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Real-time kinetics of restriction–modification gene expression after entry into a new host cell

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ABSTRACT

Most type II restriction–modification (R–M) systems produce separate restriction endonuclease (REase) and methyltransferase (MTase) proteins. After R–M system genes enter a new cell, protective MTase must appear before REase to avoid host chromosome cleavage. The basis for this apparent temporal regulation is not well understood. PvuII and some other R–M systems appear to achieve this delay by cotranscribing the REase gene with the gene for an autogenous transcription activator/repressor (the 'C' protein C.PvuII). To test this model, bacteriophage M13 was used to introduce the PvuII genes into a bacterial population in a relatively synchronous manner. REase mRNA and activity appeared ~10 min after those of the MTase, but never rose if there was an inactivating *pvuIIC* mutation. Infection with recombinant M13pvuII phage had little effect on cell growth, relative to infection with parental M13. However, infection of cells pre-expressing C.PvuII led to cessation of growth. This study presents the first direct demonstration of delayed REase expression, relative to MTase, when type II R–M genes enter a new host cell. Surprisingly, though the C and REase genes are cotranscribed, the *pvuIIC* portion of the mRNA was more abundant than the *pvuIIR* portion after stable establishment of the R–M system.

INTRODUCTION

Most bacteria and archaea possess restriction–modification (R–M) systems (1), in part for defense against DNA bacteriophages. PvuII, like most type II R–M systems, includes two independently active enzymes: a restriction endonuclease (REase) that cleaves DNA at a target sequence, and a methyltransferase (MTase) that modifies the same sequence to protect it from the REase (2). These two activities must be carefully balanced to ensure protection of the host chromosome. Moreover, this balance must achieve both appropriate steady-state levels and have suitable kinetics of expression following horizontal transfer of the R–M genes (3). The protective MTase must be expressed in advance of the REase, so as to protect the new host before REase appears. Where this does not occur, there is strong selection for mutation of the REase gene [e.g. in R^+M^- subclones (4)].

The mechanisms of R–M gene expression and regulation are still unclear. R–M systems consisting of just the REase and MTase genes do not rely on passive regulation, determined by fixing the relative strengths of promoters for the two genes (5–8), suggesting that the appropriate expression ratios change with conditions. Several R–M systems contain a third gene (C, for 'controlling'), including PvuII (9), BamHI (10), BclI (11), BglII (12), Esp1396I (13), EcoO109I (14), EcoRV (15), Eco72I (16), HgiAI (17), BstLVI (18), Kpn2I (19), SmaI (20), BclI (11) and AhdI (21). The C proteins are small, dimeric proteins that bind DNA via a helix–turn–helix domain (11,21–23), and have an activation patch resembling that on CI protein from bacteriophage lambda (22–25). These C genes are well conserved, and even interchangeable in some cases, despite the different DNA specificities of their associated R–M genes and the genetic distances between their host bacteria (10,26). They fall into several complementation groups (26).

In all C-dependent R–M systems studied to date, the REase gene is tightly linked to (and often overlaps) the upstream C gene, and in all examined cases but one (27) the REase gene is completely dependent on transcription from the C promoter. Autogenous activation of the *pvuIICR* promoter occurs via the C protein binding to upstream operators called 'C-boxes' (24,28,29) (Figure 1A). Recently, the C protein from the PvuII R–M system was shown to act not only as an autogenous

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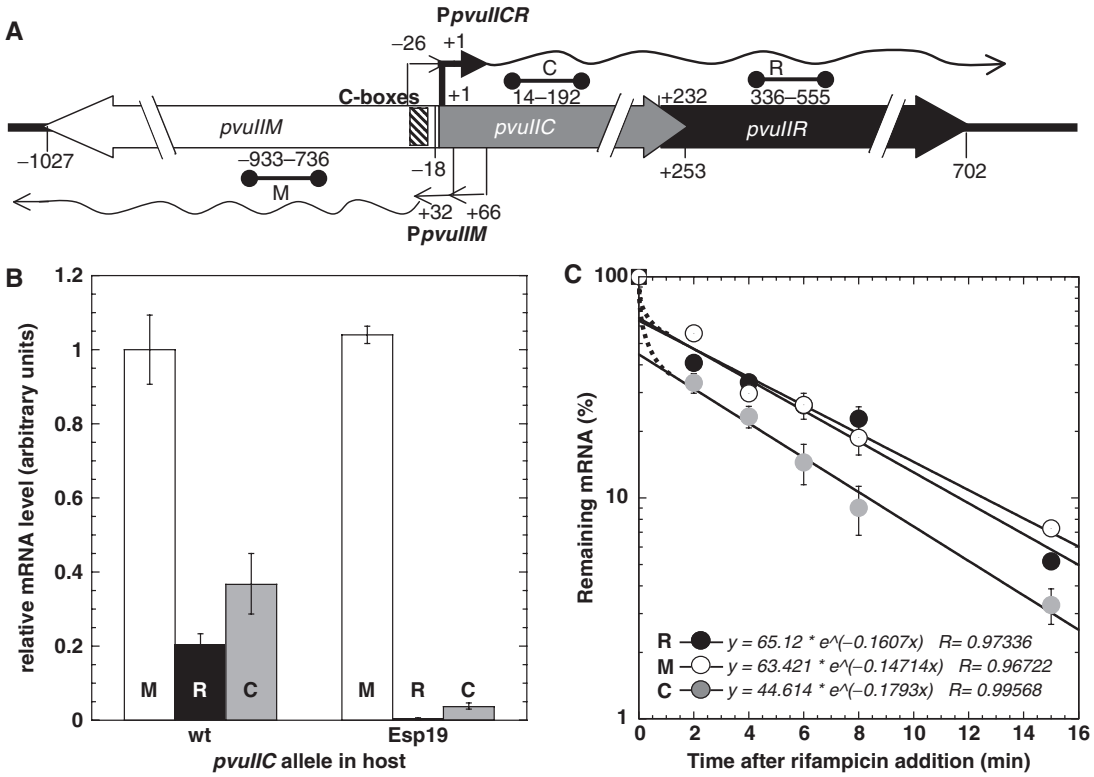

Figure 1. The PvuII R–M system and its transcripts. (**A**) Genetic structure of PvuII R–M system. Numbering is relative to the initiation codon of *pvuIIC*, which is also the start of a leaderless transcript for this gene. Promoters are indicated by bent arrows, and the monocistronic mRNA for *pvuIIM* (MTase, −18 to −1027) and polycistronic mRNA for *pvuIICR* (C = regulator, +1 to +253; R = REase, +232 to +702) are indicated by wavy lines. Two alternative transcription initiation sites for *pvuIICR* are located at positions +1 (for the C.PvuII-dependent promoter; thick arrow) and −27 (for the C.PvuII-independent promoter; thin arrow). The positions of PvuII mRNA segments amplified by QRT–PCR are indicated by the dumbbell shapes. (**B**) Relative steady-state levels of PvuII mRNAs. The accumulation of *pvuIIM* (white bar), and of the *pvuIIC* (gray bars) or *pvuIIR* (black bars) segments of *pvuIICR* mRNA, were measured via QRT–PCR. These levels used *recA* mRNA as the internal standard, as described in 'Materials and methods' section. All results were normalized to the wt level of *pvuIIM* mRNA and standard error bars from three determinations are shown. (**C**) PvuII transcript stability. At time = 0, rifampicin was added to an exponential culture of *E. coli* TOP10F' growing in LB medium. The mRNA levels were determined via QRT–PCR, using stable 5 S rRNA as the internal standard as described in 'Materials and methods' section. The circles represent *pvuIIM* (white), *pvuIIC* (gray) and *pvuIIR* (black). All three mRNA levels are normalized to 100% at time = 0 (the three points overlap). The data were fitted to an exponential decay, after a short initial phase of more rapid decay (see text). Standard error bars from three determinations are shown.

transcriptional activator, but also as an efficient repressor (29), as predicted on theoretical grounds for C.AhdI (21). Subsequent experimental and modeling studies suggest that, for C.AhdI, the repression results from binding competition between the regulator and RNA polymerase (30). In the studied systems, C protein has either little or negative effect on MTase expression; for example, in the Eco72I system the C protein reduces MTase expression 10-fold (16).

As no C protein is present when the R–M genes first enter a new host cell, REase gene transcription is very limited, but is believed to increase rapidly due to the positive feedback loop. This view is consistent with observations that, first, pre-expressing C protein in a cell prevents transformation by the intact R–M system, presumably due to premature expression of the REase; and second, the absence of active C protein is associated with very low or nonexpression of the REase (24,26,28). However, the expected delay in REase versus MTase expression has to date been inferred from steady-state behavior of various mutants or clones, and never directly demonstrated or measured.

We present here the first direct evidence for a C protein role in temporal regulation of the REase gene, following the entry of a C-dependent R–M system into a new host cell. Our approach was to use bacteriophage M13 to introduce the PvuII genes into a bacterial population. This allowed the gene transfer to be carried out over a short period of time and with minimal physiological disturbance of bacterial culture (compared to transformation, for example). PvuII gene expression was measured via quantitative real-time RT–PCR and enzyme activity assays. Our results indicate a significant lag between MTase and REase expression, and show that—despite their cotranscription—REase transcript levels roughly follow those of the C gene but at a lower level.

MATERIALS AND METHODS

Bacterial strains, phages and plasmids

The *Escherichia coli* K-12 strains used in this study are described below, and plasmids and phages used are listed in Table 1. All strains into which *pvuIIM* is introduced

Table 1. Plasmids and phages used in this study

Name	Relevant feature(s)	Reference
Phages		
M13mp19/18	*E. coli* bacteriophage DNA vector	(82)
M13cat	*cat* (chloramphenicol acethyltransferase) gene cloned into M13mp18	This study
M13pvuIIwt	wt PvuII R-M system from pPvuRM3.4 cloned into M13mp19	This study
M13pvuIIEsp19	PvuII R-M system from pPvuRM3.4-Esp19 (inactive C)	This study
Plasmids		
pACYC177	Cloning vector (Amp^R, Kan^R)	(83)
p200	wt *pvuIIC* gene with its own promoter, △*pvuIIM*, △*pvuIIR*, KanR, p15A ori	This study
p201	As p200, but *pvuIIC*-Esp19 mutation (see below)	This study
pDK435	Transcriptional fusion of *pvuIIC* promoter including wt C boxes (positions −93 to +88) in front of promotorless *lacZ* reporter gene in pKK232-8, Tet^R, p15A ori	D. Knowle, unpublished data
pKK232-8	Promotorless *cat* reporter gene (Amp^R)	(84)
pPvuRM3.4	wt PvuII R-M system in pBR322 backbone	(4)
pPvuRM3.4–Esp19	As pPvuRM3.4, but C protein mutant (insertion mutation – Leu codon in HTH motif of C gene, unable to bind DNA)	(22)

Table 2. Oligonucleotides used in this study

Pair of oligonucleotides	Sequence (5′ → 3′)	Used for
PV	AGTGGATCCGTCATTAC GGC GATGAATTCTGATGGTGACTG	Cloning PvuII R-M system into M13mp19
cat	TGGGATCCCTGTTGATACCGGG GCGGAGCTCCAGGCGTTTAAGGGC	Cloning *cat* gene into M13mp18
H1	AAGTCTGCCATTTGCCGATAACGC AAGTTTGACGATTGGTGCAGGCAG	MTase activity assay
H3	CGCCATTCTGTTGGCTCGGTTATT TTGCCATTGTGGCGATGCTTCTTG	MTase activity assay
pvuIIM	AAACGCCGATGCCGCAACATATTC TTGATGGGTATTAAAGCGCATCCCG	QRT–PCR
pvuIIR	TGGTGGAAAGTTGCTTCAAGTCCT TGCGATACCACGGTATATGGCAAA	QRT–PCR
pvuIIC	CAAATCCTTTATCAGCCCGATTAACCC AGGCATTTGCTATTCGCTCAATGT	QRT–PCR
M13gII	ATAGCTACCCTCTCCGGCATGAAT CGGGAGAAGCCTTTATTTCAA CGCA	QRT–PCR
M13gVIII	AAAGCCTCTGTAGCCGTTGCT TGATACCGATAGTTGCGCCGACA	QRT–PCR
recA	TAACCTGAAGCAGTCCAACACGCT TTGTTCTTCACCACTTTCACGCGG	QRT–PCR
5SrRNA	TAGCGCGGTGGTCCCA CACTACCATCGGCGCTACG	QRT–PCR

must lack the *mcrBC* restriction system (4,31–33). *Escherichia coli* TOP10F′ [F′{*lac*I^q, Tn*10*(Tet^R)} *mcr*A (*mrr-hsd*RMS-*mcr*BC) 80*lac*ZM15 *lac*X74 *rec*A1 *ara*D139 (*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G′] (Invitrogen) was used as the host strain for M13 phage infections. *Escherichia coli* TOP10 (without the F' plasmid; Invitrogen) was used for all other purposes including cloning steps. The recombinant M13 bacteriophages M13pvuIIwt (C^+ phenotype) and M13pvuIIEsp19 (C^- phenotype; contains an in-frame insertion of one Leu codon into the upstream helix of the C.PvuII helix–turn–helix motif) were generated by cloning the entire PCR-amplified 2.04-kb PvuII R–M system into BamHI- and EcoRI-linearized M13mp19 replicative form (RF) DNA, using 'PV' primers (Table 2). Plasmids pPvuRM3.4 (4) and pPvuRM3.4–Esp19 (22) were used as template. M13cat was generated by cloning a SacI- and BamHI-cleaved, PCR-amplified *cat* gene ('cat' primers, Table 2), using pACYC184 as the template and M13mp18 RF as vector (linearized with the same enzymes). All phage lysates were made following transfection of *E. coli* TOP10F' cells in accordance with standard methods (34). Infectious phage titers were determined by plaque formation on the same strain by the top agar overlay technique (34). Due to instability of M13 clones (35), the phage stocks were prepared from nonpassaged (original preparation) M13 RF DNA via transfection.

M13 infection experiments

Cells were grown overnight in LB medium supplemented with tetracycline (15 μg/ml) to maintain the F′ episome,

as M13 infection can lead to loss of F factors (36). After dilution, the culture was grown to $OD_{600nm} = 0.23$, then split into four equal portions. Of these, one was infected with M13pvuIIwt, one with M13pvuIIEsp19, one with M13mp19 (vector control) and the fourth was an uninfected control (Figure S1). The multiplicity of infection (MOI) was 5 PFU/CFU if not otherwise indicated. Triplicate samples were collected over a period of 70 min. The first replicate sample in each case was used for total RNA isolation, the second was used for genomic DNA isolation and the third was sonicated for use in endonuclease activity assays. Efficiencies of plaquing (EOPs) were calculated by dividing the phage titers on the tested strain [*E. coli* TOP10F' (p200; *pvuIIC* wt) or (p201; *pvuIIC*-Esp19) by the titers on reference strain [*E. coli* TOP10F' (pACYC177)].

RNA extraction and cDNA synthesis

Culture sample volumes were corrected for OD to maintain similar cell numbers per sample. Samples were immediately mixed with 3 ml of RNA Protect reagent (Qiagen) and total RNA was isolated using the RNeasy Mini kit (Qiagen). cDNAs were obtained, after RQ1 RNase-free DNase (Promega) treatment, by using random hexamers (Invitrogen) and ImProm-II Reverse Transcriptase (Promega). For the mRNA stability experiment, culture samples were taken starting 30 s prior to addition of rifampicin (500 μg/ml), and total RNA was extracted and cDNAs prepared as above. The half-lives of transcripts were determined by fitting the percentage of mRNA remaining versus time to an exponential decay function.

QRT–PCR

Primers were designed with PrimerQuest software (Integrated DNA Technologies) to ensure the same T_m and similar PCR product size. The following primers were used to amplify the segments of the following genes: *pvuIIM*, *pvuIIR*, *pvuIIC*, M13gII, M13gVIII, *recA*, 5SrRNA (Figure 1A; Table 2). A LightCycler (Roche) was used with LightCycler software. Real-time PCR was performed in triplicate, and final products were confirmed by agarose gel electrophoresis. Each reaction contained 2 μl of diluted cDNA template (1:10) and 8 μl of mixture: dNTPs (0.2 mM), PCR buffer (1×), primers (1 μM), SYBR Green I dye (1:20 000) and Platinum *Taq* DNA polymerase (0.5 U) (Invitrogen). The PCR employed the following cycling parameters: 95°C for 2 min, followed by 40 cycles of 94°C for 5 s, 59°C for 5 s, 72°C for 15 s each; and finally the melting curve (59–94°C) program for quality control, and cooling to 40°C.

The mRNA levels for the target genes were quantified from the *Ct* value, which is the PCR cycle number at which the fluorescence crosses a predefined threshold. The threshold value is selected on the basis of being well above the background noise level, but less than half of the level at which fluorescences eventually plateaus. The levels of gene expression for each gene were normalized to the level of the reference housekeeping gene *recA* in all studies except the mRNA stability assay, where 5 S rRNA was used as the internal, stable marker. RecA expression is not affected by M13 infection (37,38). Furthermore, the strains used to carry the *recA1* allele, preventing induction of an SOS response (in which *recA* itself would be induced) (39). Data were averaged for each set of replicates. For M13 infection experiments, the relative fold-change mRNAs ratios were obtained by normalizing each time point data in reference to the earliest measurements. The calculations also included PCR efficiency, where each cDNA was serially diluted and its *Ct* was plotted to calculate the slope corresponding to PCR efficiency (40,41). Investigated transcripts showed optimal PCR efficiencies: 2.019 for *pvuIIR*, 2.053 for *pvuIIM*, 2.18 for *pvuIIC*, 1.99 for M13gII, 2.12 for M13gVIII and 2.09 for *recA* (internal reference gene) with high linearity ($R^2 > 0.98$).

MTase protection activity assay

The MTase protection test analyzed the methylation status of selected PvuII sites on the *E. coli* K-12 chromosome, following the M13 phage infection. Genomic DNA from each sample, isolated from culture samples at various times after infection, was purified using a Wizard Genomic DNA Purification Kit (Promega). Portions of genomic DNA (20 μg) were digested overnight with HindIII and PvuII, separated by electrophoresis on 0.8% agarose gels, and transferred onto nylon membranes (Millipore) in 20× SSC buffer. The DNA was then fixed to the membrane by UV irradiation. The two specific probes H1 (733 bp; primers H1) and H3 (857 bp; primers H3) (Table 2) were prepared by PCR, and labeled with biotin using a Biotin High Prime Kit (Roche). Membranes were hybridized with the H1 and H3 probes at the same time, at 55°C for 12 h in hybridization buffer (each probe at 30 ng/ml of buffer). Membranes were developed using a North2South Chemiluminescent Hybridization and Detection Kit (Pierce) as recommended.

REase activity assay

Samples were harvested by centrifugation, and the pellets were rinsed with PBS. After centrifugation, the cells were resuspended in 100 μl of buffer A (10 mM K/PO_4 pH 7.0, 30 mM KCl, 1 mM Na_2EDTA, 10 mM 2-mercaptoethanol and 5% glycerol v/v) and disrupted by sonication (10 bursts of 5 s) at 4°C in a cup horn probe. Cellular debris was removed by centrifugation (3000*g*, 10 min). PvuII REase activity was assayed in a 20-μl reaction mixture containing: 0.5 μg of bacteriophage λ DNA, 10 mM Tris–HCl pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and 5 μl of the clarified cell lysate. Reactions were incubated for 10 min at 37°C, and the DNA was resolved on 0.8% agarose gels.

Chloramphenicol acetyltransferase (CAT) assay

The CAT ELISA kit (Roche) was used to colorimetrically quantitate CAT reporter levels based on a sandwich ELISA method. The recommended protocol was followed with some modifications. The culture was grown in LB medium, infected with M13cat phage and time-point samples were collected until 70 min of postinfection. Samples (1 ml each) were removed, pelletted and

resuspended in 0.5 ml of lysis buffer (100 mM K/PO_4 pH 7.8, 1% Triton X-100, 5 mg/ml BSA, 1 mM DTT, 5 mg/ml lysozyme) and kept for 20 min at RT. The centrifugally cleared cell extracts were diluted 1:25 and loaded onto microplate modules with anti-CAT antibodies prebound to the surface. CAT concentration was calculated as nanograms per milliliter of culture at OD_{600nm}.

RESULTS

Steady-state level of PvuII transcripts

Before studying the kinetics of PvuII gene expression, following introduction into new host cells, we first analyzed the steady-state behavior of these genes. We grew two *E. coli* K-12 (TOP10F') strains in parallel, each of them bearing a pBR322-derived plasmid. One plasmid carried the intact PvuII R–M system [pPvuRM3.4, active C.PvuII protein, 'C^+' phenotype (4)], and the second plasmid differed only in having an in-frame insertion in *pvuIIC* (pPvuRM3.4-Esp19). This mutation adds a Leu codon into the helix–turn–helix motif, reducing PvuII restriction to background levels (22), and eliminating detectable DNA binding by C.PvuII (28) ('C^-'phenotype). The strain pair was grown to mid-exponential phase in LB medium, total RNA was isolated and gene expression was monitored by quantitative real-time reverse transcriptase PCR. We used probe sets that measured *pvuIIM* and polycistronic *pvuIICR* transcripts (dumbbell shapes in Figure 1A). Previous studies have shown that there are two alternative promoters for *pvuIICR* transcription (Figure 1A). One promoter leads to initiation within the C-boxes, is independent of C.PvuII but very weak, and is believed to provide necessary initial amounts of *pvuIIC* transcription to prime the positive feedback loop; while the second promoter yields a leaderless message that is only produced in the presence of C.PvuII (24). Transcription of *pvuIIM* occurs from a pair of promoters within *pvuIIC*, both of which appear to be minimally affected by C.PvuII (22).

The quantitative analysis confirms earlier observations (22,28) that C.PvuII has little or no effect on *pvuIIM* expression (Figure 1B, white bars). In the presence of active C.PvuII, *pvuIIM* transcript levels are 5-fold (4.9 ± 0.7) more abundant than those of *pvuIIR*. Despite being cotranscribed (28), *pvuIIC* mRNA is twice (1.8 ± 0.4) as abundant as *pvuIIR* mRNA (Figure 1B). In $C.PvuII^-$ cells, where the mutation consists of a one-codon (sense) insertion, *pvuIICR* mRNA is about 10-fold less abundant than in $C.PvuII^+$ cells when measured within the C segment, and about 50-fold less abundant when measured in the R segment (Figure 1B). This low-level transcription presumably relies on the weak C-independent *pvuIICR* promoter (24). These measurements are consistent with earlier assays of *pvuIIC-cat* transcriptional fusions (28).

To test whether the differences in mRNA abundance are due to differential stability, we measured the rate of disappearance of each mRNA species following culture treatment with the transcription initiation inhibitor rifampicin (Figure 1C). Both *pvuIIM* and *pvuIICR* transcripts have similar decay patterns. The PvuII mRNAs appear to undergo rapid initial decay, within 2 min decaying to 55% for *pvuIIM*, 41% and 33% for *pvuIICR* probed separately in the R and C segments, respectively. A possible explanation for this rapid initial phase of decay is discussed below. The initial rapid turnover is followed by slower exponential decay that was used to calculate the half-lives. MTase mRNA was slightly more stable, with a half-life of ~4.7 min. The bicistronic *pvuIICR* mRNA, probed independently within *pvuIIC* and *pvuIIR*, yielded half-lives of ~3.8 min for the C segment, and ~4.3 min for the R segment (Figure 1C). These half-lives are in the average range of transcript stability, with the half-life of bulk *E. coli* mRNA being 2.4 min at 37°C (32,42). Combining these results, neither the rapid decay of ~60% of *pvuIIR* versus ~70% of *pvuIIC*, nor the longer-term half-life ratio of 1.1 (R/C) can explain the presence of about twice as much *pvuIIC* as *pvuIIR* mRNA during steady-state growth (Figure 1B).

M13 bacteriophage infection model

To study the timing role of the C.PvuII feedback circuit, and to test its inferred delaying effect on REase gene expression, we developed a system for relatively synchronous introduction of the PvuII R–M genes into the bacterial population via infection with recombinant bacteriophage M13. We first performed pilot M13 infection studies, in which actively growing *E. coli* F' cells were infected with M13mp19 or M13cat (carrying a *cat* gene for chloramphenicol acetyltransferase) at a multiplicity of infection (MOI) of 5. Samples were taken every 5 min, through 70 min postinfection (Figure 2).

When cells were infected with M13cat phage, CAT protein was detectable by ELISA about 20 min postinfection (Figure 2C), which agrees with measurements of the M13 infection-to-release time of 15–20 min (43,44) (Supplementary Data; Figure S2). As *cat* expression does not rely on accumulation of an activator, this result suggested an appropriate time range for subsequent experiments. At the same time, we monitored culture growth, confirming the expected slowdown for the M13-infected culture (Figure 2A).

M13 phage transcript levels were determined for the growing culture infected with M13mp19 phage (Figure 2B). Total RNA from the culture samples was isolated and prepared for quantitative analysis via QRT–PCR (Materials and methods section). We probed for M13 mRNA from genes II and VIII. M13 gII is one of the earliest transcripts, the product of which is involved in DNA replication (45), while gVIII is the most abundant mRNA and specifies the major capsid protein (46). We measured the relative mRNA fold change with respect to the very initial level (1 min after infection) and normalized to *E. coli recA*. Previous microarray analysis indicated that M13 infection does not significantly alter *recA* expression (37). Phage transcripts (gII and gVIII) rose detectably about 20 min after infection, as was found for *cat* expression, and the levels of phage transcripts kept

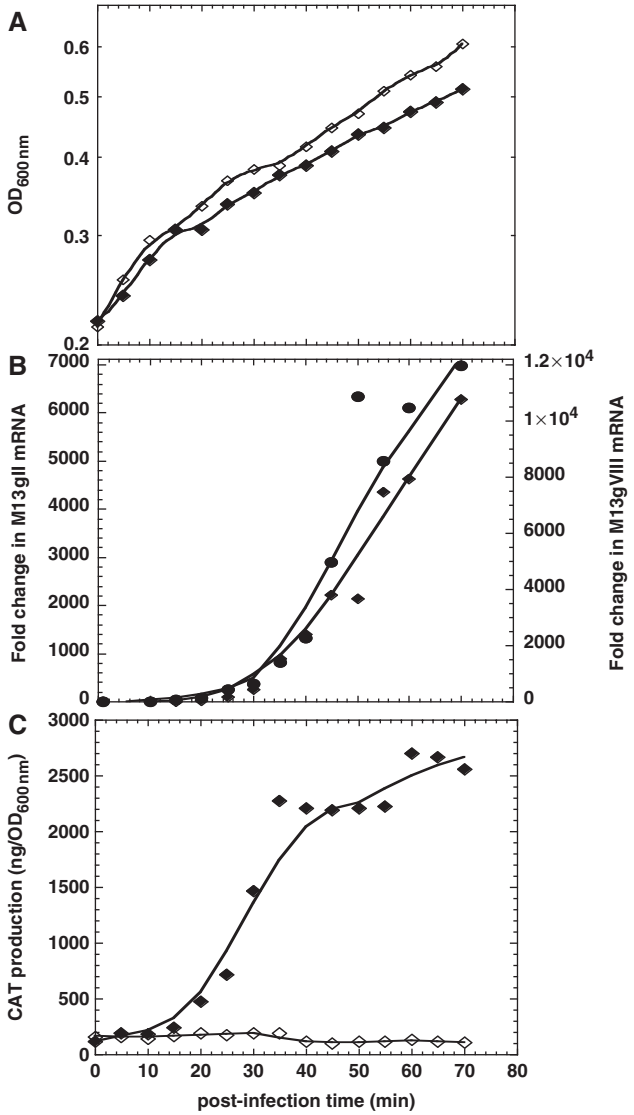

Figure 2. M13mp19 and M13cat infection of *E. coli* TOP10F'. Cultures were infected at an MOI of 5 PFU/CFU in LB medium, supplemented with tetracycline to maintain the F' episome. (**A**) Growth was monitored at OD_{600nm}. Open symbols are not-infected cells, and closed symbols represent cells infected with M13cat phage. (**B**) The relative levels of M13 phage transcripts for gII (circles) and gVIII (diamonds) for the infection with phage M13mp19. The RNA isolation and QRT–PCR were performed as described in 'Materials and methods' section. (**C**) Chloramphenicol acetyltransferase (CAT) production after infection with M13cat phage (filled diamonds), at MOI = 5, versus uninfected cells (open diamonds). CAT levels, determined via immunoassay as described in 'Materials and methods' section, are expressed as nanograms of protein per OD_{600nm} of culture.

increasing over the monitored time course of 70 min (Figure 2B).

We found it necessary to carry out these experiments in the presence of tetracycline, which selects for maintenance of the F' plasmid that makes the cells infectable by filamentous phage such as M13 (47,48). M13-infected cells have a tendency to eliminate F plasmids (36). Whatever the mechanism may be, in the absence of tetracycline the levels of M13 gene expression (phage-specific or cloned), M13 DNA, and M13 plaque-forming units all show a pronounced cycling behavior (Figure S2) that made relative temporal measurements more difficult.

'Short-circuiting' effect of C.PvuII pre-expression

One of the forms of evidence suggesting a key role for the C.PvuII feedback circuit in controlling the delayed expression of REase relative to MTase is that pre-expression of C.PvuII in cells prevents their transformation by the intact R–M system (26,28). Since C.PvuII activates transcription of *pvuIIR*, this observation was interpreted as indicating that pre-expression of C.PvuII led to immediate expression of *pvuIIR*, and lethally premature appearance of REase activity. To test the physiological relevance of the M13 model, we determined whether the M13-mediated transfer of PvuII genes was sensitive to pre-expression of C.PvuII. We used host cells carrying plasmids with no *pvuIIC* (pACYC177 vector control), wt *pvuIIC* (pIM200) or *pvuIIC*-Esp19 (pIM201) (neither *pvuIIM* nor *pvuIIR* was present in any of these plasmids). We then measured the EOP (Materials and methods section) in all possible host–phage pairings of the three *E. coli* strains with three M13 phage stocks carrying the intact PvuII R–M system (M13pvuIIwt), the PvuII system with a defective *pvuIIC*Esp19 allele (M13pvuIIEsp19), or with no *PvuII* genes (M13mp19; phage vector control). Each set of EOPs was normalized to the titer obtained for the vector control *E. coli* host with that phage stock.

The results obtained for host cells with pre-expressed wt C.PvuII indicate that, as expected, the EOP of M13 carrying the intact PvuII R–M system is reduced (about 3-fold relative to cells having no pre-expressed C.PvuII; Figure 3A). Surprisingly, pre-expression of the defective mutant *pvuIIC* allele (that has an in-frame one-codon insertion) also reduced the EOP of M13pvuIIwt, by about 3-fold. One possible explanation of this result is that $C.PvuII^{Esp19}$ can form heterodimers with wt C.PvuII, and that these heterodimers can activate transcription of $P_{pvuIICR}$ thus leading to premature REase expression. This interpretation is consistent with the result of the reciprocal experiment (Figure 3A) that M13 bearing the *pvuIIC*-Esp19 allele shows reduced EOP on cells that have pre-expressed wt C.PvuII from the plasmid, but not on cells that have produced $C.PvuII^{Esp19}$ and have no source of wt C.PvuII subunits.

The reductions in EOP due to 'short circuiting' were correlated with much smaller plaque size than were seen in control infections. The smaller plaques also appeared to be less turbid than control M13 plaques (Supplementary Data; Figure S3). Recombinant M13 coding for EcoRI REase infects and kills the host cells, forming clear plaques in comparison to the turbid growth zones produced by plain M13 (49). These observations suggest that more-severe growth restriction (and possibly cell death) occurs after infection of cells with pre-expressed *pvuIIC*, limiting the production of progeny phage. To test this possibility, we determined the effect of *pvuIIC* pre-expression on the growth of *E. coli* TOP10F' cells following M13pvuIIwt infection (Figure 3B). We monitored growth in LB medium to mid-log phase (OD_{600nm} = 0.25)

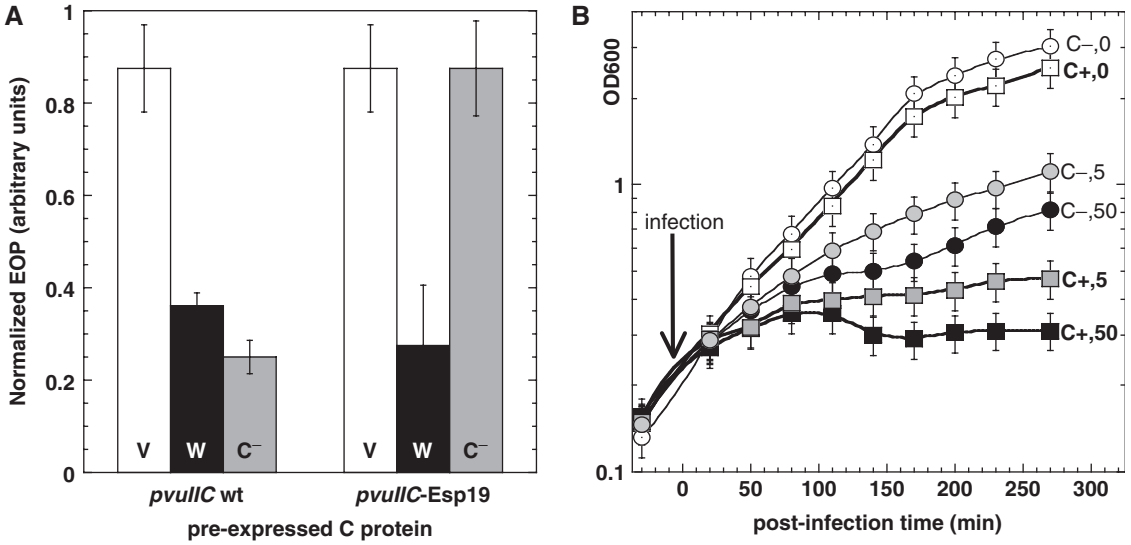

Figure 3. Effect of pre-expression of wt *pvuIIC* on PvuII R–M establishment. (**A**) Three *E. coli* TOP10F' host strains were used that carried no *pvuIIC* gene (pACYC177 vector control), carried wt *pvuIIC* (pIM200), or carried the defective *pvuIIC*-Esp19 (pIM201). The efficiency of plaquing (EOP) was measured after infecting each strain with each of three M13 phage stocks: one with the wt PvuII R–M system (M13pvuIIwt; black bars, marked 'W' for 'wt'), one with *pvuIIC*-Esp19 in the PvuII R–M system (M13pvuIIEsp19; gray bars, marked 'C^-') and one with no *PvuII* genes (M13mp19; white bars, marked 'V' for 'vector'). Each dataset was normalized to the titration result obtained for that phage stock on the vector control host *E. coli* TOP10F' (pACYC177). These normalization values would yield three bars at height 1.0 and are not shown (the actual values were very close: M13mp19 = 1.6×10^{11}, M13pvuIIwt = 1.4×10^{11} and M13pvuIIEsp19 = 1.4×10^{11} PFU/ml). The error bars represent the standard deviations from triplicate measurements. (**B**) Two cultures of *E. coli* TOP10F' were used, that harbored either no *pvuIIC* (pACYC177; circles) or wt *pvuIIC* (pIM200; squares). When their growth in LB medium reach mid-log phase (OD_{600nm} = 0.25), the cultures were divided into three portions. One portion was infected with M13pvuIIwt at an MOI of 50 (black symbols), one at an MOI of 5 (gray) and the third portion remained uninfected (white). The growth of the culture was monitored as OD_{600nm}. The bars represent standard deviations from two experiments.

and then infected at an MOI of 5 or 50 PFU/CFU. Two cultures were tested: with *pvuIIC* present on a plasmid (pIM200) or absent (pACYC177 vector control). We saw significantly lower culture densities for the infected cells, and this effect was enhanced where wt C.PvuII was pre-expressed (Figure 3B). These observations support the interpretation that pre-expression of C.PvuII prevents transformation by the intact PvuII R–M system by killing the host cell (26,28), and are consistent with the physiological relevance of the M13-based model for R–M system gene expression.

Introduction of PvuII R–M genes into the new host cells via recombinant M13 bacteriophage infection: kinetics of mRNA appearance

To study PvuII gene establishment in a new host, we used the two M13 recombinant stocks carrying the PvuII genes: M13pvuIIwt (C^+ phenotype) and M13pvuIIEsp19 (C^- phenotype due to an insertion of one sense codon) (Table 1). The host cells for infection (which contained no PvuII genes) were grown to exponential phase, and split into four equal portions. Of these, one was infected with M13pvuIIwt, one with M13pvuIIEsp19, one with M13mp19 (vector control), each with an MOI of 5 PFU/CFU and the fourth was an uninfected control (Supplementary Data; Figure S1). All three infected cultures grew more slowly than the uninfected culture, but the restricting phage did not cause worse growth than the others (Figure S1).

Using RNA prepared from these culture samples, the accumulation of PvuII mRNAs was measured as described in 'Materials and methods' section. In addition to *pvuIIM*, *pvuIICR* was probed separately for the *pvuIIC* and *pvuIIR* segments (see Figure 1B). As might be expected for the infection with M13pvuIIwt (C^+ phenotype), *pvuIIM* mRNA levels rose above background about 20 min postinfection, similar to what had been seen for *cat* and M13 phage genes in pilot experiments (Figure 2). In contrast, the appearance of *pvuIIR* mRNA was delayed to about 30 min postinfection (Figure 4A). The levels of *pvuIIM* and *pvuIIR* transcripts eventually reached about the same 5:1 ratio seen in steady-state cultures (Figure 1B), by about 45 min postinfection. Although *pvuIIC* and *pvuIIR* share the same transcript (28), the level of mRNA probed for C or R separately (Figure 1A) was not the same through the time course of the experiment. By about 40 min postinfection, the C/R ratio reached the value of ~2 that was observed for transcripts from the plasmid-borne PvuII genes (Figure 1B, and see 'Discussion' section).

When cells were infected by phage carrying a defective in-frame (sense) mutation in *pvuIIC* (M13pvuII-Esp19), the REase transcript level never rose above the background level (Figure 4B). The small rise in the low *pvuIIC* transcript level, about 40 min after infection, is presumably due to the weak C-independent promoter (24). Surprisingly, while C.PvuII had no effect on the steady-state 'level' of *pvuIIM* mRNA (Figure 1B), it did have effects on the 'kinetics' of *pvuIIM* expression

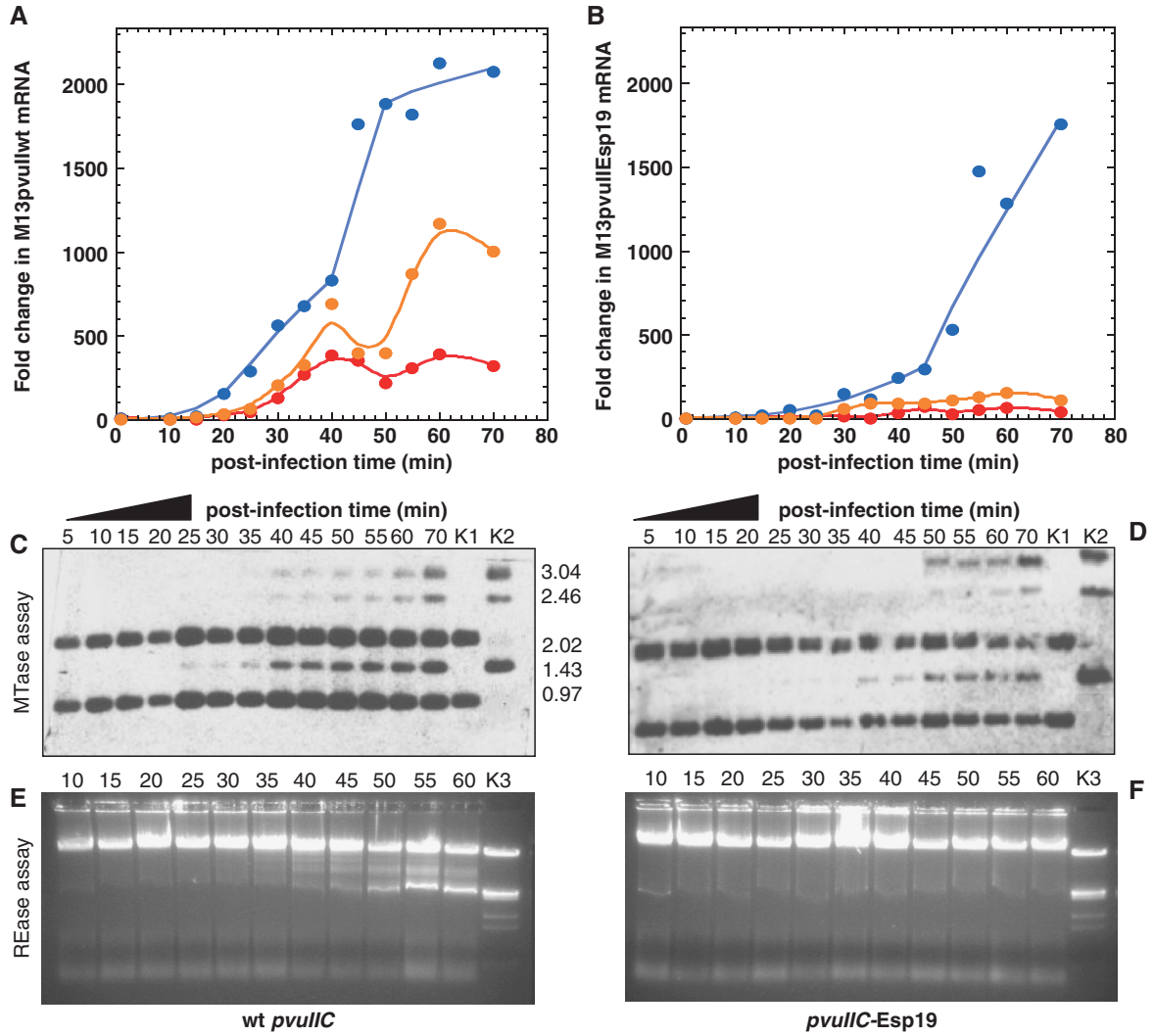

Figure 4. *In vivo* kinetics of PvuII restriction-modification gene expression after entering a new host cell. The experiment was performed as described in Figure 2 and in 'Materials and methods' section. (**A, B**) Relative levels of PvuII transcripts determined by QRT–PCR (Materials and methods section). TOP10F' cells were infected with M13 carrying wt *pvuIIC* (A) or the null *pvuIIC*-Esp19 allele (B) in the context of the full PvuII R–M system. The various mRNAs are shown as *pvuIIM* (blue), *pvuIIC* (orange), *pvuIIR* (red). (**C, D**) *In vivo* kinetics of *E. coli* chromosomal DNA methylation. Chromosomal DNA was isolated at various times following entry of the wt PvuII R–M system (C) or that system with the *pvuIIC*-Esp19 allele (D), digested with HindIII, and then with PvuII REase as described in 'Materials and methods' section. Protection from *in vitro* digestion with PvuII REase was detected by probing Southern blots with specific biotinylated PCR products. The control lanes contained DNA from uninfected cells (K1; gives the pattern expected for unprotected DNA), or digested with only HindIII (K2; gives the pattern expected for fully methylated DNA). (**E, F**) *In vivo* kinetics of REase activity following entry of the PvuII R–M system. The M13pvuII phages carried either wt *pvuIIC* (E) or its inactive *pvuIIC*-Esp19 variant (F). Crude cell extracts were isolated as described in 'Materials and methods' section, and incubated with DNA from bacteriophage λ. Lane K3 shows the same DNA digested by commercial PvuII REase (NEB).

(compare Figure 4A and B). After infection with the C.PvuII$^-$ M13, MTase mRNA started to accumulate about 30 min postinfection, rising more rapidly after 40 min; in both cases this is ~10 min later than following infection with M13pvuIIwt.

As a control, the accumulation of M13 DNA was also measured for the infection with M13pvuIIwt (data not shown). At all times tested, the ratio between the two identically oriented genes *pvuIIC* and M13gII was close to 1 (averaging 1.46, and ranging from 0.96 to 2.01) consistent with stability of the DNA insert in M13 genome. This had been a concern because many longer M13 inserts are subject to accumulation of spontaneous deletions during replication (50).

Introduction of PvuII R–M genes into the new host cells via recombinant M13 bacteriophage infection: kinetics of activity appearance

The 10-min delay between appearance of *pvuIIM* and *pvuIIR* transcripts is important to our understanding of R–M system mobility. However, this delay does not provide information about the actual rate of protection of the host chromosome. In other words, it is not clear (except by inference) whether a 10-min delay is sufficient. The average *E. coli* cell growing exponentially in rich medium has about two full-chromosome equivalents (51), or about 9 Mbp of DNA, that would have to be protected during the delay before REase appearance.

To address this question, we determined the rate of host chromosome protection from restriction, and the rate of appearance of active REase, following infection with M13pvuII. The MTase protection test analyzed the methylation status of selected PvuII sites from among the ~1430 (52) on the *E. coli* K-12 chromosome [GenBank U00096 (53)]. Genomic DNA, isolated from culture samples at various times after infection, was digested with HindIII (to generate defined methylation-independent fragments) and PvuII REase, and resolved on an agarose gel. Southern blots were probed with biotin-labeled DNA specific to regions adjacent to selected PvuII sites (Materials and methods section). If the tested PvuII sites were being methylated, then longer (uncut) DNA fragments would appear over time (Figure 4C and D, as in control lanes K1 versus K2).

In vivo PvuII methylation activity was observed and, as with the *pvuIIM* transcription data, the rate of appearance was slightly different between the C.PvuII$^{\pm}$ cultures (Figure 4C and D). DNA from samples taken 5–20 min postinfection was unmethylated (Figure 4C and D, lanes 5–20), as expected since *pvuIIM* transcripts were not expressed at that time (Figure 4A and B). About 25 min after infection, the methylated pattern started to appear, and was pronounced by 40 min for C.PvuII^{+} infections (Figure 4C; lanes 40–70). The same was observed, but on a delayed basis, for C.PvuII^{-} infections, where methylation started to appear about 40 min postinfection and was pronounced 10 min later (Figure 4D). Late in the infection, samples showed the partial methylation expected for a mixed population of infected and uninfected cells (Supplementary Data).

One possible contributing factor to these results derives from the fact that we are measuring population averages, and not methylation in individual cells. It is possible in theory that a subset of cells does not sufficiently protect its chromosomal DNA before REase appears, and is killed as a result. Even if these cells did not lyze, their cleaved DNA might be rapidly degraded (54,55), selectively removing unmethylated DNA from our assessment. Consistent with this possibility, the relative amount of protected DNA appears to be lower in the absence of restriction (Figure 4C versus 4D). This would not, however, affect determination of the time of onset of methylation.

The REase activity assay was based on crude extract digestions of DNA from bacteriophage λ. For M13pvuIIwt (C^{+} phenotype), REase activity became evident about 35 min postinfection (Figure 4E), corresponding to its mRNA profile (Figure 4A). In contrast, for M13pvuIIEsp19 (C^{-} phenotype), REase activity was not detected over the 70-min course of the experiment (Figure 4F).

DISCUSSION

Bacteriophage M13 model system

To study PvuII gene establishment in a new host, we chose to use a nonlytic phage model. This presents substantial advantages over transformation- or conjugation-based approaches. For transformation (including electroporation), the cells are profoundly disturbed physiologically by the preparatory washing procedures, heat or electrical shock, and shift into a rich medium (56,57). Gene expression studies in such cells would thus be very difficult to interpret (58). Transfer into a population of new hosts via conjugation occurs over a longer time period, so that gene entry would be relatively asynchronous, and there would also be complications due to PvuII gene expression in the already established donor cells. With the M13-based system, we could sensitively monitor the real-time the kinetics of PvuII gene expression following fairly synchronous introduction into new cells, and with much less physiological disturbance of the recipients than with transformation.

The choice of phage was also important. As opposed to lyzing the host cell, M13 phage is continually released from infected cells following assembly and extrusion through the cell wall (43,59). M13 has been used successfully to deliver antimicrobial agents in phage therapy trials (60,61). Microarray analysis of M13-infected versus uninfected *E. coli* (37) indicates a minimally disturbed transcriptional response, even though growth rates of infected cells are reduced (62) and such reduction itself can have substantial effects on gene expression (63,64). In addition, M13 DNA enters the cell in single-stranded form (43). The PvuII genes naturally reside on a mobilizable plasmid (4), and would presumably enter new cells via conjugation in the form of ssDNA (43). We therefore believe that the PvuII gene expression patterns observed, following M13-mediated entrance, reflect the responses of a relatively normal entry into a physiologically relevant environment.

Several of our results with the M13-PvuII model system are also consistent with its physiological relevance. First, as with transformation-based systems, pre-expression of the C protein leads to reduced plaquing efficiency by M13 phage that carries the PvuII R–M system (Figure 3A). This result supports earlier inferences that pre-expressing C proteins leads to lethally premature expression of the linked REase gene (26,28), including the observation that such 'short circuiting' was associated with DNA damage (55,65,66). The second result supporting the physiological relevance of this model system is the similar expression kinetics of the MTase (*pvuIIM*) gene to that of other constitutive genes (such as *cat*) introduced via M13 (compare Figures 2C and 4A). The third such observation is that, at longer times after infection, the ratio of PvuII-specific transcripts reaches the same value as in established plasmid-borne PvuII genes (Figure 5B shows this for *pvuIIM* and *pvuIIR*).

C.PvuII feedback-dependent delay of *pvuIIR* expression

R–M systems appear to undergo horizontal gene transfer at a substantial rate (3,67). It has been proposed, by inference from steady-state results, that (where present) C proteins are responsible for temporal control of R–M systems. This control was suggested to allow MTase production and protection of host DNA before potentially lethal REase activity appears (22,26,28). However, the

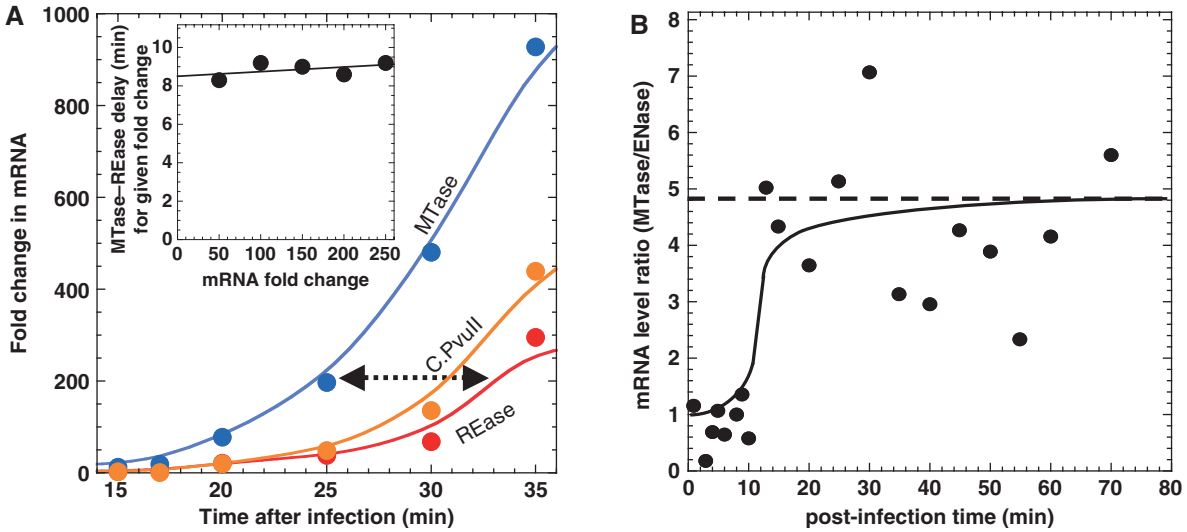

Figure 5. Delayed expression of REase versus MTase. (**A**) The mRNAs for REase (red), MTase (blue) and C.PvuII (orange) were measured following infection with M13 carrying wt *pvuIIC* as in 'Materials and methods' section. The data are from an experiment identical to that shown in Figure 4A, but focusing on the initial time period. The inset shows the time between when *pvuIIM* and *pvuIIR* mRNA reach given fold increases; the dotted double arrow in the main panel illustrates this delay at the 200-fold increased level. (**B**) Ratios of PvuII transcript levels (MTase/REase) over time after infection. The dotted horizontal line indicates the ratio seen in the steady-state plasmid-borne wt PvuII R–M system (from Figure 1B). The ave ± SE is 0.8 ± 0.4 for points within 10 min of infection, and 4.3 ± 1.3 for the later points. The curve is not fitted to the data, but shows the pattern expected for an initial period of no differential expression (ratio = 1.0) followed by a hyperbolic approach to the steady-state ratio.

proposed temporal control was never directly demonstrated or measured.

Using bacteriophage M13 to introduce the PvuII genes into a bacterial population, we showed here that a delay in expression of PvuII REase (relative to that of the MTase) actually occurs and is just <10 min in length (under the growth conditions used) (Figure 5A inset). This delay is reflected in both quantitation of mRNAs and of enzyme activity data (Figure 4). This implies, and our *in vivo* methylation assays confirm (Figure 4C and D), that 10 min is enough time to protect the roughly 3000 PvuII sites per average growing cell (51,52). Modification would have to be at least on one strand per site, though PvuII REase can still cut hemimethylated sites (*in vitro*) at a low rate (68). Presumably, selection for PvuII regulatory mechanisms would not require complete protection, but only a delay sufficient to limit the amount of damage to the extent that it can be repaired (55,66,69) in a reasonably high fraction of the new host cells.

To our knowledge, there has been no previous study of expression kinetics in a type II R–M system. The type I EcoK R–M system was studied kinetically (70,71), using conjugation as the route of transfer. In that study, restriction activity (from *hsdR*) appeared about 15 generations after transfer, while modification activity (from *hsdM*, *hsdS*) was detected almost immediately (70). The kinetics of promoter activity for restriction (P_{res}) and modification (P_{mod}) subunits were also tested, and indicated simultaneous expression. This result indicates a major role for posttranscriptional regulation for the EcoK R–M system (71), now known to include controlled assembly/disassembly, together with HsdR subunits that are degraded if free (72), and phosphorylated only if complexed with HsdMS (73). This regulatory approach may be less feasible for type II systems, where unlike type I enzymes the REase is active independently of the MTase (74).

The C.Pvu-dependent regulatory switch

The C-protein-dependent control systems of type II R–M systems mediate regulatory decisions that mean life or death to the host cell. In this respect, the type II R–M systems resemble proteic toxin–antitoxin addiction systems (26), and the latter are often controlled by an autogenous regulator (75). C.PvuII both activates and represses transcription of its own gene, and both activities are required for mobility and stability of a functional (restricting) system (29). On the one hand, selectively impairing the repression portion of C.PvuII action significantly reduces the efficiency of R–M system transfer (by transformation) into new host cells (76). On the other hand, no restriction activity appears in the absence of functional C.PvuII (9,22,28), and this would presumably also reduce the maintenance stability of the plasmid (26).

The roles of the C protein do not rule out additional posttranscriptional regulatory mechanisms. We have previously shown that the C and REase genes are on a polycistronic mRNA (*pvuIICR*), with no evidence whatsoever for an independent *pvuIIR* promoter (24,28). However, in this study we found that there is about twice as much C segment as R segment mRNA (Figure 1B). In nonpolar defective mutants of *pvuIIC*, there is still significantly (~8×) more *pvuIIC* than *pvuIIR* mRNA (from the weak C-independent promoter). This indicates that whatever is causing the apparent attenuation of *pvuIICR* transcription does not depend on the presence of active C protein.

The difference between *pvuIIC* and *pvuIIR* mRNA levels does not appear to be due to differential stability.

Post-rifampicin decay kinetics show two phases (Figure 1C), but in both phases the *pvuIIC*-specific portion of the *pvuIICR* transcripts is less stable than the *pvuIIR* portion. This pattern, where the 5′ portion of the transcript is degraded more quickly than the 3′ portion, appears to be common in *E. coli* (77). Thus, the actual drop in transcription rate between *pvuIIC* and *pvuIIR* must be >2-fold (at least for an established system in rapidly growing exponential cells). One possible explanation for the rapid initial drop in post-rifampicin mRNA levels might derive from the fact that the *pvuIICR* and *pvuIIM* mRNAs are perfectly complementary to one another at their 5′ ends (for 32 or 66 nt, depending on which *pvuIIM* promoter was used; see Figure 1A). Hybridization would mutually and differentially affect a subset of the mRNAs in either of two ways. The duplexed mRNAs might be more stable, due to protection of the 5′ ends responsible for initiating decay (78), or less stable, as is the case in many sRNA–mRNA interactions or when translation initiation is blocked (79,80).

This study also revealed one interesting feature of the C protein itself. An inactive variant of C.PvuII (*pvuIIC*-Esp19), when pre-expressed prior entry of M13 carrying the wt PvuII genes, was able to reduce the EOP as efficiently as wt C.PvuII (Figure 3A). The in-frame insertion for that mutant adds a Leu codon to the upstream helix of the helix–turn–helix motif (22), and homodimers of this protein are defective for DNA binding (28). The results shown in Figure 3A suggest that the mixed heterodimers (of wt and Esp19) both bind DNA and activate transcription. Activation by mutant/wt heterodimeric regulatory proteins has been observed previously (81), and the Esp19 mutation in *pvuIIC* is well away from the subunit interface [based on alignment to C.AhdI; see Figure 5 in (21)].

This model system should allow exploration of the effects of promoter, operator and activator/repressor mutations on the temporal behavior of type II R–M system, to better understand the process of their establishment, and of toxin–antitoxin addiction modules in general.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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