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Cloning and Structural Characterization of the *mcrA* Locus of *Escherichia coli*

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***Escherichia coli* has DNA restriction systems which are able to recognize and attack modified cytosine residues in the DNA of incoming bacteriophages and plasmids. The locus for the McrA/RglA system of modified cytosine restriction was located near the *pin* gene of the defective element, e*14*. Hence, loss of the e*14* element through abortive induction after UV irradiation caused a permanent loss of McrA restriction activity. e*14* DNA encoding McrA restriction was cloned and sequenced to reveal a single open reading frame of 831 bp with a predicted gene product of 31 kDa. Clones expressing the complete open reading frame conferred both McrA and RglA phenotypes; however, a deletion derivative was found which complemented RglA restriction against nonglucosylated T6*gt* phage but did not complement for McrA restriction of methylated plasmid DNA. Possible explanations for this activity and a comparison with the different organization of the McrB/RglB restriction system are discussed.**

Escherichia coli has restriction systems which act against 5-methyl- and 5-hydroxymethylcytosines at specific DNA sequences (22). Such processes were first recognized phenomenologically in T-even bacteriophages lacking glucosylation of their 5-hydroxymethylcytosine residues (15, 27, 28). These phages are sensitive to so-called RglA and RglB restriction systems; usually the 5-hydroxymethylcytosine is protected by glucosylation and is not attacked (28). Later, similar restriction activities were manifested by difficulties in interspecific cloning of certain bacterial methylase genes in *E. coli* (17, 18, 25). It appeared that cells with these clones died through the suicidal activity of their own restriction systems acting against the atypically methylated cytosine in their genomes. By treating transforming DNA in vitro, or producing DNA from cells expressing cloned methylase genes, it was possible to create methylated target plasmids and phages to determine the genetic and contextural parameters of modified cytosine restriction (Mcr) (25).

In this way, McrA and McrB restriction systems active against 5-methylcytosine were identified and equated with the RglA and RglB activities discovered earlier (24, 25). McrB recognizes N^4- and C5-methylcytosine produced by a broad range of DNA methylases and appears to act against 5-methylcytosine preceded by a purine residue (21, 25). The *mcrB* locus is at 99 min on the genetic map near *hsdRMS* encoding the more familiar *Eco*K system of restriction and modification (24, 26, 32). Molecular examination of this locus revealed that it is composed of the *mcrB* and *-C* genes (8, 30, 31).

Compared with the McrB system, McrA restriction has a narrower sequence specificity, being active against $C^{me}CGG$ (25). The locus encoding McrA restriction was placed near *purB* in the 25-min region of the chromosome (26) and then located more precisely on the defective element, e*14* (9, 11, 24). This work confirms the conclusion that *mcrA* is near *pin* in e*14* (24), presents a molecular characterization of the gene, and provides an example in which McrA and RglA activities are not fully equivalent.

(A preliminary account of some of this work is presented in the proceedings of the conference RecA and Related Proteins, which was held in Saclay, France, on 17–21 September 1990 [12].)

MATERIALS AND METHODS

Bacteria. *E. coli* AB1157 contains e*14* and is $McrA^+$ as well as being multiply auxotrophic (13). PP1303 is an $e14^-$ derivative of AB1157 (38), and MH757 is PP1303 made recombination deficient by transduction of *srl*::Tn*10 recA1*. WA802 (40) and DH5 (Life Technologies Ltd.) have been characterized as $McrA^-$ $RglA^-$ and $McrA^+$ $RglA^+$, respectively (23).

Plasmids and phages. pGP62 consists of e*14* cloned in pBR322 with duplication of the *P*-invertible region and *pin* gene of e*14* (38). pBR322 (3) and Bluescript plasmid pKS^- (Stratagene Inc.) were used for cloning and analysis of *mcrA* sequences. pMH503 is an Amp^s derivative of pHSG415 (37) and confers kanamycin and chloramphenicol resistance; it was chosen for its sensitivity to McrA restriction after appropriate methylation. Wild-type T6 phage and nonglucosylated T6*gt* were used to test the Rgl phenotype (27).

Bacterial culture. Cultures were routinely grown in LB broth or agar (16) with 50 μg of kanamycin, 15 μg of ampicillin, or 15 μg of chloramphenicol per ml when needed for plasmid maintenance. Plasmid transformation used the $CaCl_2$ method (16). For UV irradiation, cultures were resuspended in 10 mM $MgSO_4$ and exposed to UV light from a Hanovia mercury vapor lamp. Doses were determined with a UVX Radiometer (Ultraviolet Products Inc., San Gabriel, Calif.).

Plasmid DNA. Plasmid DNA was prepared by alkaline lysis (2) and purified, when necessary, by CsCl equilibrium centrifugation with ethidium bromide (16). DNA fragments were purified by using Geneclean (Bio 101 Inc.) or by electroelution. DNA dephosphorylation and ligation used standard methods (16). Plasmid DNA was digested with restriction endonucleases (Bethesda Research Laboratories) in accordance with the manufacturer's instructions.

Mcr restriction of plasmid DNA. pMH503 DNA was methylated with *Hpa*II methylase (BCL Ltd.) for McrA restriction assays and with *Hha*I methylase (BCL Ltd.) for assays

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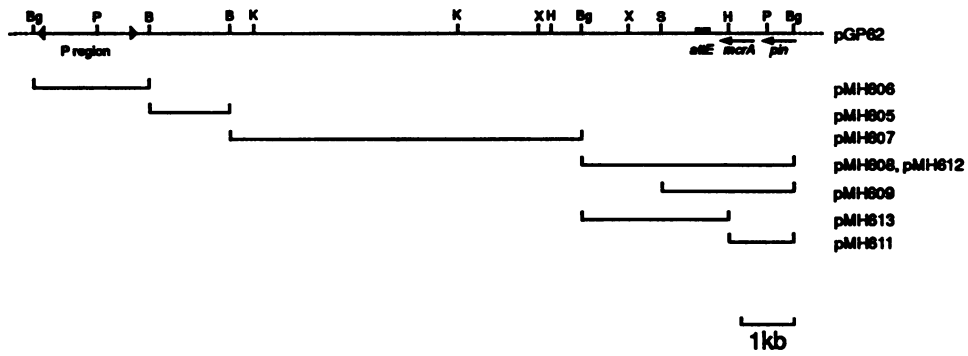

FIG. 1. Physical map of the *mcrA* region and showing the circularized form of e*14* cloned in pGP62. Segments of pGP62 were subcloned into the plasmids listed on the right. pMH612 and pMH613 contain segments of pGP62 DNA subcloned into pBR322; pMH605 to -611 were derived from segments of pGP62 DNA subcloned into pKS$^-$. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; P, *Pst*I; K, *Kpn*I; H, *Hin*dIII, S, *Sal*I; X, *Xba*I.

of McrB restriction. The Mcr status of bacteria was determined from the relative efficiency of transformation with pMH503 plasmid DNA which had been treated with methylase compared with unmethylated controls. The relative transformation efficiency of methylated DNA was the ratio of transformation frequencies of methylated versus nonmethylated DNA. To compensate for minor differences in the concentration of input DNA, the relative transformation efficiency was normalized by the ratio of methylated and nonmethylated DNA transformation efficiencies in nonrestricting McrA$^-$ McrB$^-$ WA802 or McrA$^-$ PP1303.

Rgl phenotype tests. The Rgl phenotype was determined from the relative plating efficiency of T6*gt* and T6 phages. To compensate for minor differences in the titers of the two phages, the relative plating efficiency was normalized by the ratio of phage titers determined in nonrestricting RglA$^-$ *E. coli* WA802.

DNA hybridization. For colony hybridization (35), we used Whatman 541 paper with stringent conditions and 50% formamide washing at 65°C (1), omitting polyethylene glycol and NaCl from the hybridization buffer. Probes were labeled with [α-^{32}P]thymidine triphosphate (Dupont New England Nuclear Research Products), using a Random Oligonucleotide Labeling Kit (Amersham Corp.) in accordance with the manufacturer's instructions.

DNA sequencing. Dideoxy DNA sequencing was done with a Sequenase II kit from U.S. Biochemical Corp. and ^{35}S-ATP (34). For analyses of nucleotide sequence, we used the University of Wisconsin Genetics Computer Group package of programs (6).

Nucleotide sequence accession number. The *E. coli mcrA* sequence has been assigned the GenBank accession number M-76667.

RESULTS

Subcloning *mcrA* from plasmid pGP62 containing genetic element e*14*. Previous work found that McrA restriction was encoded by the defective element e*14* (11, 24); prior to that, e*14* had been cloned (38) to produce plasmid pGP62. As a first step in locating the *mcrA* locus in e*14*, a restriction map of pGP62 was constructed with respect to restriction enzymes *Bgl*II, *Bam*HI, *Hin*dIII, *Kpn*I, and *Xba*I. The four e*14* DNA fragments produced by digestion of pGP62 with *Bam*HI and *Bgl*II were subcloned into the Stratagene Bluescript vector pKS$^-$ (Fig. 1).

Subclones were examined for their ability to complement *E. coli* PP1303 for its deficiency in McrA restriction, using a plasmid transformation assay. In this assay the recognition sequence for McrA restriction, C^{me}CGG, was created in vitro by using commercially purified *Hpa*II methylase (25). The test plasmid selected for this work was pMH503, an ampicillin-sensitive derivative of the low-copy-number vector pHSG415 (37). pMH503 has several advantages; it is compatible with plasmid ColE-based cloning vectors being tested, its resistance to kanamycin and chloramphenicol does not impair selection of the ampicillin resistance of the cloning vectors, and compared with a number of other plasmids, it is relatively sensitive to attack by McrA restriction after methylation with *Hpa*II methylase. In parallel transformations, the relative transformation efficiency of *Hpa*II-methylated versus nonmethylated pMH503 DNA gives a clear indication of McrA restriction status. Accordingly, *Hpa*II methylation reduced the transformation efficiency of pMH503 to approximately 1% relative to unmethylated DNA in *mcrA*$^+$ AB1157 (Table 1). In *mcrA* PP1303,

TABLE 1. McrA and RglA phenotypes of plasmids with cloned *mcrA* sequences

Host[a]	Plasmid	Transformation efficiency (methylated/nonmethylated)[b]	McrA	Plating efficiency of T6*gt*[c]	RglA
mcrA$^+$		0.008	+	$<10^{-4}$	+
mcrA		1	−	1	−
	pGP62	0.011	+		
	pMH605	0.5	−		
	pMH606	1.1	−		
	pMH607	0.7	−		
	pMH608	No transformants			
	pMH609	0.02	+	$<10^{-4}$	+
	pMH611	0.76	−	$<10^{-4}$	+
	pMH612	0.01	+	$<10^{-4}$	+
	pMH613	0.88	−	0.89	−

[a] Hosts for the transformation assay were *mcrA*$^+$ AB1157 and *mcrA* PP1303, except pGP62, which used the *recA1 mcrA* MH757 derivative of PP1303 for plasmid stabilization (38). Hosts for the RglA assay were *mcrA*$^+$ DH5 and *mcrA* WA802.

[b] Transformation efficiency of methylated pMH503 versus nonmethylated pMH503 in the test strain normalized to the transformation efficiency in control McrA$^-$ *E. coli* PP1303.

[c] Plating efficiency of nonglucosylated T6*gt* on the test strain versus WA802 RglA$^-$.

there was no discrimination in transformation efficiency between *Hpa*II-methylated and nonmethylated DNAs, which transformed with equal efficiency.

Subclones pMH605, pMH606, and pMH607 did not complement PP1303 for McrA restriction, as shown by the approximately equal transformation efficiencies of *Hpa*II-methylated and nonmethylated pMH503 (Table 1). However, when the 4-kb *Bgl*II fragment cloned into pKS$^-$, to give pMH608, was introduced into PP1303, no transformants arose with either *Hpa*II-methylated or nonmethylated pMH503. The presence of pMH608 was found to cause difficulties in growth for a number of *E. coli* K-12 strains such that the McrA phenotype conferred by this plasmid could not be determined by using the normal transformation assay. The same 4-kb *Bgl*II fragment, cloned into the single *Bam*HI site of pBR322 and transformed into *E. coli* PP1303, did not cause the same growth abnormalities observed for pMH608. This plasmid was designated pMH612 and was found to complement *E. coli* PP1303 for its deficiency in McrA restriction assayed by transformation with *Hpa*II-methylated plasmid DNA.

***mcrA* is situated between *pin* and *attE* within e*14*.** The position of *mcrA* was mapped more finely by using deletion derivatives of plasmids pMH608 and pMH612 in assays of McrA restriction. This cloned DNA was bisected by cleavage at a *Hin*dIII site between the *attE* and *pin* loci; subcloning the *pin*-containing portion gave pMH611, while the larger subclone on the other side of the *Hin*dIII site gave pMH613 (Fig. 1). Neither pMH611 nor pMH613 was able to complement PP1303 for its deficiency in McrA restriction of *Hpa*II-methylated DNA, indicating that the *Hin*dIII site of the cloned DNA is situated within the *mcrA* locus. Plasmid pMH609 was constructed from pMH608 by deleting the 1.5-kb *Sal*I fragment extending from the Bluescript polylinker to the single *Sal*I site in the cloned insert. pMH609 fully complemented *mcrA* in *E. coli* PP1303. Thus, *mcrA* must lie in the 2-kb region between the *Sal*I site at the end of the cloned insert of pMH609 and the *Pst*I site of pMH609, known to be situated within the *pin* gene of e*14*.

Complemention the RglA$^-$ phenotype. To date, the McrA and RglA phenotypes have been usually taken as synonymous. Thus, the *mcrA* locus would encode the RglA phenotype describing restriction of nonglucosylated T-even phage (24). T6*gt* phages lack the glucosyltransferase required to glucosylate their 5-hydroxymethylcytosine DNA and are restricted by RglA. The plating efficiency of T6*gt* phage on RglA$^+$ *E. coli* DH5 was less than 10^{-4} of that of wild-type phage on the same strain, whereas mutant and wild-type phages plated with equal efficiency on RglA$^-$ *E. coli* WA802 (Table 1).

Just as it complemented McrA restriction, pMH612 complemented RglA$^-$ WA802, reducing the plating efficiency of T6*gt* to the low levels found in wild-type cells (Table 1). Similarly, pMH609, which was McrA$^+$, was also RglA$^+$. Furthermore, the *Hin*dIII deletion derivative pMH613, which had lost e*14* sequences between the *Hin*dIII site and the vector polylinker, was McrA$^-$ and also RglA$^-$. However, pMH611, with deletion in the opposite direction from the same *Hin*dIII site, still gave a RglA$^+$ phenotype even though the transformation assay showed it to be McrA$^-$ (Table 1). Thus, assays of RglA and McrA were not completely equivalent. These data indicate that the gene encoding RglA must be located between *pin* and the *Hin*dIII of pMH611 (Fig. 1). However, in the transformation assay of McrA restriction, the putative *mcrA* locus appeared to be interrupted by the same *Hin*dIII site.

One possible explanation for the apparent discordance between McrA and RglA assays could be that there are two separate genes lying adjacently in the area between *pin* and *attE*, one of which spans the *Hin*dIII restriction site and is required for McrA restriction and the other of which is sufficient to encode RglA. An alternative explanation would be that a single gene encodes both restriction activities and that a terminal deletion affects only McrA activity. In an attempt to resolve these possibilities, the segment of DNA from the end of *pin* to *attE* was sequenced.

Sequencing of *mcrA*. Genetic analysis of clones containing the whole of e*14* had indicated that the locus encoding McrA and RglA was located somewhere between the end of *pin* and *attE*, the e*14* attachment site. Published DNA sequences were available for both of these areas (4, 20), which enabled suitable primers to be designed for sequencing both strands of the intervening region thought to encode *mcrA*. The DNA sequence of this area between *pin* and *attE* is made up of 991 bp (Fig. 2). Within this region there is a 831-bp open reading frame, and this bridges the *Hin*dIII restriction site which interrupts McrA activity. Upstream from this open reading frame are consensus −10 and −35 promoter sequences, indicating that expression may be possible from this sequence, which we therefore define here as the *mcrA* locus. The base pair composition of the *mcrA* gene is 61% AT, which compares with an AT richness of 55% for the e*14* invertible region and 61% for the *mcrBC* genes.

The translated product of the *mcrA* gene contains 277 amino acids and would have a molecular weight of 31,389. The deletion plasmid, pMH611, which displays an McrA$^-$ RglA$^+$ phenotype, would encode the first 197 of these amino acids, but may in practice form a fusion product from transcription continuing into 21 codons of flanking vector DNA. Codon usage is not typical of highly expressed *E. coli* genes, indicating that this sequence may be expressed poorly. Indeed, our failure to detect any protein products from McrA$^+$ plasmids in maxicells (33), other than β-lactamase (data not shown), also suggests that the *mcrA* sequence is poorly expressed.

SOS-induced alleviation of McrA restriction. Since SOS induces loss of e*14* from the chromosome (9), SOS-induced alleviation of McrA restriction might also be expected. Hence, *mcrA*$^+$ *E. coli* AB1157 cells were irradiated with 40 J of UV per m^2 and assayed for subsequent McrA restriction of *Hpa*II-methylated pMH503. After 1 h of postirradiation growth, restriction of *Hpa*II-methylated pMH503 was comparable to that of control, unirradiated *E. coli* AB1157, with transformation of *Hpa*II-methylated pMH503 at 2.5% of unmethylated levels (Table 2). However, in a sample of the irradiated cells which were subcultured into fresh medium and grown overnight (15 h), the transformation efficiency of *Hpa*II-modified plasmid DNA rose to 45% (Table 2). Thus, UV irradiation of *E. coli* alleviated McrA restriction after an extended period of postirradiation growth. By using colony hybridization to an e*14* DNA probe, it was shown that 25 of 50 colonies originating from the irradiated culture had lost e*14* from their chromosomes (Fig. 3). Furthermore, in tests of individual e*14*$^+$ and e*14*$^-$ UV stocks, those which had lost e*14* had become McrA$^-$, while those which had retained e*14* continued to be McrA$^+$ (data not shown). Thus, the percentage of cells which had totally lost McrA restriction by abortive excision of e*14* matched the overall reduction in McrA restriction in the whole population of irradiated cells.

Plasmid DNA treated with *Hha*I methylase was used in parallel transformation assays to examine the effects of UV irradiation on McrB restriction (25). However, unlike McrA,

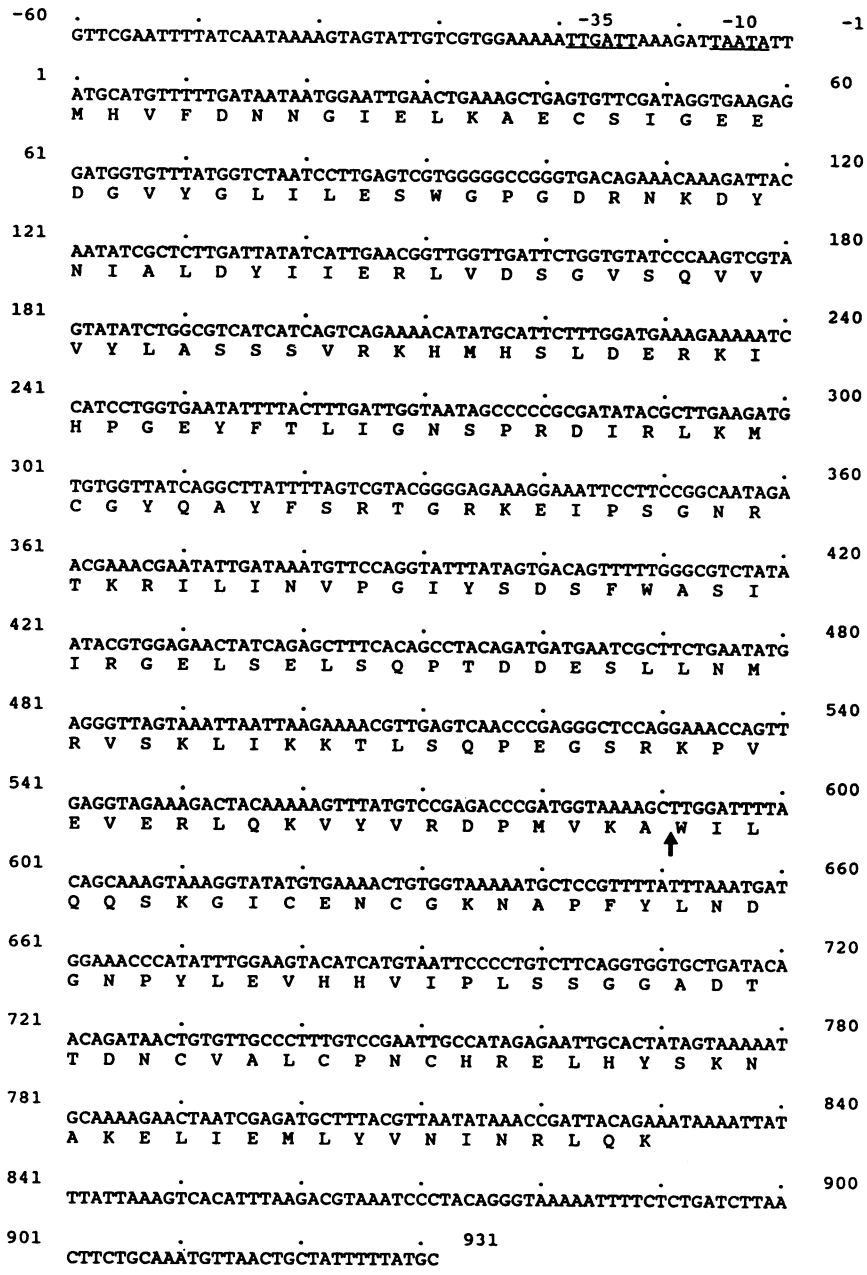

FIG. 2. Nucleotide sequence of the *mcrA* locus. Putative regulatory elements are underlined. The interruption to the protein sequence by *Hin*dIII cutting to produce pMH611 is indicated by the vertical arrow after amino acid 98.

McrB restriction of *Hha*I-methylated plasmid did not change during up to 15 h of growth after UV irradiation (Table 2) nor was there any large-scale loss of *mcrB* genomic DNA (data not shown). Thus, UV irradiation did not alleviate McrB restriction.

DISCUSSION

The locus specifying the *E. coli* McrA DNA restriction system has been localized to a single gene in the *att-pin* interval of the defective element, e*14*. The presence of *mcrA* on e*14* was based first on earlier work (11, 24) and then on the demonstration here that SOS-induced loss of e*14* is coupled with loss of McrA restriction of *Hpa*II-methylated plasmid. The actual position of *mcrA* came from the ability of subcloned fragments of e*14* to provide McrA restriction activity in a nonrestricting host cell. In one of these complementing subclones, a single open reading frame was found which traversed an McrA-inactivating *Hin*dIII site. In other words, when this sequence was interrupted by cleavage at the *Hin*dIII site, McrA restriction activity was lost. Furthermore, this open reading frame is preceded by potential −10 and −35 consensus promotor sequences. These results therefore confirm the position of *mcrA* in e*14* determined independently by Raleigh et al. (24). The restriction pattern of the *mcrA* region is consistent with the genomic restriction map of *E. coli* (14).

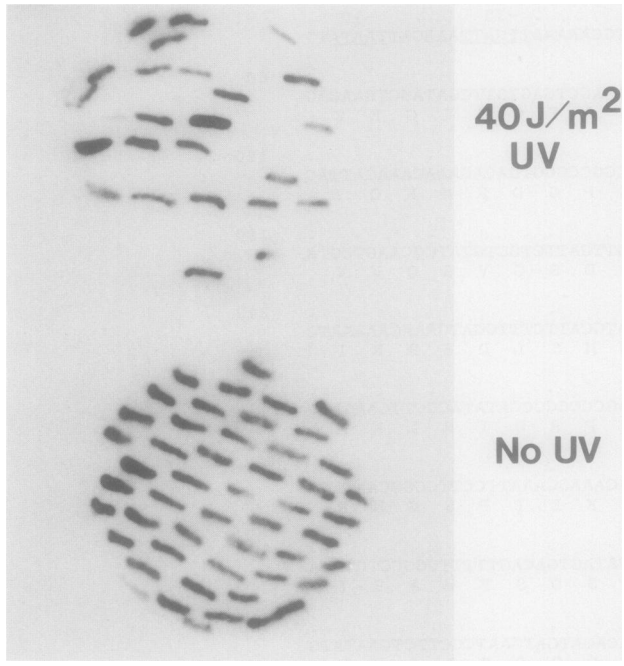

FIG. 3. Loss of e*14* from *E. coli* after UV irradiation. Colony nucleotide hybridization was used to detect the presence of e*14* in 50 single-colony isolates of unirradiated *E. coli* AB1157 (lower) and 50 single colonies grown from cells surviving a UV dose of 40 J/m^2 (upper). The probe was the whole pGP62 plasmid carrying cloned e*14*.

As discussed earlier, RglA and McrA phenotypes have been seen as manifestations of the same gene activity assayed in different ways (24, 25). However, the phenotype conferred by a plasmid encoding a 3′-terminal deletion of the *mcrA* sequence proved to be an exception to this rule. This plasmid, pMH611, was able to complement its e*14*$^-$ host for restriction of nonglucosylated T6*gt* but did not complement for restriction of *Hpa*II-methylated plasmid. pMH611 has a truncated *mcrA* sequence encoding 197 amino acids followed by 21 more vector codons before reaching a stop codon. Hence, there does appear to be some difference in the way in which the products of this sequence deal with methylated and hydroxymethylated DNA. This difference may reflect either different affinities in the residual activity of the truncated peptide for the two types of cytosine modification or the existence of different domains for interaction with 5-methyl- and 5-hydroxymethylcytosines. A separation of RglA and McrA phenotypes has also been reported in a mutant which showed a temperature-sensitive McrA phenotype but was RglA$^-$ at both permissive and restrictive temperatures (19). A Rgl$^-$ McrA$^+$ phenotype has also been found by Ramalingam et al. (cited in reference 22).

TABLE 2. UV-induced alleviation of McrA but not McrB restriction

Strain	UV dose (J/m^2)	h post-UV growth before transformation	Transformation efficiency (methylated/nonmethylated)[a]	Mcr phenotype
PP1303	0		1	McrA$^-$
AB1157	0		0.025	McrA$^+$
AB1157	40	1	<0.001	McrA$^+$
	40	15	0.45	45% McrA$^-$
WA802	0		1	McrB$^-$
AB1157	0		<0.007	McrB$^+$
AB1157	40	1	<0.007	McrB$^+$
	40	15	0.012	McrB$^+$

[a] Methylation with *Hpa*II methylase for McrA assays or *Hha*I for McrB assays. Given is the transformation efficiency of methylated pMH503 versus nonmethylated pMH503 in the test strain normalized to the transformation efficiency in control McrA$^-$ *E. coli* PP1303 or McrB$^-$ WA802.

The equivalent RglB and McrB phenotypes of *E. coli* encoded by *mcrBC* have been separated and shown to rely on different combinations of the McrB and -C proteins (7, 8, 29, 30). However, this explanation for the separation of McrA and RglA phenotypes is not consistent with the single gene structure of the *mcrA* locus found here.

The permanent loss of McrA restriction activity after SOS-inducing treatments would be predicted because of the abortive induction and loss of e*14* in an event reminiscent of lysogenic induction. The extended period of outgrowth needed to lose McrA restriction stems from the time needed to lose e*14* by unilinear inheritance in the descendants of the induced cells. However, McrB restriction was not alleviated nor was there any loss of *mcrB* chromosomal material. Thus, the responses of McrA and McrB restriction systems to SOS induction differ from one another and from the transitory alleviation of *Eco*K restriction elicited by SOS induction of *E. coli* K-12 (5, 10, 36).

The single gene of the *mcrA* locus produces a product with a predicted weight of 31 kDa. This contrasts with the McrB system in which more than one gene product is involved (7, 8, 29–31). The mechanism of action of these gene products in recognizing and removing methylated cytosine from DNA is not yet known. However, the marked structural and size differences of the *mcrA* and *mcrBC* loci suggest different mechanisms of McrA and McrB action. A similar conclusion might also be drawn from the narrow sequence specificity of McrA for removal of methylated cytosine compared with McrB (25). This situation may have parallels with bacterial excision repair systems for removing other forms of modified or damaged bases. The *E. coli* nucleotide excision repair system relies on a complex of relatively large UvrA, -B, and -C proteins to recognize and remove a wide range of damage. In contrast, other specific forms of damage are removed in base excision events which rely on single, small DNA glycosylases with very narrow substrate ranges (39). Resolution of the mechanisms of modified cytosine removal obviously awaits purification and characterization of the McrA protein.

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