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Identification and Characterization of a Gene Responsible for Inhibiting Propagation of Methylated DNA Sequences in *mcrA mcrB1 Escherichia coli* Strains

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Identifying and eliminating endogenous bacterial enzyme systems can significantly increase the efficiency of propagation of eukaryotic DNA in *Escherichia coli*. We have recently examined one such system which inhibits the propagation of lambda DNA rescued from transgenic mouse tissues. This rescue procedure utilizes lambda packaging extracts for excision of the lambda DNA from the transgenic mouse genome, as well as *E. coli* cells for subsequent infection and propagation. This assay, in combination with conjugal mating, P1 transduction, and gene cloning, was used to identify and characterize the *E. coli* locus responsible for this difference in efficiency. It was determined that the *E. coli* K-12 *mcrB* gene when expressed on a high-copy-number plasmid can cause a decrease in rescue efficiency despite the presence of the *mcrB1* mutation, which inactivates the classic McrB restriction activity. (This mutation was verified by sequence analysis.) However, this McrB1 activity is not observed when the cloned *mcrB1* gene is inserted into the *E. coli* genome at one copy per chromosome. A second locus was identified which causes a decrease in rescue efficiency both when expressed on a high-copy-number plasmid and when inserted into the genome. The data presented here suggest that this locus is *mrr* and that the *mrr* gene product can recognize and restrict cytosine-methylated sequences. Removal of this DNA region including the *mrr* gene from *E. coli* K-12 strains allows high rescue efficiencies equal to those of *E. coli* C strains. These modified *E. coli* K-12 plating strains and lambda packaging extract strains should also allow a significant improvement in the efficiency and representation of eukaryotic genomic and cDNA libraries.

Several restriction systems that are capable of inactivating foreign DNA by endonuclease cleavage have been identified in *Escherichia coli* strains. The most widely known systems are *Eco*K, Mrr, McrA, and McrBC. The EcoK (Hsd) system works by selectively restricting DNA that is not protected by adenine methylation at the N-6 position in the sequence, A^{N6me}ACNNNNNNGTGC or GC^{N6me}ACNNNNNNGTT (3, 53). The second system, Mrr (methylated adenine recognition and restriction), has also been shown to involve adenine methylation; however, in this case the methylation does not serve to protect the DNA, but makes the DNA vulnerable to the restriction system. Methylation of adenine in the sequence G^{N6me}AC or C^{N6me}AG is recognized and subsequently restricted by Mrr (17). The systems McrA and McrBC (previously known as RglA and RglB) are similar to Mrr in that they recognize and restrict methylated DNA (28, 33–37). However, these two systems differ from Mrr in that they recognize methylated cytosine. If a cytosine is methylated in the sequence 5′-C^{5me}CGG, it will be restricted by McrA (modified cytosine restriction) (34). The McrBC system restricts DNA in the methylated sequence 5′-G^{5me}C (10, 28, 33, 34), G^{5hme}C (36, 37), or G^{N4me}C (4). The *mcrC* gene is a contiguous, downstream gene within the *mcrB* operon under the control of the *mcrB* promoter (39, 40). The *mcrC* gene has been reported as being essential for McrB restriction of most of the identified methylated DNA sequence specificities (11, 27, 39). It should be noted that the recognition sequences for Mcr and Mrr nucleases are suggested in the literature, but precise sequence requirements are unknown.

These restriction activities, when present in lambda packaging extracts or plating strains, reduce the cloning efficiency of methylated DNA (8, 12, 16, 22, 23, 30, 32, 51, 54, 55). It has also been shown that some of these activities inhibit the transfer of lambda shuttle vector DNA from transgenic mice to *E. coli* cells (14, 20, 21, 45). These inhibitory effects on lambda phage rescue result at least partially from the high levels of eukaryotic cytosine methylation occurring within the DNA (21). We had previously developed restriction-free lambda packaging extracts and plating strains in order to remove the inhibitory effect and increase the efficiency of these processes (21, 22, 23). During the development of these extracts and strains, it was observed that strains containing the *mcrA mcrB1* genotype did not rescue methylated lambda DNA genomes as efficiently as did *E. coli* C strains. This observation was consistent with other reports indicating that *E. coli* C has a high tolerance for propagation of eukaryotic DNA (14, 55). To determine the cause of these *E. coli* K-12 and *E. coli* C effects, experiments were performed to identify the locus responsible for the inhibitory activity. This report establishes that the inhibitory activity on lambda rescue is due to the expression of a gene localized within the 99-min region of *E. coli* K-12 strains. In addition, the genes contributing to methylation intolerance within this region of *E. coli* K-12 are identified.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phage. Table 1 lists the *E. coli* strains, plasmids, and phage used.

Media and chemicals. Bacterial strains were routinely grown in LB liquid medium or on NZYM plates (24). Antibiotics (Sigma Chemical Co., St. Louis, Mo.) were added when necessary as follows: ampicillin, 100 μg/ml; tetracycline, 15 μg/ml; chloramphenicol, 30 μg/ml; and ka-

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TABLE 1. Bacterial strains, plasmids, and phages

Strain, plasmid, or phage	Relevant properties	Source or reference[a]
Strains		
C-130	Hfr-5	G. Bertani; 43
C-131	Hfr-6	G. Bertani; 43
C-1091	Hfr-12	G. Bertani; 43
CH1371	*e14-1272*::Tn*10*	C. Hill
E. coli C	*E. coli C4506*; *lacZ*; has no known restriction activity	G. Christie via R. Calendar; 52
E. coli C(pBR)	*E. coli* C4506; pBR322 Amp^r Tet^r	SCS, P. Kretz
E. coli Tn*10* mapping strains	Tet^r	C. Gross; 49
ED8767	*mcrA mcrB1 hsdS3*	N. Murray; 26
ER1451	*hsdR2* or *hsdR17 mcrB1*	NEB; 34
HB101	*mcrB mrr hsdS20* (phenotypically $HsdR^-$); carries the 98.4-min region of *E. coli* B	Laboratory collection; 24
JH122	*mcrB4*::Tn*10 mrr2*::Tn*5*	16a
K-40	HfrH	R. Calendar
K802	*metB1 galK2 galT22 hsdR2 mcrB1 mcrA*	Laboratory collection; 34
LCK8	K802 *zjj-202*::Tn*10*	L. Comai via B. Bachmann; 34
LE392	*hsdR514 mcrA*	Laboratory collection; 24
NM554	*mcrA mcrB hsdR*; MC1061 derivative	SCS; 32
NM621	*mcrA mcrB hsdR*; C600 derivative	N. Murray; 51
PLK-18	K802 *lacZ*; $RecA^+$ pUC18 Amp^r; recipient in interrupted matings	SCS, P. Kretz
RA1	RR1 *mcrA*; carries the 98.4-min region of *E. coli* B	SCS, P. Kretz
S17-1(λpir)	RP4-2-Tc::Mu	K. Timmis; 18, 48
SCS-7	K802 Δ(*mcrBC-hsdRMS-mrr*)*151* Δ*lacZ*	SCS, P. Kretz
Y1088	*hsdR514 mcrA*	Laboratory collection; 56
Y1090	Hsd^+	Laboratory collection; 56
Plasmids		
pBC KS(−), SK(−)	Cloning vectors; pBluescript II Cm^r Amp^s; substrate in restriction assays	SCS; B. Rose, Calgene
pBluescript II SK(−)	Amp^r; cloning vector	SCS; 1
pBR322	Tet^r Amp^r; substrate in restriction assays	Laboratory collection; 6
pBS	Amp^r; cloning vector	SCS; 44
pMS*pst*IMR	pBR322 harboring *Pst*I methylase gene	SCS, M. Simcox
pOU61cos	Amp^r; cosmid vector; temperature-dependent low copy number	V. Knott; 19
pUC18*Sfi*	Amp^r; cloning vector	K. Timmis; 18
pUTKm	Amp^r Km^r; Tn*5*-based delivery plasmid	K. Timmis; 18
Phages		
λL2B	Lambda shuttle vector	SCS; 21
Lambda ZAP II	Substrate in restriction assays	SCS; 44
P1	Generalized *E. coli* transducing phage	D. Smith, UCSD

[a] NEB, New England BioLabs; UCSD, University of California, San Diego.

namycin, 50 μg/ml. Various selective medium plates were used to determine strain phenotypes, including M9 minimal medium (24) and MacConkey medium (Difco Laboratories, Detroit, Mich.). Supplements included glucose, lactose, or galactose (0.2%), thiamine (0.34 μg/ml), and amino acids (0.02 μg/ml), all obtained from Sigma. Fusaric acid and chlortetracycline hydrochloride for Bochner medium were obtained from Sigma. Restriction enzymes used in cloning experiments were obtained from Stratagene Cloning Systems (SCS), La Jolla, Calif.

Restriction phenotype assays. *Eco*K restriction activity was identified by using Lambda ZAP II vector propagated on HB101 (unmethylated) and Lambda ZAP II vector propagated on Y1090 (*Eco*K modified = adenine methylated). The bacterial strain to be tested was infected with Lambda ZAP II vector and plated as a top agar lawn on NZYM. An $HsdR^+$ ($EcoK^+$) strain would allow efficient plaque formation when infected with methylated Lambda ZAP II vector but would restrict unmethylated Lambda ZAP II vector, resulting in at least 100-fold less efficient plaque formation.

Mrr restriction activity was identified by using Lambda ZAP II vector propagated on LE392pMS*pst*IRM (adenine methylated, $CTGC^{N6me}AG$). The bacterial strain to be tested was infected with adenine-methylated Lambda ZAP II vector and plated as a top agar lawn on NZYM. An Mrr^+ strain would allow efficient plaque formation when infected with unmethylated Lambda ZAP II vector but would restrict methylated Lambda ZAP II vector, resulting in two- to fivefold less efficient plaque formation.

McrBC restriction activity was identified by a transformation assay as described by Ross and Braymer (41). Either pBR322 (Amp^r Tet^r) or pBC (Cm^r) was used, depending on the antibiotic resistance of the strain to be tested. The plasmid was methylated in vitro by using *Alu*I methylase ($AG^{5me}CT$) (New England BioLabs, Beverly, Mass.) and then checked for complete methylation by using *Alu*I restriction endonuclease (SCS). The strain to be tested was transformed separately with methylated and unmethylated plasmid, using a standard $CaCl_2$ protocol (24). An $McrBC^+$ strain would restrict the methylated plasmid, resulting in a 10- to 100-fold less efficient transformation compared with the unmethylated plasmid transformation.

McrA restriction activity was identified by the same transformation assay used for McrBC determination except that *Hpa*II methylase ($C^{5me}CGG$) (New England BioLabs) and *Hpa*II restriction endonuclease (SCS) were used instead of *Alu*I enzymes. A 5- to 50-fold less efficient transformation of methylated plasmid was obtained for $McrA^+$ strains.

Rescue assay. λL2B DNA was stably integrated into the mouse genome via microinjection (20, 21, 29, 45). For the experiments presented here, high-molecular-weight genomic DNA was isolated from the testes of a transgenic mouse and allowed to resuspend overnight in buffer. Approximately 10 μg of the genomic DNA was then added to an in vitro lambda packaging extract, Transpack (SCS), for 1 h 45 min at room temperature in order to rescue the lambda DNA from the genome. The packaging reaction was then diluted to 1 ml with SM buffer (24). A 200-μl sample of an overnight culture of the *E. coli* strain of interest was then infected with 5 μl of the diluted reaction by preadsorption for 15 min at 37°C, followed by the addition of 3 ml of molten top agar (24) and plating on NZYM. Plaques were counted after overnight incubation at 37°C. A control was performed in the same manner, using λL2B which had been propagated in *E. coli*. Approximately equal numbers of phage particles were used for the control and experimental platings. The plating efficiencies of *E. coli*-derived λL2B and mouse-derived λL2B were then compared. The plating strain was considered to have high efficiency if the mouse-derived λL2B phage plated equivalent to *E. coli*-derived λL2B phage. The plating strain was considered to have low efficiency if the mouse-derived λL2B plated <5% as efficiently as the *E. coli*-derived λL2B. This would constitute at least a 20-fold difference in plating efficiency between eukaryotic modified DNA and unmodified DNA.

Mapping experiments. Conjugal matings were performed as described by Silhavy et al. (47). Bacteriophage P1 transductions were performed as described by Miller (25).

Cosmid library and deletion mutant construction. A cosmid library of *E. coli* K-12 strain LCK8 was made by partial digestion of the genomic DNA with *Sau*3AI restriction endonuclease. Following ligation into the compatible *Bam*HI site of the temperature-dependent low-copy-number cosmid vector pOU61cos, the DNA was packaged with the size-selective Gigapack II XL lambda packaging extract (SCS; 22, 46). *E. coli* C was infected with the packaged cosmids and grown at 30°C to maintain the low copy number of the cosmid. Clones containing the 99-min region were identified by colony hybridization (24) using an oligonucleotide probe homologous to the *E. coli* K-12 *hsdS* region, bp 227 to 244 (15). Clone pC-CA, containing a 40-kb insert extending approximately from min 98 to 99, was restriction mapped and used for construction of deletion subclones.

Deletion mutants were generated by using selected restriction sites as described below (see Fig. 3). Clone pC-CA was digested with *Bam*HI to release an 8.2-kb fragment which was gel isolated, purified, and ligated into the *Bam*HI site of pBS for the generation of pSC-17. For pSC-5, the 8.2-kb *Bam*HI fragment of pSC-17 was further digested with *Bgl*II and *Eco*RI, allowing isolation of a 5.8-kb fragment, which was then ligated into pBS cut with *Bam*HI and *Eco*RI. An *Eco*RV partial digestion of pSC-5 was performed to obtain a 4.0-kb fragment. This fragment was isolated and ligated into pBC SK(−) cut with *Eco*RV to create pSC-T. pSC-T was restricted with *Hpa*I and *Stu*I to generate a 2.7-kb fragment containing *mcrB* and *mcrC*. This fragment was ligated into the *Eco*RV site of pBluescript II SK(−) plasmid vector to generate pSC-23. The deletion subclone pSC-a was made by digesting pSC-23 with *Nsi*I to generate two fragments, isolating the 5.0-kb fragment, and recircularizing the plasmid. The deletion subclone pSC-H was made by digesting pSC-23 with *Sty*I and *Hin*dIII, generating three DNA fragments, and then blunting the ends by using a Klenow fill-in kit (SCS). Subsequently, the 3.3- and 1.4-kb fragments were isolated and religated to generate a plasmid lacking the *mcrB* gene. pSC-8 was constructed by isolating the 2.4-kb *Hin*dIII fragment of pSC-23 and ligating it into the *Hin*dIII site of pBC KS(−). This construct placed expression of the *mcrC* gene under the control of the β-galactosidase promoter within pBC KS(−). All other clones used the intact *mcrB* promoter for expression of cloned genes.

The cosmid clone pC-Ca was further subcloned by digestion with *Eco*RI, gel isolation, and purification of the single 2-kb fragment. This fragment containing DNA from the *mrr* region was ligated into the *Eco*RI site of pUC18*Sfi* to give pSC-RI.

Plasmid copy number. Plasmid copy number was determined by the method of Taylor and Brose (50). Strains were grown in LB containing [^{3}H]thymidine for 5 h. Then a modified Birnboim-Doly alkaline lysis miniprep procedure was performed, resulting in separate chromosomal and plasmid DNA pellets. Each was acid hydrolyzed and measured for incorporation of radioactivity, which was used to estimate copy number based on the formula $n = [(Gh)(R1)]/[(Gp)(R2)]$, where Gh is the genome size of the host bacteria in base pairs, Gp is the size of the plasmid in base pairs, R1 is the counts per minute of the plasmid fraction, and R2 is the counts per minute of the chromosomal fraction.

Sequence analysis of *mcrBC*. The *mcrBC* genes of clone pSC-23 were sequenced by a dideoxy method with T7 DNA polymerase as instructed by the supplier (United States Biochemical Corp.). Primers (17-mers) were designed to anneal at ~200-bp intervals of both DNA strands based on the sequence data of Ross et al. (40) and Dila et al. (11).

Construction of SCS-7. A P1 lysate made on strain JH122 was used to transduce K802, selecting for a Kan^r Tet^r phenotype. The 99-min region was then deleted by first selecting for loss of tetracycline resistance on Bochner medium (5). Tetracycline-sensitive colonies were then screened for kanamycin sensitivity, indicating that a portion of the 99-min region containing the *mcrBC* and *mrr* genes had been deleted [Δ99 or Δ(*mcrBC-hsdRMS-mrr*)]. To remove McrA activity, the strain was then transduced with a P1 lysate of strain CH1371. Tetracycline-resistant colonies were selected, purified, and then selected for loss of tetracycline resistance on Bochner medium. Restriction assays for McrA, McrBC, Mrr, and *Eco*K were performed to verify the successful deletion of each restriction gene. The resulting strain was named SCS-7.

Insertion of genes into the *E. coli* chromosome. Genes were inserted into the *E. coli* chromosome by the suicide delivery method of Herrero et al. (9, 18). Briefly, clone pSC-a was digested with *Kpn*I and *Eco*RI to allow gel isolation of the *mcrB1* gene and the partial *mcrC* gene. This 2-kb fragment was then ligated into the *Kpn*I and the *Eco*RI sites of the auxiliary plasmid pUC18*Sfi*, allowing the isolation of pSC-a2. The *mcrBC* genes were then excised from this plasmid by using *Sfi*I and ligated into the unique *Sfi*I site of Tn*5*-based suicide delivery plasmid pUTKm. *E. coli* host S17-1(λpir) was transformed with this ligation mixture while Kan^r Amp^r isolates were selected. An isolate found to carry *mcrBC* (pUTKm-a3) was used as the donor strain to conjugally mate this plasmid into the recipient strain SCS-7. Transconjugants were selected on M9 minimal medium containing glucose

TABLE 2. Comparison of plating efficiencies

Strain	Phenotype[a]			Plating efficiency[b] of lambda phage derived from:	
	McrA	McrB	HsdR	*E. coli* K-12	Mouse
ED8767	(−)	(−)[c]	(−)	High	Low
ER1451	(−)	(−)[c]	−	High	Low
HB101	+	−	−	High	Low
K802	−	−[c]	−	High	Low
LCK8	−	−[c]	−	High	Low
LE392	−	+	−	High	Low
NM554	−	−	−	High	Low
NM621	(−)	(−)	−	High	Low
Y1088	(−)	(+)	−	High	Low
E. coli C	−	−	−	High	High

[a] Determined as described in Materials and Methods; agree with published literature. ( ), phenotype is based on previously published literature only; +, restriction activity is present; −, restriction activity is absent.

[b] Plaque formation of mouse-derived λL2B as defined in Materials and Methods.

[c] Carries the *mcrB1* mutation (33).

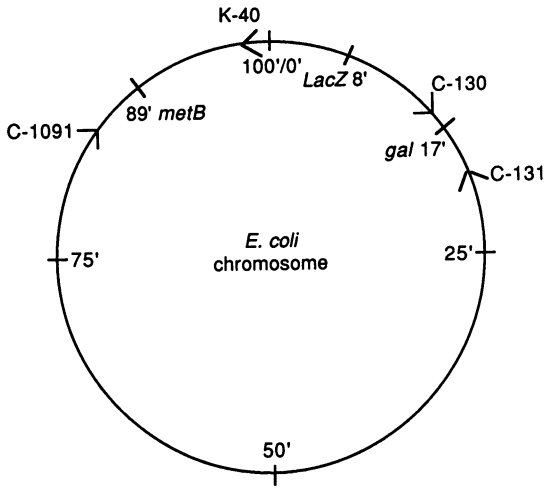

FIG. 1. Minute map of the *E. coli* chromosome. The point of origin and direction of transfer of each *E. coli* Hfr strain used in conjugal mating experiments are indicated by an arrow. Locations of genes used as selectable markers are shown. Previous publications suggested that *E. coli* C and *E. coli* K-12 genetic maps were not identical (52); however, they are similar enough to allow mapping experiments using the markers shown.

and kanamycin. Isolates were then screened for Amp sensitivity, indicating that the entire pUTKm-a3 plasmid was not present but that the Tn*5-mcrBC* region of the plasmid had been successfully transposed into the chromosome. The presence of these genes was further verified by polymerase chain reaction amplification. Two of these isolates, named SCS-7(Ins. *mcrB1*)1 and -2 (Ins. = insertion), were then tested for their effects on rescue efficiency.

The 2-kb *Eco*RI fragment of subclone pSC-RI was inserted into the chromosome of SCS-7 by the same method. However, because the sequence of the inserted fragment was not known in this case, presence of the 2-kb *Eco*RI fragment in the chromosome of SCS-7(Ins. RI) was further verified by Southern blot analysis. Genomic DNAs of LCK8, SCS-7, and SCS-7(Ins. RI) were separately digested with *Eco*RI and *Bss*HII and then subjected to gel electrophoresis and Southern transfer. A random-primed 2-kb *Eco*RI fragment was used as the probe.

RESULTS

Identification of the *E. coli* locus responsible for inhibition of lambda phage rescue from transgenic mice. In original experiments to retrieve lambda phage shuttle vectors from the mouse genome, it was discovered that several *E. coli* K-12 strains did not allow significant recovery of the phage. Restriction-minus (*mcrA mcrB1 hsdR*) strains did allow recovery, but only at an efficiency equal to approximately 0.01% of theoretical (21). Numerous restriction-minus *E. coli* K-12 strains were tested, but all resulted in minimal rescue efficiency (Table 2). However, *E. coli* C, which has no known restriction activity (32, 33, 42), allowed high rescue efficiencies of up to 30% of theoretical (Table 2; 21).

To identify the locus responsible for the decreased rescue efficiency with *E. coli* K-12, Hfr mating experiments were performed. *E. coli* C Hfr strains with various points of origin (Fig. 1) were mated into *E. coli* K-12 strain PLK-18 and plated on media which would allow selection of conjugational recombinants. Since all *E. coli* C Hfr strains permitted high rescue efficiency (data not shown), mated K-12 recipients receiving the relevant locus could be screened for improved rescue efficiency. Uninterrupted mating of C-130 or C-131 into PLK-18 yielded several K-12 recombinants having high lambda rescue efficiency. This finding demonstrated that an *E. coli* K-12 strain allowing efficient rescue could be constructed. To localize the region from *E. coli* C which removed the inhibitory effect from *E. coli* K-12, C-1091 was mated with PLK-18. Several transconjugant isolates that allowed high rescue efficiency were obtained. The *metB* (89-min) or *lacZ* (8-min) locus was transferred to all of these isolates, while the *gal* (17-min) locus was not. This finding suggested that the 17-min *gal* region and flanking regions did not contain the inhibitory locus. A 40-min interrupted mating of C-130 into PLK-18 gave several $MetB^+$ transconjugants that allowed high rescue, suggesting that the essential locus was positioned between min 80 and 15. To narrow the region further, the reverse experiment was performed, in which *E. coli* K-12 strain K-40 (HfrH) was mated into *E. coli* C(pBR), selecting for $LacZ^+$ conjugates. Of the 10 $LacZ^+$ recipients obtained, none reduced the lambda rescue efficiency of *E. coli* C(pBR). Since the K-40 (HfrH) point of origin was positioned at approximately min 100 and *lacZ* is an early transfer gene at min 8, it was concluded that the inhibitory locus was not contained between min 100 and 8 and most likely was positioned in the latter portion of the *E. coli* map. These data suggested that the inhibitory locus was contained between min 80 and 100.

On the basis of the frequency of association of the *metB* (89-min) locus with high rescue efficiency of *E. coli* K-12/*E. coli* C recombinants, P1 transduction of the *metB* (89-min) region was used to define further the inhibitory locus. Donor strains were chosen from a mapping library in which each strain contained a Tn*10* transposon (encoding tetracycline resistance) at a known position in the K-12 chromosome (49). Strains were chosen that resulted in transductants of the region from 75 to 100 min during selection for Tet^r in the recipient strain. Strains which could potentially result in the transfer of nonmutant *mcrB* or *hsdR* genes were not used. Instead, LCK8 was chosen as a donor strain for the 99-min region since it was known to be $HsdR^-$ and to lack McrB

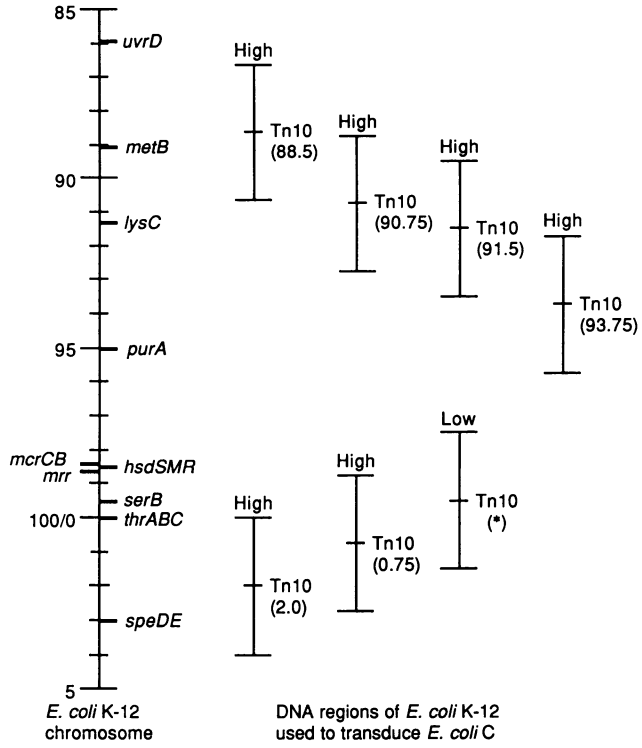

FIG. 2. *E. coli* K-12 chromosome min 85 to 5 and the DNA regions selected in transduction experiments. Not all genes are shown. DNA fragments shown represent possible region transduced when one is selecting for Tn*10*, but actual regions transduced were ~1.8 min in length. (*), Precise location of this Tn*10* was unknown but was believed to be between *mrr* and *serB*; this region was from the donor strain LCK8. All other regions were from donor strains of the Tn*10* mapping library (49). High and Low indicate efficient and inefficient plaque formation of mouse-derived λL2B (as defined in Materials and Methods) on *E. coli* C transductants that received the indicated region of DNA.

activity as a result of the *mcrB1* mutation yet was shown to inhibit efficient rescue of mouse derived λL2B. In addition, it contained a Tn*10* transposon encoding Tet^r near min 99 which could be used as a selectable marker. *E. coli* C was used as the recipient strain because it allowed high rescue efficiency and thus permitted the screening of Tet^r *E. coli* C transductants for decreases in plating efficiency.

Only when LCK8 was used as the donor strain were the Tet^r transductants found to have the inhibitory effect. This finding indicated that DNA between min 96.5 and 1.5 of K-12 had recombined into the *E. coli* C chromosome, causing a decrease in plating efficiency (Fig. 2). Transductions of DNA overlapping this region were then performed using additional donor strains from the Tn*10* mapping library to further map the locus. The limits of the transduced regions and their effect on lambda rescue efficiency are shown in Fig. 2. Other than LCK8-derived *zjj-202*::Tn*10* (~99 min), no DNA regions transferred the inhibitory activity. These data indicated that the inhibitory locus was contained within the 99-min region.

Characterization of the 99-min region by deletion analysis. A cosmid DNA library was constructed from LCK8 DNA, and clones containing the 99-min region were selected by screening with an oligonucleotide probe complementary to the *hsdS* gene. *E. coli* C was chosen as the host strain for the library since previous reports indicate that this strain contains no homology to the *E. coli* K-12 *hsd* region (32, 42). One of the clones identified from this library, pC-CA, was found to inhibit efficient plating of mouse-derived λL2B. After restriction mapping, pC-CA was determined to contain ~40 kb of LCK8 genomic DNA from the region between min 98 and 99 (Fig. 3).

To identify the gene(s) responsible for the inhibitory activity, several subclones were generated from the pC-CA cosmid clones and tested for inhibition. The level of inhibition was determined by testing the plating efficiencies of the lambda DNA recovered from the transgenic mice (Table 3). The largest subclone, pSC-17, has ~8.2 kb of insert DNA containing the *mcrBC* genes (Fig. 3). The presence of this subclone in *E. coli* C cells significantly reduced plating efficiency compared with that of *E. coli* C cells containing only the negative control pBluescript II SK(−) plasmid. Two additional subclones of pC-CA, pSC-5 (~5.8-kb insert) and pSC-T (~4.0-kb insert), were generated to make stepwise deletions from both the 5′ and 3′ ends of the DNA insert. These clones exhibited similar inhibitory activities. An additional subclone, pSC-23, containing only the *mcrB* and *mcrC* genes, also displayed the inhibitory activity. These data indicate that despite the presence of the *mcrB1* mutation, this *mcrBC* locus was capable of inhibiting propagation of lambda phage DNA recovered from transgenic mice.

To determine whether the *mcrB* or *mcrC* gene was independently responsible for this inhibitory effect, additional deletion mutants were generated. pSC-H, containing a complete *mcrC* gene with a 1-kb deletion of *mcrB* gene, did not produce the inhibitory effect. This finding indicated that the *mcrC* gene did not independently generate the inhibitory effect. To evaluate the role of *mcrB* independent of *mcrC*, pSC-a was created by deleting 0.6 kb of the 1-kb *mcrC* gene, leaving the *mcrB* gene complete. The inhibitory effect was not reduced by this deletion, suggesting that the *mcrC* gene was not essential for this activity. However, since this deletion left the truncated *mcrC* gene in the proper translational reading frame, an additional deletion subclone was constructed which eliminated all but 49 bp of the *mcrC* gene. As with the previous construct, the inhibitory effect was not reduced by this deletion (data not shown). It has been reported that the *mcrB* gene may code for 33- and 51-kDa proteins in which the DNA sequence coding for the 33-kDa protein is contained within the DNA sequence of the 51-kDa protein (Fig. 3; 38, 40). For this reason, we investigated the effect of truncating the *mcrB* gene 51-kDa protein on rescue efficiency. The subclone pSC-8, containing an in-frame deletion of the upstream sequences which pertain only to the 51-kDa protein, was tested and found not to display the inhibitory effect (Table 3). It is concluded that the *mcrB* gene without *mcrC* is responsible for an inhibitory effect when expressed from a plasmid and that the 51-kDa protein made by the *mcrB* gene is required, while the 33-kDa protein is not sufficient.

It was also noted from these data that the level of inhibitory effect caused by the various subclones fluctuated from clone to clone. To determine whether this fluctuation was the result of variance in the plasmid copy number, the method of Taylor and Brose (50) was used to determine the plasmid copy number of each clone. When one directly compares the plating efficiency of mouse-derived lambda phage with plasmid copy number, a correlation is evident between decreased plating efficiency and increased plasmid copy number (Table 3).

Sequence analysis of *mcrBC*. To prove that clone pSC-23

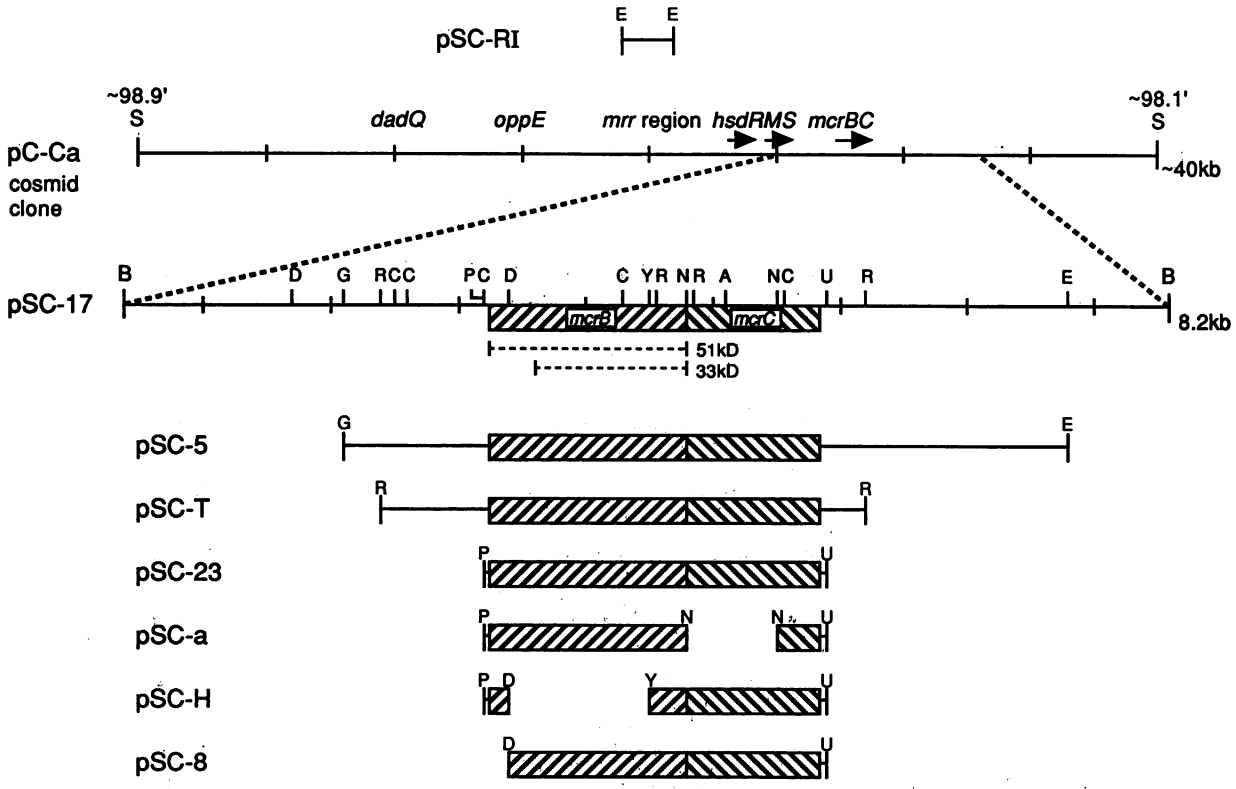

FIG. 3. Subcloning of the cosmid clone pC-Ca. Gene locations and map minutes shown for pC-CA are approximations based on the *E. coli* K-12 genetic map of Bachmann (2) and based on the assumption that *hsd* is located at ~98.4 min (15). Arrows indicate direction of transcription. The possible *mcrB* protein sizes (in kilodaltons) are shown as estimated by Ross et al. (38, 40). Enzymes: B, *Bam*HI; D, *Hin*dIII; G, *Bgl*II; R, *Eco*RV; P, *Hpa*I; C, *Hin*cII; Y, *Sty*I; N, *Nsi*I; A, *Sca*I; U, *Stu*I; E, *Eco*RI; S, *Sau*3AI.

contained the previously uncharacterized *mcrB1* mutation, both strands of the ~2.7-kb insert containing *mcrB* and *mcrC* were sequenced, and the data were compared with the published sequences of Dila et al. (11) and Ross et al. (40). The data obtained here were determined to precisely match those of Dila et al. with the exception of a single base change at bp 807 of the Dila sequence (bp 924 of the Ross sequence). This point mutation results in the conversion of a glutamine codon to a chain-terminating codon in the *mcrB* gene.

Relationship of the *Alu*I methylase assay for McrBC restriction activity to the lambda phage rescue assay. DNA sequences within clones pSC-23 and pSC-a were derived from LCK8, which carries the *mcrB1* mutation and has been shown not to express the classic McrBC restriction activity. This classic restriction activity is defined here as that which recognizes the specific methylated sequences conferred by the methylases *Alu*I, *Dde*I, *Hae*III, *Hha*I, *Msp*I, and *Pvu*II as well as the methylated sequences of unknown specificity conferred by approximately 10 other methylases (34). Despite derivation of these clones from the McrBC$^-$ strain, these clones display an inhibitory activity toward lambda phage rescue (Table 3). To confirm that the subclones do not contain the classic McrBC$^+$ phenotype, the McrBC transformation assay was performed. As predicted, *E. coli* C containing pSC-23 or pSC- a did not restrict the *Alu*I-methylated plasmid (Table 4). However, the control LE392

TABLE 3. Plating efficiencies of clones in *E. coli* C

Clone	Plating efficiency (PFU/plate)[a] of lambda phage derived from:		Plasmid copy number
	E. coli K-12	Mouse	
pC-Ca	278	0	5
pSC-17	389	148	35
pSC-5	309	70	69
pSC-T	302	46	Not tested
pSC-23	257	12	128
pSC-a	331	15	134
pSC-H	386	316	122
pSC-8	194	199	186
pBluescript II	270	271	160

[a] Average of three to five independent platings.

TABLE 4. McrB restriction activities

Strain	*mcrB* genotype	Transformation efficiency of *Alu*I-methylated plasmid[a]	Restricts *Alu*I methylated plasmid
LE392	*mcrB*$^+$	0.02	Yes
LCK8	*mcrB1*	1.2	No
E. coli C	Not present	0.9	No
E. coli C(pSC-23)	—[b]	0.8	No
E. coli C(pSC-a)	—[b]	1.3	No

[a] Relative to that of unmethylated plasmid (set at 1.0).

[b] *E. coli* C4506 carrying a plasmid subclone of the *mcrB1* region of strain LCK8. pSC-23 contains complete *mcrB* and *mcrC* genes. pSC-a contains a complete *mcrB* gene and a deletion in the *mcrC* gene.

TABLE 5. Plating efficiencies of SCS-7 strains carrying *mcrB1*

Strain[a]	Plating efficiency (PFU/plate)[b] of lambda phage derived from:	
	E. coli K-12	Mouse
LCK8	290	0
SCS-7	328	359
SCS-7(pUC18*Sfi*)	356	308
SCS-7(pSC-a2)	225	1
SCS-7(Ins. *mcrB1*)1	350	385
SCS-7(Ins. *mcrB1*)2	301	370

[a] pSC-a2 is pUC18*Sfi* carrying the *mcrB1* gene. SCS-7(Ins. *mcrB1*)1 and -2 are two separate isolates of SCS-7 carrying the *mcrB1* gene inserted into the chromosome.
[b] Average of at least two independent platings.

TABLE 6. Plating efficiencies of SCS-7 strains carrying the *Eco*RI fragment

Strain[a]	Plating efficiency (PFU/plate)[b] of lambda phage derived from:	
	E. coli K-12	Mouse
LCK8	276	1
SCS-7	322	265
SCS-7(pUC18*Sfi*)	356	308
SCS-7(pSC-RI)	311	0
SCS-7(Ins. RI)	300	0

[a] pSC-RI is pUC18*Sfi* carrying the 2-kb *Eco*RI fragment. SCS-7(Ins. RI) is SCS-7 carrying the 2-kb *Eco*RI fragment in the chromosome.
[b] Average of at least two independent platings.

(*mcrB*$^+$) caused an ~50-fold decrease in transformation efficiency, indicating that the methylated plasmid had been restricted. All strains transformed the unmethylated control plasmid efficiently. It can be concluded from these results that the inhibitory effect on the plating efficiency of mouse-derived λL2B caused by these subclones is not the result of the classic McrBC restriction activity but is the result of what we define as McrB1 activity.

Insertion of *mcrB1* into the *E. coli* chromosome. To determine whether the inhibitory effect observed with pSC-a was dependent on expression from a high-copy-number plasmid, the *mcrB1* gene of this construct was stably inserted into the chromosome of SCS-7. SCS-7 was chosen as the recipient strain because it contains a deletion of the 99-min region and also because it is isogenic to LCK8 with the exception of the Tn*10* insertion near *serB* in LCK8. After screening the genotypes of two independent transconjugants to prove that they were SCS-7 and carried the *mcrB1* insertion as opposed to the freely replicating suicide vector, we tested the isolates for inhibitory effect using the lambda phage rescue assay. As shown in Table 5, no inhibitory effect on rescue efficiency was observed in SCS-7 or in either of the SCS-7 isolates carrying the *mcrB1* insertion. SCS-7pSC-a2 caused the expected decrease in efficiency, while SCS-7 carrying the vector with no insert (pUC18*Sfi*) did not cause a decrease. Use of LCK8, from which the *mcrB1* gene was cloned, resulted in the expected decrease in efficiency. All strains tested allowed equally efficient plating of *E. coli*-derived lambda.

Because two independent transconjugants were tested, the lack of inhibitory activity in these isolates is not believed to be due to the region in which the *mcrB1* gene integrated into the chromosome because the method of insertion has been shown by Herrero et al. (18) to be random. The lack of activity upon insertion is also not due to the host strain, since six independent transconjugants in which *E. coli* C was the recipient also did not display the inhibitory effect. Thus, it was concluded that although the *mcrB1* gene can inhibit efficient lambda rescue when expressed on a high-copy-number plasmid (~150 copies), this gene is not responsible for the significant decrease in rescue efficiency observed in LCK8 and other strains carrying the same restriction genotype (*mcrA mcrB1 hsdR*).

Subcloning of the *mrr* region from the pC-Ca cosmid. To identify the gene(s) which results in an inhibitory effect on lambda rescue when expressed from a high-copy-number plasmid as well as when inserted into the chromosome of SCS-7, the original cosmid clone pC-Ca was further subcloned (Fig. 3). The restriction map of pC-Ca indicated that the *mrr* region was contained on the cosmid, and it had previously been shown that a Tn*5* insertion in a 2-kb *Eco*RI fragment of this region would inactivate Mrr adenine methylation restriction activity (17). Therefore, this 2-kb *Eco*RI fragment was cloned. Clone pSC-RI was constructed and determined to decrease rescue efficiency (Table 6). The 2-kb *Eco*RI insert of this plasmid containing the *mrr* region was then inserted into the chromosome of SCS-7. After genotype screening, one transconjugant was found to be SCS-7 carrying the *Eco*RI fragment in the chromosome and was named SCS-7(Ins. RI). This was further verified by Southern blot analysis of the genomic DNA from this isolate (data not shown). The isolate was then tested for effect on lambda rescue efficiency (Table 6). SCS-7 and SCS-7 carrying the vector with no insert (pUC18*Sfi*) allowed high rescue efficiency. SCS-7(pSC-RI) expressed the inhibitory effect as previously shown. SCS-7(Ins. RI) resulted in a decrease in efficiency equal to the decrease observed in LCK8. All strains tested allowed equally efficient plating of lambda derived from *E. coli*. Thus, it is concluded that the 2-kb *Eco*RI fragment causes the inhibitory effect both when expressed on a high-copy-number plasmid and when inserted as a single copy into the *E. coli* chromosome.

Clone pSC-RI was tested for the standard *mrr* activity as defined in Materials and Methods and determined to cause a twofold decrease in plating efficiency of adenine-methylated substrate (data not shown). Because LCK8 (Mrr$^+$) only showed a 2.5-fold decrease and SCS-7 (Mrr$^-$) showed no decrease, it is concluded that this 2-kb *Eco*RI fragment does express the standard Mrr activity as defined by Heitman and Model (17).

Comparison of *E. coli* K-12 and *E. coli* C. SCS-7 was constructed to determine whether deletion of the entire *mcrBC-hsdRMS-mrr* region was sufficient to allow *E. coli* K-12 strains to rescue mouse-derived λL2B as efficiently as *E. coli* C. LE392 (*mcrA*), LCK8 (*mcrB1 mcrA*), SCS-7 (Δ99 *mcrA*), SCS-11 (Δ99 *mcrA*), and *E. coli* C all allowed efficient plating of λL2B which had been propagated in *E. coli* K-12 (Table 7). Consistent with previous experiments, LE392 and LCK8 inhibited efficient plating of mouse-derived λL2B. However, deletion of the *mcrBC-hsdRMS-mrr* region in *E. coli* K-12 resulted in a 1,000-fold increase in plating efficiency. This efficiency was at least equal to that of *E. coli* C. This result confirmed that no *E. coli* C genes were required for high lambda rescue efficiency.

DISCUSSION

E. coli restriction systems are thought to have evolved as a protection system against invading foreign DNA. Although

TABLE 7. Comparison of *E. coli* K-12 and *E. coli* C

Strain	Relevant genotype	Relative plating efficiency of lambda phage derived from:		Inhibits rescue of phage from mouse	Restricts *Alu*I-methylated plasmid
		E. coli K-12	Mouse		
LE392	*mcrA*	570	1	Yes	Yes
LCK8	*mcrB1 mcrA*	580	1	Yes	No[a]
SCS-7	Δ(*mcrBC-hsdRMS-mrr*) *151 mcrA*	570	960	No	No
E. coli C	Not present	500	950	No	No

[a] No, McrB activity is not present.

beneficial to the cell in a natural setting, these systems are deleterious to numerous molecular biological experiments. One of these systems, McrB, was identified by its ability to cleave DNA methylated by any one of 16 different characterized cytosine methylases (34). The classic McrB activity toward these methylated sites can be inactivated by *mcrB1*, a small mutation within the *mcrBC* locus. The exact location of this mutation has been identified here and determined to result in the conversion of a glutamine codon to a chain-terminating codon near the middle of the *mcrB* coding region. Most McrBC$^-$ laboratory strains are believed to carry this mutation (34). Although no detectable activity is present when the *mcrB1* locus is at one copy per cell, this mutation does not completely inactivate the McrBC restriction activity toward propagation of mouse-derived lambda phage when it is expressed from a high-copy-number plasmid. The activity of the *mcrB1* region when expressed from a high-copy-number plasmid reduces plating efficiency of lambda phage DNA isolated from transgenic mouse tissues by more than 20-fold. Although no activity has previously been shown for the *mcrB1* mutant, it was determined by deletion subcloning that this reduced plating efficiency is due to the *mcrB1* gene independent of the *mcrC* gene. Of the numerous methylases known to confer sensitivity to restriction by the *mcrBC* locus, most are believed to require both the *mcrB* and *mcrC* genes. The nonmutant *mcrB* has been shown to act independent of *mcrC* in only one case, that being the restriction of sequences methylated by *Msp*I methylase (11). This activity is inactivated by the *mcrB1* mutation when expressed at low copy number. However, McrB1 activity is detected at high copy number with use of the mouse-derived lambda substrate; therefore, the *mcrB1* mutation most likely acts to lower the efficiency of the *mcrC*-independent restriction activity. The *mcrC*-dependent restriction activity is lost with the *mcrB1* mutation even at high copy number, as judged by the lack of restriction of *Alu*I-methylated DNA. In addition, truncation of 57 amino acids from the amino terminus inactivates the McrB1 activity toward mouse-derived lambda DNA. Together, these data suggest that the amino terminus of the protein contains the catalytic domain of the *mcrB* gene while the carboxy terminus is important for catalytic efficiency and the broad restriction specificity mediated through *mcrC*.

Since the *mcrB1* gene did not inhibit plating efficiency at a low copy number, experiments were continued to identify a gene(s) which would account for the entire inhibitory effect at single copy. Because an additional restriction locus, *mrr*, was also known to be within the 99-min region of the *E. coli* chromosome, the restriction map of Heitman and Model (17) was used to determine that the cosmid clone pC-Ca carried a 2-kb *Eco*RI fragment which coincides with the *mrr* restriction activity toward adenine-methylated sequences. This *Eco*RI fragment was shown to inhibit the plating efficiency of mouse-derived lambda phage by at least 250-fold. Insertion of this fragment into the chromosome of a strain carrying a deletion of the 99-min region, SCS-7, resulted in a similar inhibitory effect on plating efficiency, at least 250-fold. This is equal to the decrease observed in the nondeleted isogenic parent strain LCK8 and demonstrated that the inhibitory effect of the 2-kb *Eco*RI fragment is not DNA copy number dependent.

Mrr restriction activity has previously been associated with restriction of methylated adenines. However, we have shown that this region is capable of inhibiting rescue of lambda DNA from eukaryotic cells. Since only cytosine methylation has been identified in murine cells, it is likely that this *mrr* region contains a gene responsible for restriction of methylated cytosines with specificity differing from that of *mcrA* and *mcrBC*. This activity may be identical to the proposed McrF activity of *mrr* (31).

The complete removal of the 99-min region containing the *mrr* gene from *E. coli* K-12 results in strains that do not inhibit propagation of mouse-derived lambda phage. We have observed that *E. coli* K-12 Δ99 strains [mcrA (Δ*mcrBC-hsdRMS-mrr*)] plate mouse-derived lambda phage as efficiently as do restriction-minus *E. coli* C strains and that conversely, *E. coli* C strains containing the *E. coli* K-12 *mrr* gene plate inefficiently. These observations indicate that the *mrr* gene can independently account for the entire difference in rescue efficiencies between *E. coli* K-12 and *E. coli* C strains. However, we have not ruled out the possibility that other genes in the 99-min deletion region may also cause an inhibitory effect.

This work underscores the importance of using appropriate plating strains and lambda packaging extract strains for genomic cloning as well as for lambda and cosmid rescue by in vitro packaging of DNA from eukaryotic sources. It is likely that the efficiency and representation of many libraries could be increased by using *E. coli* strains that are *mcrA* Δ(*mcrBC-hsdRMS-mrr*). The degree of restriction which results from genes within the 99-min region is dependent on the size and extent of methylation of the target DNA. Because of the magnitude of the inhibitory effect observed in these experiments (~1,000-fold), future characterization of restriction loci may benefit from the use of the convenient lambda rescue assay.

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