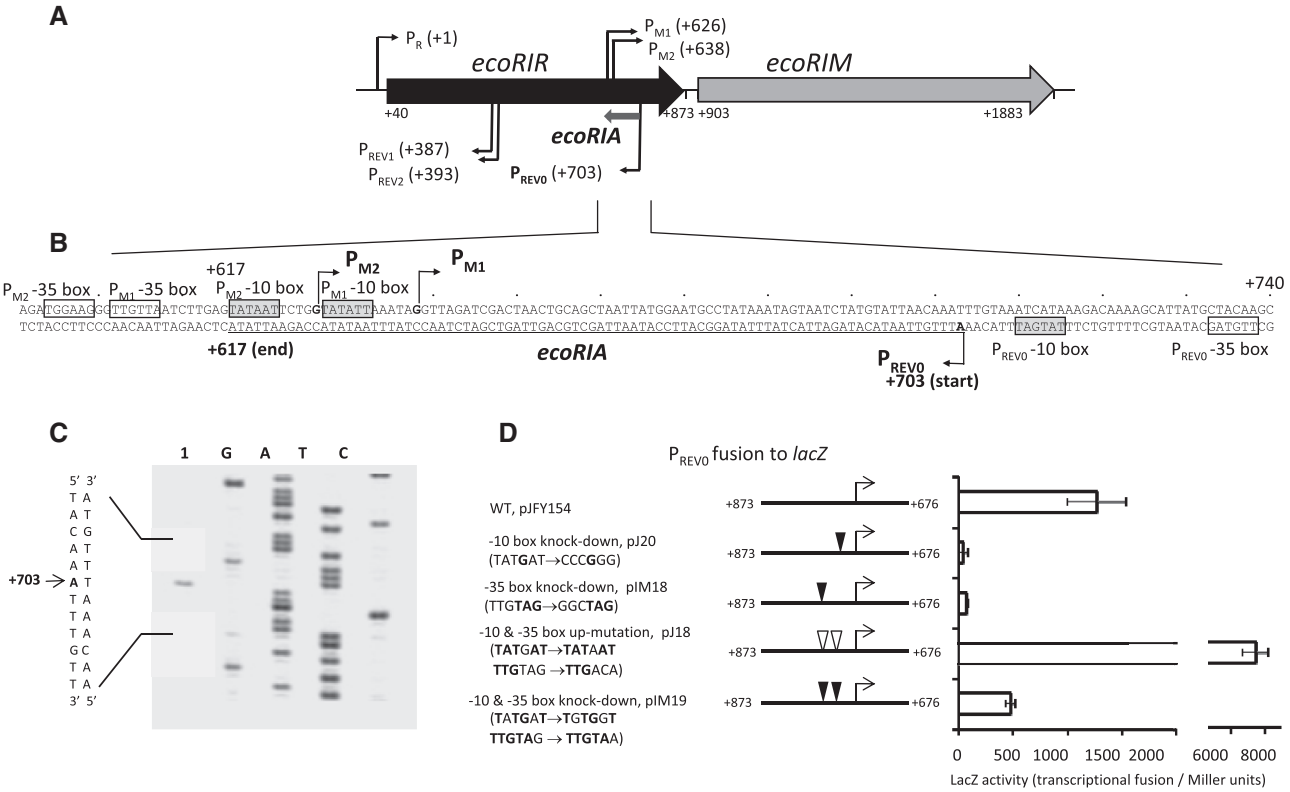

Figure 1. The antisense RNA (Rna0) encoded by the *ecoRIA* gene. (**A**) EcoRI restriction–modification system. Coordinates are relative to the transcription initiation site for the bicistronic *ecoRIRM* mRNA from P_R promoter. P_{M1} and P_{M2}, two overlapping promoters for *ecoRIM* transcription (28); P_{REV1} and P_{REV2}, two overlapping reverse promoters associated with negative effects on expression from the P_R promoter (29); P_{REV0}, a reverse promoter for an antisense RNA studied in this report. (**B**) *ecoRIA* antisense RNA gene. Transcription initiation sites are indicated by arrows. The *ecoRIA* sequence is underlined. Promoter hexamers are boxed in gray (−10 box) and white (−35 box) (11, this work). (**C**) Mapping the transcription initiation site by primer extension. Extension from a *lacZ* gene-specific primer (lacP) (Supplementary Table S1) was carried out using total RNA from *E. coli* harboring pJFY154 (lane 1, see below). Lanes G, A, T and C represent products of the dideoxy sequencing reactions carried out with the same primer. The initiation site is indicated by an arrow. (**D**) Promoter activity of P_{REV0}. DNA fragment carrying P_{REV0} was fused to the *lacZ* gene (pJFY154). Substitutions were made at the −35 (pIM18) or −10 box (pJ20) of the P_{REV0} promoter to knock down promoter activity, as well as to increase its activity, by matching them to the consensus sequences for *E. coli* σ^{70} RNA polymerase (pJ18). P_{REV0} activity with the 3 nt changes present in pIM24 (Table 1) was also measured (pIM19) (see Figure 4 below). These changes did not affect the amino acid sequence. Each value represents an average of four measurements, along with their standard deviations.

Table 1. Plasmids

Name	Relevant features/genotype	References
pIK163	pBR322 carrying *ecoRIRM* operon	(10)
pHSG415	pSC101 derivative, thermo-sensitive replication, Kan^R Ap^R, Cm^R	(41)
pIK172	pHSG415 carrying the WT *ecoRIRM*, Ap^R, Cm^R	(11)
pIK173	pHSG415carrying *ecoRIR–M+*, Ap^R, Cm^R	(11)
pGEM-T	*E. coli* TA cloning vector, Ap^R	Promega
pGEM-T2	pGEM-T carrying an *ecoRIRM* operon fragment (+431 – +612)	(29)
pGEM-T4	pGEM-T carrying an *ecoRIRM* operon fragment (+536 – +777)	(29)
pLY2	pACYC184 carrying promoter-less *lacZ* gene	(29)
pLY66	Transcriptional fusion of a WT *ecoRIRM* operon fragment with P_R promoter (−67 to +400) fused to *lacZ* in pLY2	(29)
pBLY18	pJFY161 derivative, double mutations in −10 boxes of P_{M1M2} promoters (TATAAT to TAGCGG and TATATT to TAAAGC)	This study
pJFY35	Transcriptional fusion of a WT *ecoRIRM* operon fragment (+873 to +660) to *lacZ* in pLY2	This study
pJFY47	Transcriptional fusion of a WT *ecoRIRM* operon fragment (+873 – +474) to *lacZ* in pLY2	This study
pJFY153	Transcriptional fusion of a WT *ecoRIRM* operon fragment (+873 to +535) to *lacZ* in pLY2	This study
pJFY154	Transcriptional fusion of the WT *ecoRIRM* operon fragment (+873 to +676) to *lacZ* in pLY2	This study
pJFY185	Transcriptional fusion of a WT *ecoRIRM* operon fragment (+873 – +633) to *lacZ* in pLY2	This study
pJFY161	Transcriptional fusion of a WT *ecoRIRM* operon fragment (+873 – +585) to *lacZ* in pLY2	This study
pJ18	pJFY154 derivative, up mutations in the −10 and −35 boxes of P_{REV0} (TATGAT→TATAAT; TTGTAG to TTGACA)	This study
pJ20	pJFY154 derivative, down mutations in the −10 box of P_{REV0} (TATGAT→CCCGGG)	This study
pIM13	Transcriptional fusion of a WT *ecoRIRM* operon fragment with promoter $P_{M1,M2}$ (+585 to +784) fused to *lacZ* in pLY2	This study
pIM15	Transcriptional fusion of a WT *ecoRIRM* operon fragment with promoter $P_{M1,M2}$ (+406 to +873) fused to *lacZ* in pLY2	This study
pIM16	Transcriptional fusion of a *ecoRIRM* operon fragment with P_{M1}/P_{M2} promoter (+406 to +709) fused to *lacZ* in pLY2; P_{REV0} promoter hexamers are not present	This study
pIM18	pJFY154 derivative, mutations in the −35 box of P_{REV0} (TTGTAG→GGCTAG)	This study
pIM19	pJFY154 derivative, mutations in the −10 and −35 boxes of P_{REV0} (TATGAT→TGTGGT and TTGTAG→TTGTAA); no change in R.EcoRI amino-acid sequence	This study
pIM11	Transcriptional fusion of a WT *ecoRIR* fragment with P_R and $P_{M1,M2}$ promoters (−67–+784) fused to *lacZ* in pLY2	This study
pIM21	Derivative of pIM11, mutation as in pJ20 to inactivate P_{REV0} promoter	This study
pIM30	Derivative of pIM11, mutations as in pBLY18 to inactivate $P_{M1,M2}$ promoter	This study
pIMRM	Entire WT EcoRI system in pACYC184 backbone; Cm^R	This study
pIM24	pIMRM derivative, 3-nt substitutions as in pIM19 in the −10 and −35 boxes of P_{REV0} (TATGAT→TGTGGT and TTGTAG→TTGTAA); no change in R.EcoRI amino acid sequence	This study
pIM27	pIMRM derivative; R–M+, deletion of HindIII–BglII fragment of *ecoRIR*	This study
pIM-REV0	pUC18 derivative carrying *ecoRIA* gene for the antisense RNA (Rna0) from P_{REV0} promoter; 4-nt up mutations in $P_{M1,M2}$ reverse promoter (TGGAAG→GGGATG; TTGTTA→AAGTTA)	This study
pIM-ΔRNA	pUC18 derivative with a part of *ecoRIR* gene (−67 – +130), used as a negative control for testing antisense RNA	This study

R, restriction; M, modification; WT, wild-type; Cm, chloramphenicol; Ap, ampicillin; Kan, kanamycin.

translation initiation or to promote RNA degradation (30–33). A proportion of the small RNAs acting preferentially *in cis* were discovered mainly in plasmids, phages and transposons (34).

In this work, we discovered another intragenic reverse promoter near the 3′ end of the restriction gene and identified a small antisense RNA transcribed from this region. We demonstrate roles of this antisense RNA gene in the regulation of transcription in this R–M system and in the attack on invading DNA and on host bacteria.

MATERIALS AND METHODS

Bacterial strains and plasmids

Escherichia coli K-12 strains used in this study included MG1655 (35) (from Don Biek, National Cancer Institute, NIH, Bethesda, USA) for the post-segregational cell killing assay and MC1061 [*araD139 Δ(ara-leu)7696 galE15 galK16 Δ(lac)X74 rpsL hsdR2 mcrA mcrB1*] (36) for the measurement of LacZ activity. Strain DH10B [F^- *mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 Deo*^{R} *araD139 Δ(ara-leu)7697 galU galK rpsL (Str*^{R}*) endA1 nupG*] (Invitrogen) was used for plasmid construction.

Construction of the promoter-less reporter, pLY2, was reported previously (28,29). All the *lacZ* transcriptional fusions were constructed in a similar way (Table 1). Corresponding regions of the EcoRI operon from pIK163 (10) were amplified by PCR (primer list in Supplementary Table S1) using KOD plus polymerase (Toyobo), and PCR products were digested with XbaI and cloned into pLY2 linearized with XbaI. All of the substitution mutants were constructed by the megaprimer method (37) and their sequences were confirmed.

The p15A replicon plasmids carrying the entire EcoRI R–M system and its variants were constructed as follows. The wild-type EcoRI system in pIMRM was amplified by PCR using pIK163 plasmid template and primers Mr and Rf (Supplementary Table S1), then PCR products were digested with HincII and XbaI and ligated to pACYC184 linearized with the same restriction enzymes. Its R^-M^+ variant (pIM27) was constructed by cleavage of pIMRM with HindIII and BglII, sites present within the *ecoRIR* gene, followed by T4 DNA polymerase treatment to blunt the overhangs, and finally plasmid self-ligation. The variant of the EcoRI R–M system, pIM24, containing P_{REV0} promoter down mutations, was constructed by site-directed mutagenesis (Stratagene).

Plasmids to deliver antisense RNA *in trans* were generated as pUC18 derivatives as follows. Plasmid pIM-REV0 carrying the antisense gene *ecoRIA* under the P_{REV0} promoter was constructed by PCR amplification of an *ecoRIR* gene fragment (+590–+873) with the 1588bf and 1801r primers, followed by XbaI digestion and cloning into XbaI-linearized pUC18. The 1588bf primer introduced 4 bp substitutions into the composite $P_{M1,M2}$ promoter (−35 hexamers TGGAAG →GGGATG; TTGTTA→AAGTTA) to overcome transcriptional interference and obtain a higher level of antisense RNA. A negative control plasmid carrying no antisense RNA (pIM-ΔRNA) was constructed by cloning part of the *ecoRIR* gene (the same length DNA fragment) closer to its 5′ end, into the pUC18 vector. The sequences of all of the constructs were confirmed.

LacZ activity measurement

LacZ activity was determined using exponentially growing cells in LB medium at 37°C as previously described (38). Duplicate measurements were obtained using two clones of each strain. Error bars indicate the standard deviation of four measurements.

Preparation of RNA probes

The oligonucleotides used for probes and primers are listed in Supplementary Table S1. The PCR product amplified from pGEM-T4 using the 'Colef' and '1800r' primers was used as a template for 'probe a' synthesis by T7 RNA polymerase. 'Probe b', previously designated as the 'right probe' (29), was complementary to *ecoRIR* mRNA from +431 to +612. The PCR product amplified from pIK163 (*ecoRIRM*) by the '1128f' and '1748r' primer pair was used as a template for generating a probe with SP6 RNA polymerase to detect the *ecoRIR* gene by northern blot analysis. Similarly, the PCR product amplified from pIK163 (*ecoRIRM*) by the '1612f' and '1748r' primer pair was used as a template for generating a probe with SP6 RNA polymerase to detect the *ecoRIM* gene by northern blot analysis. *In vitro* transcription with SP6 or T7 RNA polymerase was performed according to the manufacturer's standard transcription protocol (Promega). After 1 h, 10 U of RQ DNase I (Promega) was added to digest the DNA template. The unincorporated ribonucleotides were removed by passage through a Sephadex G-50 column, and transcribed RNAs of the expected sizes were purified by electrophoresis through an 8% polyacrylamide/8 M urea gel.

Total RNA extraction

Escherichia coli MC1061 cells harboring a plasmid of interest were harvested while growing in exponential phase. For all of the small RNA experiments, total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions. In other cases, total RNA was extracted using the RNeasy Protect Bacteria Mini Kit (Qiagen) according to the manufacturer's instructions. After purification, total RNA was treated with DNase I and its concentration and purity was checked by measuring the absorbance at 260/280 nm and by electrophoresis through a 1.2% formaldehyde-agarose gel.

Primer extension

For the primer extension reaction, 20 μg of total RNA was reverse-transcribed with 1 U of AMV Reverse Transcriptase from the Primer Extension System (Promega) in the presence of 1 pmol of [γ-^{33}P] end-labeled primer, according to the manufacturer's protocol. The sequencing reaction with the fmol ®DNA Cycle Sequencing System (Promega) was performed with the same end-labeled primer and template used for the primer extension, according to the manufacturer's protocol. The reaction products were resolved through an 8% sequencing gel, dried and visualized by exposure to a PhosphorImager screen.

RNase protection

Total RNA annealed to a corresponding complementary RNA probe was digested with *E. coli* RNase I (Promega), with a minor modification to the manufacturer's protocol (39). Briefly, the same quantity of total RNA was ethanol co-precipitated using an excess of purified RNA probe and the same quantity of loading control (a [γ-^{33}P] end-labeled single-stranded DNA probe, see Supplementary Table S1). The precipitate was dissolved, hybridized overnight at 45°C, and digested according to the manufacturer's protocol. Finally, the 'RNase-protected' fragments were resolved in a 10% denaturing polyacrylamide gel, dried and visualized by exposure to a PhosphorImager screen or an X-ray film.

Phage restriction

Restriction activity *in vivo* was estimated by the ability of cells with an R–M system to restrict plaque formation of λ*vir* bacteriophage. The media used for these experiments contained selective antibiotics. Phage titer was determined by plaque formation using the top agar overlay technique (40).

Post-segregational killing

LB media containing two selective antibiotics was inoculated with a single colony of *E. coli* MG1655 bearing two plasmids. One plasmid carried a thermo-sensitive replication unit with a wild-type EcoRI R–M system

(pIK172), its restriction-negative variant (pIK173) or no EcoRI R–M system (vector control) (pHSG415) (41). The other compatible plasmid was a pUC18 derivative carrying a gene (*ecoRIA*) for the antisense RNA. After 10–12 h of incubation at 30°C, the cultures were diluted and spread quantitatively either on LB agar containing the appropriate selective antibiotics for both plasmids or on LB agar without any antibiotics, and were incubated at 30°C or 42°C, respectively. Subsequently, the colonies were counted and the ratio of colony-forming units under the two conditions was determined. In parallel, a qualitative spot test was carried out by dropping the same volume of diluted culture on to agar plates.

Bioinformatic analyses

In silico promoter prediction and terminator prediction were performed using the BPROM and FindTerm software, respectively (http://www.softberry.com/all.htm). RNA secondary structure was predicted with RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

RESULTS

Novel reverse promoter (P_{REV0}) at the 3′-end of the restriction endonuclease gene

Organization of the EcoRI system along with its promoters is shown in Figure 1A. We previously demonstrated that a down-mutation in the composite reverse promoter, $P_{REV1,REV2}$ within the restriction gene (Figure 1A) can increase expression of the restriction gene from the P_R promoter (29). In the present work, we tested another reverse promoter candidate, revealed by *in silico* analysis, located at the 3′-terminus of the *ecoRIR* gene (Figure 1A and B). The corresponding DNA fragment (position +873–+676) was placed in front of the promoter-less *lacZ* gene. The resulting transcriptional fusion indeed showed high activity (pJFY154, Figure 1D). We designated this reverse promoter as P_{REV0}.

To identify the transcription initiation site of this reverse promoter P_{REV0}, a *lacZ* gene-specific primer (lacP; Table S1) was end-labeled with [γ-^{33}P]ATP. Extension of this primer hybridized to the total RNA from *E. coli* harboring pJFY154, revealed that the adenine at coordinate +703 functions as the transcription initiation site (Figure 1B and C).

Based on the location of this transcription initiation site and the consensus sequence of *E. coli* σ^{70} RNA polymerase promoters (42,43), we deduced −10 and −35 boxes of P_{REV0} as TATGAT (consensus TATAAT) and T TGTAG (consensus TTGACA), respectively (Figure 1B). An optimal spacer length of 6 bp was found between the putative −10 box and the transcription initiation site. A 17-bp distance between putative −10 and −35 boxes (Figure 1B) is considered optimal in *E. coli* promoters (43,44).

To verify the functionality of these promoter elements, we generated a set of transcriptional fusion constructs containing mutations (Figure 1D). Down mutations in the putative −10 or −35 boxes greatly decreased transcriptional activity (pJ20 and pIM18, respectively, Figure 1D), whereas up mutations, matching the consensus sequence in both the −10 and −35 boxes, increased activity about 6-fold (pJ18, Figure 1D).

Antisense RNA (Rna0) from the reverse promoter

To map the termination site of the antisense RNA from P_{REV0}, P_{REV0}-carrying DNA fragments of increasing downstream length (Figure 2A) were fused to the promoter-less *lacZ* gene. The sudden drop in *lacZ* transcription activity between the +873/+633 fragment and the +873/+585 fragment (Figure 2A) suggested termination between +585 and +633, although molecular mechanisms other than termination should also be considered and will be explored in the 'Discussion' section.

To detect antisense RNA expressed from the P_{REV0} promoter *in vivo* in the context of the EcoRI R–M system, a sensitive RNase protection assay was performed. A 242-nt ^{32}P-labeled RNA probe (complementary from +536 to +777) was synthesized *in vitro*. The excess probe was hybridized with the total RNA extracted from the cells and cleaved with RNase I to remove RNA overhangs. A specific protected transcript was detected from *E. coli* harboring a plasmid carrying WT EcoRI R-M system (pIK163) or a plasmid carrying 3′ half of the restriction gene (pJFY47) (Figure 2B).

We compared the lengths of the protected signals in the RNase protection assay between *E. coli* harboring pJFY161 (+873–+585) and pJFY47 (+873–+470) to define the transcription termination site in detail (Figure 2C). The comparable size of the protected signals revealed that the transcript could not be read through position +585. The estimated length of the antisense RNA was about 88 nt, based on the size of RNA markers (Figure 2C). This result was consistent with the above transcriptional fusion data (Figure 2A) and indicated that the apparent termination site of antisense transcription is around coordinate +617, which is located within the $P_{M1,M2}$ promoter (Figure 1B).

Neither a start codon nor an open reading frame (ORF) was detected in this RNA, suggesting that this RNA represents a noncoding, small RNA. We designated this antisense RNA gene as *ecoRIA* (A for antisense) and this RNA as Rna0. We predicted the secondary structure of this RNA (Supplementary Figure S1) and the 5′ terminus of the mRNA transcribed from $P_{M1,M2}$ (Supplementary Figure S2).

Mutual interference between the $P_{M1,M2}$ and P_{REV0} promoters

Termination of antisense transcription from P_{REV0} occurred at $P_{M1,M2}$ a composite promoter in the sense orientation (Figure 1B). We did not find any structures resembling Rho-independent-terminators using terminator prediction software. This suggested that the promoter function of $P_{M1,M2}$ interferes with P_{REV0}-initiated antisense transcription. Indeed, down mutations in the −10 boxes of the $P_{M1,M2}$ composite promoter

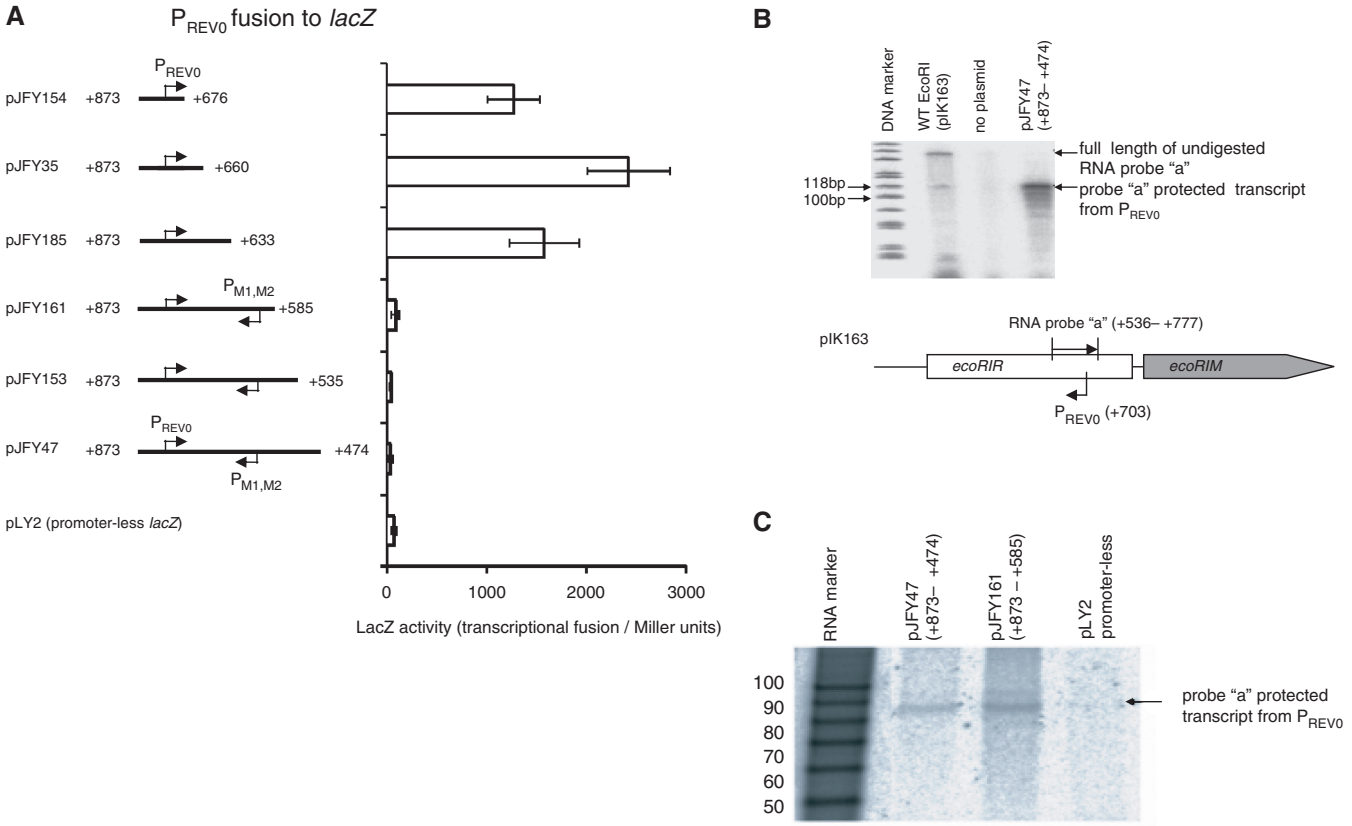

Figure 2. Mapping the antisense RNA (Rna0). (**A**) Mapping by transcriptional fusion. DNA fragments of varying sizes containing P_{REV0} were cloned upstream of the promoter-less *lacZ* gene. Each value represents an average of four measurements, along with their standard deviations. (**B**) RNase protection assay. Lane 1, ΦX174 DNA/HinfI size markers (Promega); lane 2, total RNA from *E. coli* MC1061 carrying pIK163 (wild-type EcoRI R–M system); lane 3, total RNA from the same strain without a plasmid (negative control); lane 4, total RNA from *E. coli* MC1061 carrying plasmid pJFY47. The bands below the protected band are likely a result of degradation due to unstable, double-stranded AT-rich ends. A scheme of the RNase protection assay using 'probe a', a sense RNA synthesized *in vitro*, is presented at the bottom of the figure. A map of pJY47 is shown in (A). (**C**) Length estimation by RNase protection. RNA was detected as in (B). Lane 1, RNA length marker; other lanes, detected RNA in the total RNA of *E. coli* MC1061 carrying: plasmid pJFY47 (lane 2); plasmid pJFY161 (lane 3); or plasmid pLY2 as the negative control (lane 4). Maps of the promoter fragments are shown in (A).

increased transcription about 20-fold (pJFY161 versus pBLY18, Figure 3).

Because of the symmetry of the two promoters, we examined whether $P_{M1,M2}$-initiated sense transcription was, in turn, inhibited by the antisense promoter P_{REV0}. When DNA fragments, containing the $P_{M1,M2}$ promoter, as well as the entire antisense RNA gene, and P_{REV0} were fused to the promoter-less *lacZ* gene (pIM13 and pIM15), little activity was detected (Figure 3). Whereas, deletion of the P_{REV0} promoter (pIM16) increased the activity 7-fold (Figure 3). This suggested that P_{REV0} function somehow interferes with transcription from $P_{M1,M2}$.

These *in vivo* results indicate that the P_{REV0} reverse promoter action apparently inhibits transcription from the $P_{M1,M2}$ promoters and *vice versa*. Possible underlying mechanisms for this will be considered in the 'Discussion' section. The net strength of both promoters under inhibition was comparable (pJFY161 versus pIM13, Figure 3), suggesting that they both actively contribute to maintaining fine-tuned coordination of their expression (see 'Discussion' section).

Effect of the reverse promoter mutation on P_R activity

To test whether the reverse promoter P_{REV0} affects *ecoRIR* expression from P_R, we cloned a fragment of WT restriction gene (−67–+783) in front of promoter-less *lacZ* gene (Figure 4). This restriction endonuclease gene fragment lacked the corresponding codons for 32-amino-acid residues from C-terminus and was inactive (data not shown). The inactivation of P_{REV0} promoter (plasmid pIM21) increased the net expression by 3.5-fold (Figure 4, 2nd line). $P_{M1,M2}$ is not responsible for this high expression because a double mutant of $P_{M1,M2}$ and P_{REV0} promoters (pIM32) showed a 4-fold increase (Figure 4, 3rd line). These results demonstrated that P_{REV0} plays some negative role in transcription initiated at P_R.

R–M system with a down mutation in the reverse promoter

Next, we investigated the phenotype of the entire EcoRI R–M system with a down mutation in the reverse promoter P_{REV0}. We changed the P_{REV0} promoter sequence while

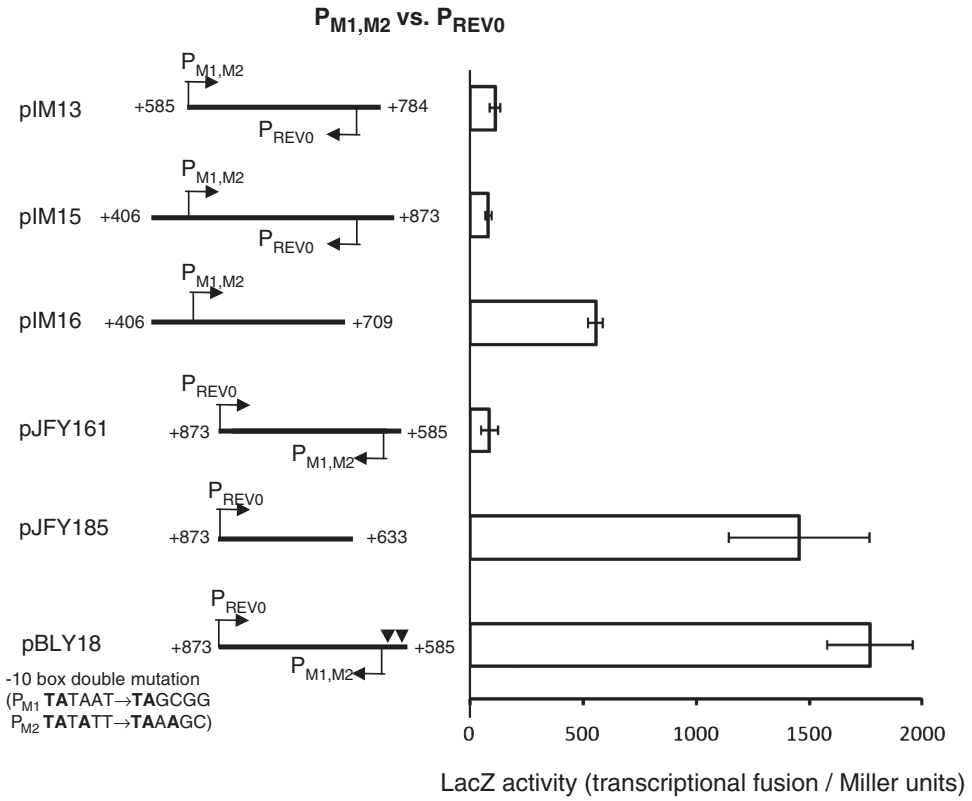

Figure 3. Interference between $P_{M1,2}$ and P_{REV0} promoters in transcriptional fusion. Each value represents an average of four measurements, along with their standard deviations.

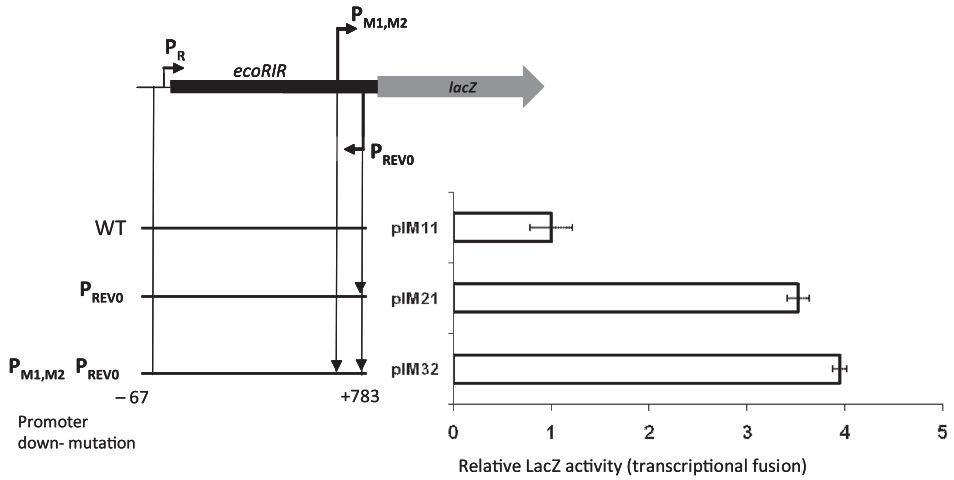

Figure 4. Effect of P_{REV0} reverse promoter on P_R activity in transcriptional fusion. Each value represents an average of four measurements, along with their standard deviations.

maintaining the amino acid sequence for the restriction endonuclease.

Based on the P_{REV0} promoter, we initially designed all possible substitutions (6 bp) for the −10 and −35 promoter hexamers. Despite many attempts, such mutants could not be generated by site-directed mutagenesis using a plasmid harboring the wild-type EcoRI R–M system as a template (pIMRM). Among the screened clones, the majority were found to be restriction-negative, i.e. the restriction gene was disrupted. A small number of restriction-positive clones were identified, which, after sequencing, were found to possess only 2- or 3-nt changes out of the intended 6-bp changes. These changes did not change the amino acid sequence of the restriction endonuclease. We selected one such clone, pIM24, with 3-bp changes in the P_{REV0} promoter: TATGAT

→TGTGGT; TTGTAG→TTGTAA. This 3-bp substitution did not abolish P_{REV0} promoter activity, but led to a 2.6-fold drop in expression of the *lacZ* fusion (Figure 1D, pJFY154 versus pIM19).

Reverse promoter mutation strengthens restriction

We examined the effect of the P_{REV0} promoter down mutation on restriction activity. We compared plaque formation with that of λ bacteriophage, which carries five EcoRI sites (Table 2). The 3-bp substitution mutation conferred almost 9-fold higher restriction than the wild type (Table 2). This result may explain why we could not obtain viable cells with the EcoRI R–M system with mutations that were expected to more severely inactivate P_{REV0} and lead to high levels of restriction, potentially killing the host bacterial cell. These results clearly indicated the biological significance of this reverse promoter, although the underlying mechanisms are yet to be elucidated (see 'Discussion' section).

Antisense RNA gene *in trans* alleviates post-segregational host killing

To further understand the biological role of the antisense RNA in modulation of EcoRI R–M system activity, we examined its role in the attack on host bacterial cells during post-segregational killing (see 'Introduction' section).

We generated a two-plasmid system in *E. coli* cells, in which the EcoRI R–M system was carried by a plasmid with a thermo-sensitive replicator, accompanied by a compatible, high-copy number, pUC18-derived plasmid containing the gene (*ecoRIA*) for the antisense RNA driven from its natural (P_{REV0}) promoter (Table 1). Host cell death was measured after induction of loss of the EcoRI R–M plasmid by blocking its replication through a temperature shift to 42°C. As shown in Figure 5A, the viability of cells with the wild-type EcoRI R–M system (pIK172) in the absence of antisense RNA *in trans,* showed a significant decrease of 10^4-fold compared with the undisturbed viability of restriction-negative variants (pHSG415 and pIK173), as reported earlier (10). However, the cells survived the post-segregational killing challenge about 15-fold better in the presence of the antisense RNA gene *ecoRIA* on a second plasmid (pIM-REV0, Figure 5A, Table 1). These results were confirmed in a qualitative spot assay (Figure 5B).

Table 2. Reverse promoter mutation increases restriction

Plasmid	Genotype	Plaque-forming units	Efficiency of plaque formation[a]	Restriction relative to WT R–M
pACYC184	R^- M^- (vector)	$(2.4 \pm 0.4) \times 10^8$	1	
pIM27	R^- M^+	$(2.2 \pm 0.3) \times 10^8$	0.9	
pIMRM	WT (R^+ M^+)	$(1.3 \pm 0.3) \times 10^6$	5.4×10^{-3}	1
pIM24	P_{REV0} down[b]	$(1.5 \pm 0.2) \times 10^5$	6.2×10^{-4}	8.7

R, restriction; M, modification; WT, wild type.
[a]Efficiency of plaque formation = plaque-forming units on tested plasmid divided by plaque-forming units on pACYC184.
[b]TATGAT→TGTGGT and TTGTAG→TTGTAA, with no change in R.EcoRI amino acid sequence.
The host bacterium was *E. coli* MG1655. The standard deviation from four measurements is indicated.

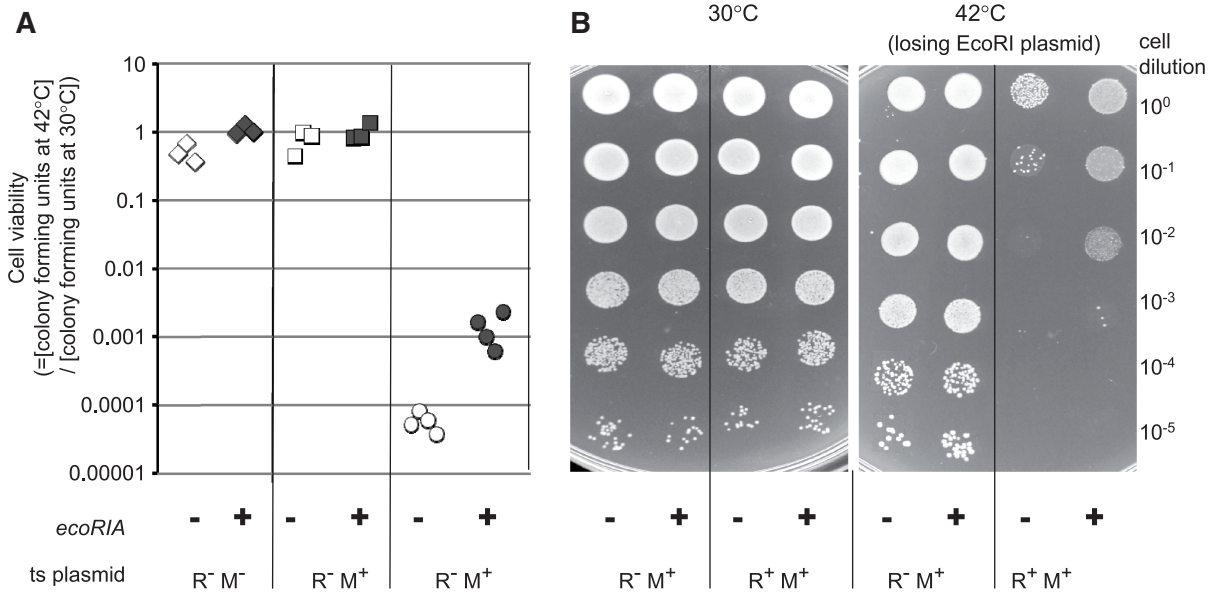

Figure 5. Effect of the antisense RNA gene *ecoRIA* on post-segregational host killing by the EcoRI R–M system. Cell survival after loss of the EcoRI R–M system was measured in the presence of the antisense RNA gene (*ecoRIA*) in a quantitative (**A**) and a qualitative assay (**B**). The *E. coli* cells carried two plasmids. One was from a series of plasmids harboring EcoRI R–M system variants on a thermo-sensitive pSC101 replicator: vector pHSG415 (diamond); pIK173 (R^- M^+ square) or pIK172 (R^+ M^+ circle). The other was from a series of pUC-derivatives to deliver antisense RNA from the P_{REV0} promoter and *ecoRIA* gene (pIM-REV0; black) or not, as the negative control (pIM-ΔRNA; white). Dilutions of the culture were spread (A) or spotted (B) on to an agar plate for incubation at a temperature permissive for replication of the RM plasmid (30°C) or at a temperature nonpermissive for its replication (42°C).

A simplest interpretation of these observations is that the antisense RNA (Rna0) transcribed from the P_{REV0} promoter was delivered *in trans* and somehow affected the activity of the EcoRI R–M system. However, other possibilities should also be considered (see 'Discussion' section).

DISCUSSION

In the present study of the EcoRI R–M system, we identified an antisense RNA (Rna0) from a novel reverse promoter (P_{REV0}) at the 3′ end of the restriction gene and demonstrated the role of this antisense RNA gene in the modulation of gene expression in this R–M system and the attack of incoming and resident DNA. In EcoRI R–M system transcription unit, there are at least six experimentally tested active promoters, where some of them act in overlapping tandems ($P_{REV1,REV2}$; $P_{M1,M2}$) and some are arranged convergently (P_R versus $P_{REV1,REV2}$; P_{REV0} versus $P_{M1,M2}$) with one promoter facing other promoter within a *ecoRIR* coding sequence (Figure 1A).

Transcriptional fusion revealed that this reverse promoter (P_{REV0}) and the promoter for the downstream modification gene ($P_{M1,M2}$) apparently inhibit each other's transcription (Figure 3). The $P_{M1,M2}$-mediated inhibition of P_{REV0}-initiated transcription resulted in its apparent termination at $P_{M1,M2}$ (Figures 1 and 2).

Such interference of transcription could take place through various mechanisms. For example (i) transcription termination by a roadblock, in which an RNA polymerase (RNAP) complex bound to a promoter displaces RNAP initiated from an opposite promoter causing termination of transcription; (ii) occlusion, in which a promoter is prevented from RNAP binding due to transient occupation by a passage of elongating RNAP from a facing promoter; (iii) collision of two elongating RNAP complexes leading to premature transcription termination; (iv) sitting duck interference, in which a transcriptional-initiation complex is hit and dislodged by the arrival of an elongation complex from a (stronger) external promoter (45–49); in addition, another possibility is (v) RNA–RNA interference, in which a transcript may be degraded or somehow prevented from being translated after interaction with its complementary RNA formed from the other promoter.

For the inhibition of P_{REV0}-initiated transcription by $P_{M1,M2}$, possibilities (ii) and (iii) seem unlikely (because the transcript is initiated). Possibility (iv) is also unlikely because the apparent termination of antisense transcription occurs past the transcription initiation site. These leave (i) (termination) and (v) (RNA–RNA) as the most likely explanations. For the inhibition of $P_{M1,M2}$-initiated transcription by P_{REV0}, however, we cannot exclude any of these five possibilities. A 'sitting duck' model has been suggested for regulation of an R–M system (50).

Our investigations have partly depended on the use of promoter mutations. However, we have to remember that other post-transcriptional events such as translation initiation and RNA stability could be affected by sequence changes. For reference, we included a prediction of the secondary structure of Rna0 and the 5′ end of the modification enzyme transcript from $P_{M1,M2}$ (Supplementary Figures S1 and S2). Because our approach involved transcriptional fusion instead of translational fusion, we do not know the effect, if any, of the reverse promoter on translation from the $P_{M1,M2}$-initiated transcript.

The presence of a linked reverse promoter might explain the failure to detect strong promoter activity in other R–M systems, such as SalI, with a similar gene organization (51,52).

Convergent promoters have been reported in the regulatory regions of several lambdoid phages, where they control the lysis/lysogeny switch (46,53). Others reported their impact on the switch between replication and conjugational transfer by plasmids (54,55). The net strength of the $P_{M1,M2}$ promoter and the P_{REV0} promoter under inhibition was comparable. This suggested that both promoters actively contributed to maintaining coordination of their expression. However, this may represent an unstable equilibrium, whereby activation of one promoter over a threshold may inactivate the other promoter, which would further activate the first promoter (56). This positive feedback loop can form a bi-stable switch, which may be involved in the lifestyle of R–M systems. For example, in the absence of previously synthesized R and M proteins after entry into a new host cell, P_M action may be dominant. Accumulation of M protein then might lead to shift toward P_{REV0} activity. This may be similar to the bi-stable switch in the life style of several lysogenic bacteriophages (57,58).

In SsoII and Ecl18kI R–M systems, the restriction and modification genes are divergent, but their promoters are convergent (R gene – P_M – P_R – M gene). Their regulation was found to be associated with interference between the two convergent promoters (25,50). In these R–M systems, as well as in MspI (59), two feedback loops have been identified. One, a negative feedback loop, is related to the autoregulation of the modification enzyme and the other, a positive feedback loop, is associated with a boost of restriction transcription after interaction of the modification enzyme with its binding site within the intergenic region.

The effect of reverse promoter mutations on the restriction of incoming bacteriophage DNA indicates the biological relevance of the present study. However, to fully understand the underlying molecular mechanisms, we have to analyze the roles of other promoters, post-transcriptional processes, R–M gene products and host factors, and assemble them into a network of interactions.

The effect of the antisense RNA gene on post-segregational host killing provides another line of evidence for the relevance of our findings to the biology of R–M systems. This *trans* effect may also provide a clue to understanding the interference between the convergent promoters. Among the four possible mechanisms considered above, (v) namely, degradation or some functional alteration of RNA triggered by its interaction with a complementary RNA, can easily explain this *trans* effect. However, the five mechanisms were proposed to explain the results of the transcriptional fusions, and

this post-segregational killing process includes other processes.

In antisense-RNA-mediated post-segregational killing systems, the antisense RNAs downregulates the toxin gene by basepairing with target toxin mRNA to modulate translation initiation or to promote mRNA degradation (13,60–62), as most small noncoding RNAs in prokaryotes do (30–33). If the antisense RNA (Rna0) alleviates the toxin (R) expression *in trans*, its action may turn out to be similar to that of these antisense RNAs. Because excess of sequence-specific anti-sense RNA molecules might result in nonspecific down-modulation, further experimental analysis is needed to prove this in the natural context.

In conclusion, our work has demonstrated a novel mechanism of R–M system regulation by noncoding, intragenic, antisense transcription. This novel regulatory system is biologically important in controlling toxic endonuclease genes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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