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 (PREV0) at the 3’ end of the restriction gene. Its antisense transcription, as measured by transcriptional gene fusion, appeared to be terminated by the PM1,M2 promoter. PM1,M2 promoter-initiated transcription, in turn, appeared to be inhibited by PREV0. Mutational inactivation of PREV0 increased expression of the restriction gene. The biological significance of this antisense transcription is 2-fold. First, a mutation in PREV0 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...
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 (ecoRII) precedes its modification enzyme gene (ecoRIIM) (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 ecoRII gene, and likely the ecoRIIM 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
and TATATT to TAA

E. coli TA cloning vector, Ap<sup>R</sup>. Promega

Table 1. Plasmids

<table>
<thead>
<tr>
<th>Name</th>
<th>Relevant features/genotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>pIK163</td>
<td>pBR322 carrying ecoRIRM operon</td>
<td>(10)</td>
</tr>
<tr>
<td>pHSG415</td>
<td>pUC19 derivative, thermosensitive replication, Kan&lt;sup&gt;R&lt;/sup&gt;, Ap&lt;sup&gt;R&lt;/sup&gt;, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(41)</td>
</tr>
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<td>pIK172</td>
<td>pHSG415 carrying the WT ecoRIRM, Ap&lt;sup&gt;R&lt;/sup&gt;, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(11)</td>
</tr>
<tr>
<td>pIK173</td>
<td>pHSG415 carrying ecoRIRM-M&lt;sup&gt;+&lt;/sup&gt;, Ap&lt;sup&gt;R&lt;/sup&gt;, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(1)</td>
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<tr>
<td>pGEM-T</td>
<td>E. coli TA cloning vector, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>pGEM-T2</td>
<td>pGEM-T carrying an ecoRIRM operon fragment (+431 – +612)</td>
<td>(29)</td>
</tr>
<tr>
<td>pGEM-T4</td>
<td>pGEM-T carrying an ecoRIRM operon fragment (+536 – +777)</td>
<td>(29)</td>
</tr>
<tr>
<td>pLY2</td>
<td>pACYC184 carrying promoter-less lacZ gene</td>
<td>(29)</td>
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<td>pLY66</td>
<td>Transcriptional fusion of a WT ecoRIRM operon fragment with Pr promoter (–67 to +400) fused to lacZ in pLY2</td>
<td>(29)</td>
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<td>pBLY18</td>
<td>pFYJ154 derivative, double mutations in –10 boxes of P&lt;sub&gt;MI,M2&lt;/sub&gt; promoters (TATAAT to TCTGCG and TATATT to TAAAGC)</td>
<td>This study</td>
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<td>pFYJ35</td>
<td>Transcriptional fusion of a WT ecoRIRM operon fragment (+873 to +660) to lacZ in pLY2</td>
<td>This study</td>
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<td>Transcriptional fusion of a WT ecoRIRM operon fragment (+873 – +474) to lacZ in pLY2</td>
<td>This study</td>
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<td>pFYJ153</td>
<td>Transcriptional fusion of a WT ecoRIRM operon fragment (+873 to +535) to lacZ in pLY2</td>
<td>This study</td>
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<td>pFYJ154</td>
<td>Transcriptional fusion of the WT ecoRIRM operon fragment (+873 to +676) to lacZ in pLY2</td>
<td>This study</td>
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<td>Transcriptional fusion of a WT ecoRIRM operon fragment (+873 – +633) to lacZ in pLY2</td>
<td>This study</td>
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<td>pFYJ161</td>
<td>Transcriptional fusion of a WT ecoRIRM operon fragment (+873 – +585) to lacZ in pLY2</td>
<td>This study</td>
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<td>pH18</td>
<td>pFYJ154 derivative, up mutations in the –10 and –35 boxes of PREV0 (TATGAT – TCTGCG)</td>
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<td>pFYJ162</td>
<td>pFYJ154 derivative, double mutations in the –10 box of Pr&lt;sub&gt;REV&lt;/sub&gt; (TATGAT – CCCGCG)</td>
<td>This study</td>
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<td>pFYJ163</td>
<td>Transcriptional fusion of a WT ecoRIRM operon fragment with promoter P&lt;sub&gt;M1,M2&lt;/sub&gt; (+585 to +784) fused to lacZ in pLY2</td>
<td>This study</td>
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<td>pFYJ164</td>
<td>Transcriptional fusion of a WT ecoRIRM operon fragment with promoter P&lt;sub&gt;M1,M2&lt;/sub&gt; (+406 to +783) fused to lacZ in pLY2</td>
<td>This study</td>
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<td>pFYJ165</td>
<td>Transcriptional fusion of a WT ecoRIRM operon fragment with promoter P&lt;sub&gt;M1,M2&lt;/sub&gt; (+406 to +790) fused to lacZ in pLY2; Pr&lt;sub&gt;REV&lt;/sub&gt; promoter hexamers are not present</td>
<td>This study</td>
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<td>pFYJ166</td>
<td>pFYJ154 derivative, mutations in the –35 box of Pr&lt;sub&gt;REV&lt;/sub&gt; (TCTGAT – GGGTAGG)</td>
<td>This study</td>
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<td>pFYJ167</td>
<td>pFYJ154 derivative, mutations in the –10 and –35 boxes of Pr&lt;sub&gt;REV&lt;/sub&gt; (TCTGAT – GGGTAGG and TCTGAT – TTGAAA); no change in R.EcoRI amino acid sequence</td>
<td>This study</td>
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<tr>
<td>pFYJ168</td>
<td>Transcriptional fusion of a WT ecoRIR operon fragment with Pr and P&lt;sub&gt;M1,M2&lt;/sub&gt; promoters (–67 +784) fused to lacZ in pLY2</td>
<td>This study</td>
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<td>pFYJ169</td>
<td>Derivative of pFYJ161, mutation as in pFYJ20 to inactivate Pr&lt;sub&gt;REV&lt;/sub&gt; promoter</td>
<td>This study</td>
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<td>pFYJ170</td>
<td>Derivative of pFYJ161, mutations as in pBLY18 to inactivate P&lt;sub&gt;M1,M2&lt;/sub&gt; promoter</td>
<td>This study</td>
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<tr>
<td>pFYJ171</td>
<td>Entire WT EcoRI system in pACYC184 backbone; Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
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<td>pFYJ172</td>
<td>pMRM derivative, 3-nt substitutions as in pFYJ19 in the –10 and –35 boxes of Pr&lt;sub&gt;REV&lt;/sub&gt; (TATGAT – GGGTAGG and TCTGAT – TTGAAA); no change in R.EcoRI amino acid sequence</td>
<td>This study</td>
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<tr>
<td>pFYJ173</td>
<td>pMRM derivative; R–M&lt;sup&gt;+&lt;/sup&gt;, deletion of HindIII–BglII fragment of EcoRI</td>
<td>This study</td>
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<tr>
<td>pFYJ174</td>
<td>pUC18 derivative carrying ecoRIA gene for the antisense RNA (Rna0) from Pr&lt;sub&gt;REV&lt;/sub&gt; promoter; 4-nt up mutations in P&lt;sub&gt;M1,M2&lt;/sub&gt; reverse promoter (TGGAAAG – CCGATG; TTGTTA – AAGTTA)</td>
<td>This study</td>
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<tr>
<td>pFYJ175</td>
<td>pUC18 derivative with a part of ecoRIR gene (–67 – +130), used as a negative control for testing antisense RNA</td>
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</table>

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

ded 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(mcr-hsdRMS-mcrBC) pho80lacZAM15 ΔlacX74 recA1 Deo<sup>R</sup> araD139 Δ(ara-leu)7697 galU galK rpsL (Str<sup>R</sup>) endA1 mup<sup>G</sup>] (Invitrogen) was used for plasmid construction.

Construction of the promoter-less reporter, pLY2, was previously reported (28,29). All the lacZ transcriptional fusions were constructed in a similar way (Table 1). Corresponding regions of the EcoRII 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_{M1,2} promoter (–35 hexamers TGGAAAG →GGGATG; TTGTGA→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 the sense 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.

### 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 RNasy 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-32P] 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-32P] 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 software, respectively (http://www.softberry.com/all.htm).

RESULTS

Novel reverse promoter (PREV0) 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, PREV1,REV2 within the restriction gene (Figure 1A) can increase expression of the restriction gene from the PR 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 promoterless lacZ gene. The resulting transcription fusion indeed showed high activity (pJFY154, Figure 1D). We designated this reverse promoter as PREV0.

To identify the transcription initiation site of this reverse promoter PREV0, a lacZ gene-specific primer (lacP; Table S1) was end-labeled with [γ-32P]ATP. Extension of this primer hybridized to the total RNA from *E. coli* harboring pJFY154, revealed that the adenine at coordinate +703 RNA 0 terminus (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 PREV0 as TATGAT (consensus TATAAT) and TTGAT (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 PREV0, PREV0-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 PREV0 promoter *in vivo* in the context of the EcoRI R–M system, a sensitive RNase protection assay was performed. A 242-nt 32P-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 PREV0 promoters

Termination of antisense transcription from PREV0 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 PREV0-initiated antisense transcription. Indeed, down mutations in the −10 boxes of the P_{M1,M2} composite promoter
increased transcription about 20-fold (pJFY161 versus pBLY18, Figure 3).

Because of the symmetry of the two promoters, we examined whether PM1,M2-initiated sense transcription was, in turn, inhibited by the antisense promoter PREV0. When DNA fragments, containing the PM1,M2 promoter, as well as the entire antisense RNA gene, and PREV0 were fused to the promoter-less \textit{lacZ} gene (pIM13 and pIM15), little activity was detected (Figure 3). Whereas, deletion of the PREV0 promoter (pIM16) increased the activity 7-fold (Figure 3). This suggested that PREV0 function somehow interferes with transcription from PM1,M2.

These \textit{in vivo} results indicate that the \textit{PREV0} reverse promoter action apparently inhibits transcription from the PM1,M2 promoters and \textit{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 PR activity**

To test whether the reverse promoter \textit{PREV0} affects \textit{ecoRIR} expression from \textit{PR}, we cloned a fragment of WT restriction gene (−67−+783) in front of promoter-less \textit{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 \textit{PREV0} promoter (plasmid pJFY47) increased the net expression by 3.5-fold (Figure 4, 2nd line). PM1,M2 is not responsible for this high expression because a double mutant of PM1,M2 and \textit{PREV0} promoters (pIM32) showed a 4-fold increase (Figure 4, 3rd line). These results demonstrated that \textit{PREV0} plays some negative role in transcription initiated at \textit{PR}.

**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 \textit{PREV0}. We changed the \textit{PREV0} promoter sequence while
maintaining the amino acid sequence for the restriction endonuclease.

Based on the \( P_{\text{REV0}} \) 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 \( P_{\text{REV0}} \) promoter: TATGAT

\[ \text{TATGAT} \]

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

Figure 4. Effect of \( P_{\text{REV0}} \) reverse promoter on \( P_{\text{R}} \) activity in transcriptional fusion. Each value represents an average of four measurements, along with their standard deviations.
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 \( \text{lacZ} \) 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 \( \lambda \) 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 (\( \text{ecoRIA} \)) 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 \( \text{ecoRIA} \) 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

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype</th>
<th>Plaque-forming units</th>
<th>Efficiency of plaque formation</th>
<th>Restriction relative to WT R–M</th>
</tr>
</thead>
<tbody>
<tr>
<td>pACYC184</td>
<td>( R^- M^- ) (vector)</td>
<td>( 2.4 \pm 0.4 \times 10^6 )</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>pIM27</td>
<td>( R^- M^- )</td>
<td>( 2.2 \pm 0.3 \times 10^6 )</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>pIMRM</td>
<td>( R^+ M+ ) (WT)</td>
<td>( 1.3 \pm 0.3 \times 10^6 )</td>
<td>5.4 \times 10^-3</td>
<td>1</td>
</tr>
<tr>
<td>pIM24</td>
<td>( P_{REV0} ) down</td>
<td>( 1.5 \pm 0.2 \times 10^6 )</td>
<td>6.2 \times 10^-1</td>
<td>8.7</td>
</tr>
</tbody>
</table>

\( R \), restriction; \( M \), modification; WT, wild type.

*Efficiency of plaque formation = plaque-forming units on tested plasmid divided by plaque-forming units on pACYC184.

\( \text{TATGAT}\rightarrow\text{TGTGGA} \) and \( \text{TGTGAG}\rightarrow\text{TGTGAA} \), 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 \( \text{ecoRIA} \) 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 \( \text{ecoRIA} \) 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 \( \text{ecoRIA} \) gene (pIM-REV0; black) or not, as the negative control (pIM-ARNA; 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).
DISCUSSION

In the present study of the EcoRI R–M system, we identified an antisense RNA (Rna0) from a novel reverse promoter (PREV0) 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 (PREV1, REV2; PM1,M2) and some are arranged convergently (PR versus PREV1, REV2; PREV0 versus PM1,M2) with one promoter facing other promoter within a coreIR coding sequence (Figure 1A).

Transcriptional fusion revealed that this reverse promoter (PREV0) and the promoter for the downstream modification gene (PM1,M2) apparently inhibit each other’s transcription (Figure 3). The PM1,M2-mediated inhibition of PREV0-initiated transcription resulted in its apparent termination at PM1,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 PREV0-initiated transcription by PM1,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 PM1,M2-initiated transcription by PREV0, 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 PM1,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 PM1,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 PM1,M2 promoter and the PREV0 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, Pm action may be dominant. Accumulation of M protein then might lead to shift toward PREV0 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 – PM – PR – 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 Mspl (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 toxigenic endonucleases genes.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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regulation of sso II restriction-modification gene expression.


