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Identification of the *merR* Gene of R100 by Using *mer-lac* Gene and Operon Fusions

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Transcriptional (operon) and translational (gene) fusions between the R100 *merR* gene and *lacZ* were constructed in vitro in a pBR322 plasmid carrying the *mer* genes derived from plasmid R100. The translational fusions were oriented in the opposite direction to and divergently from the *merTCAD* genes. This shows that the reading frame previously thought to be *merR* was incorrect. Expression of the gene fusion was repressed in *trans* by a compatible plasmid carrying the R100 *merR*$^+$ gene, as was a similarly oriented transcriptional fusion. In contrast, expression of β-galactosidase by the *lac* fragment located at the same site but in the opposite orientation was at a lower level and was not repressed by *merR*$^+$.

Resistance to mercurial compounds is a common plasmid-encoded property in bacteria (4, 16, 18). It involves detoxification of Hg^{2+} in the medium by an inducible intracellular flavoprotein called mercuric reductase, which converts the toxic Hg^{2+} to the nontoxic volatile Hg^0 (6, 14, 18, 19).

Genetic analysis and DNA sequencing studies have identified six genes in the *mer* region of R100 (5, 10–12; T. K. Misra, N. L. Brown, L. Haberstroh, A. Schmidt, D. Goddette, and S. Silver, Gene, in press). *merR* is a regulatory gene which controls transcription of the *merTCAD* operon both negatively and positively. In the absence of Hg^{2+} it acts as a repressor, while in the presence of Hg^{2+} transcription is induced. In addition, experiments with Mu *d lac* fusions showed that the *merR* product negatively regulates its own transcription (12).

DNA sequence analysis of the *mer* region of R100 revealed an open reading frame oriented in the same direction as *merTCAD* which was postulated to be *merR* (orf1 in Fig. 1) (10). However, it did not span a *Hin*cII site which genetic experiments have shown to be located in *merR* (12). Examination of the DNA sequence revealed an open reading frame in the opposite orientation which could correspond to this gene (T. K. Misra and S. Silver, personal communication; orf3 in Fig. 1). This does span the important *Hin*cII site and is oriented divergently from the *merTCAD* genes (Fig. 1).

In this report the properties of transcriptional (operon) and translational (gene) *merR-lacZ* fusions formed in vitro at the *Hin*cII site in *merR* are described. They have allowed the orientation of the *merR* gene to be identified unambiguously.

MATERIALS AND METHODS

Bacterial strains and plasmids. The plasmids used in this study are listed in Table 1. The host strain was DU5003 Δ(*lac-pro*)*XIII thi rpoB* (12).

Media and chemicals. The bacteriological media used was as described previously (12). Antibiotics and other chemicals were obtained from Sigma Chemical Co., St. Louis, Mo., or were the best grade available from British Drug Houses.

Restriction endonucleases and other enzymes. Restriction enzymes, T4 DNA ligase, and polynucleotide kinase were purchased from Boehringer Mannheim Corp. and were used according to the instructions of the manufacturer. The decameric *Bam*HI linker sequence dCCGGATCCGG was purchased from Collaborative Research, Waltham, Mass.

Isolation of plasmid DNA. The techniques for the isolation of DNA on a small scale or preparatively have been described previously (12).

β-Galactosidase assay. The β-galactosidase assay was performed as described previously (9, 12).

Construction of a linker insertion mutation in *merR*. A deletion mutation in the pBR322 *merR*$^+$ plasmid pDU1003 was constructed in vitro by removing the *Eco*RI fragment carrying the promoter-distal part of the *merA* and *merD* genes. This plasmid (pDU1102) confers hypersensitivity to Hg^{2+} and expresses *merR*$^+$ activity, as measured by complementation tests with the R100-1 *merR*::Tn*801* plasmid pDU3321.

Next, the *Hin*cII site in the *merR* region was converted into a *Bam*HI site in vitro. pDU1102 DNA was partially cleaved with *Hin*cII and fractionated on an agarose gel, and linear molecules were extracted by freeze-thawing. These were blunt-end ligated with a decameric *Bam*HI linker sequence which had been pretreated with polynucleotide kinase to provide 5′-phosphate groups. Tetracycline resistance transformants were selected in a host carrying pDU3321 (pDU202 *merR*::Tn*801*). Those transformants which failed to complement the *merR* mutation (the cells remained susceptible to Hg^{2+}) were presumed to have a linker insertion mutation in *merR*. This was confirmed by restriction enzyme analysis of the plasmids. Thus the *Hin*cII site present in pDU1102 was shown to be missing in pDU1205 (Fig. 2, lanes C and E) while pDU1205 gained a second *Bam*HI site (Fig. 2, lanes B and D). The sizes of the *Hin*cII fragments of pDU1102 (3.0 and 3.8 kilobases [kb]) and the *Bam*HI fragments of pDU1205 (2.7 and 4.1 kb) were consistent with the mutation mentioned above. Also, plasmids pDU1102 and pDU1205 were cleaved with *Taq*I (Fig. 2, lanes P and Q). The fragment patterns were identical apart from the 355-base-pair fragment of pDU1102 which carries the *Hin*cII site in *merR* (1, 10) which was slightly larger in pDU1205.

DNA sequencing. The 370-bp *Taq*I fragment of pDU1205 which carries the linker insertion mutation in *merR* was cloned into *Acc*I-cleaved M13mp9, and the nucleotide sequence was determined by the method of Sanger et al. (15).

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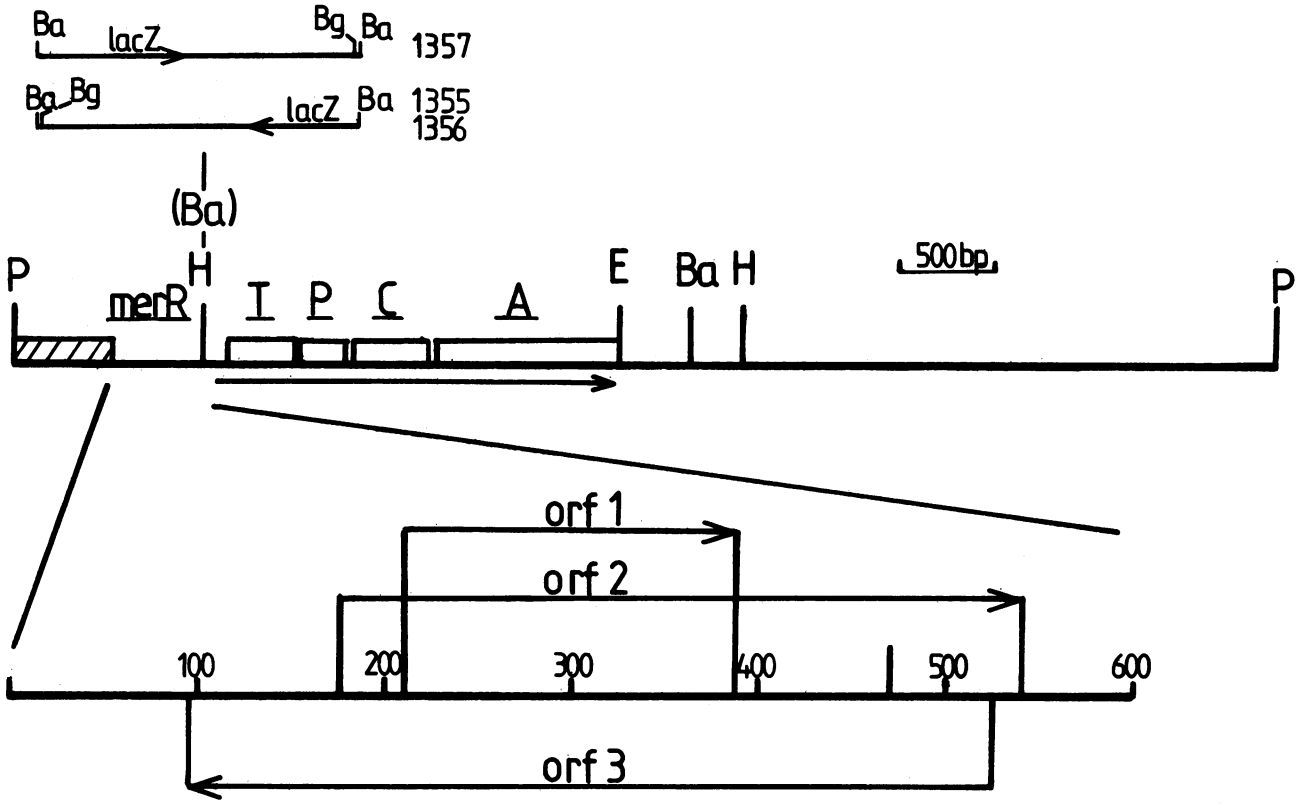

FIG. 1. Map of the *mer* region of pDU1102 and its derivatives. A map of plasmid pDU1102 is shown in the center of the figure. Restriction endonuclease cleavage sites are abbreviated as follows: P, *Pst*I; H, *Hin*cII; Ba, *Bam*HI; E, *Eco*RI; Bg, *Bgl*II. The *mer* sequences derived from R100-1 are located between the *Pst*I and *Eco*RI sites at the left of the map, while pBR322 sequences are to the right of the *Eco*RI site. The Ap^r determinant of pBR322 has been deleted, as have *mer* sequences promoter-distal to the *Eco*RI site in *merA*. The open boxes show the position of open reading frames corresponding to *merT*, *merP*, *merC*, and the promoter-proximal part of *merA*. It should be noted that the designations of these genes have been altered since the discovery of an additional open reading frame in the R100 sequence (*merC*), which is absent in the sequence of the closely related Tn*501* *mer* region (1; T. Misra and S. Silver, personal communication), and the finding that there are two genes involved in uptake of Hg^{2+} (*merT* and *merP*; N. Ni Bhriain and T. J. Foster, manuscript in preparation). The upper part of the figure shows the position and orientation of the pMC903 and pMC931 *Bam*HI *lac* fragments in the gene and operon *merR-lac* fusions. These fragments are not drawn to scale. An expanded view of the *merR* region is shown in the lower section. The numbers refer to nucleotides in the R100 DNA sequence (10) which begins at the first nucleotide to the right of the IS*1* (hatched box in the pDU1102 map). The three open reading frames have been designated orf1, orf2, and orf3. Their orientation is indicated by the arrows. The first nucleotide of the initiation codon and the last nucleotide of the termination codon for the reading frames is as follows: orf1, residues 210 and 492; orf2, residues 173 and 535; orf3, residues 526 and 92.

The sequences of the altered *Hin*cII site was shown to be GTTCCGGATCCGGGAC, with the three nucleotides at either end being derived from the *Hin*cII site and the central 10 nucleotides corresponding to the inserted linker fragment.

Construction of *merR-lac* fusions. pDU1205 DNA was cleaved partially with *Bam*HI. This was mixed with pMC903 or pMC931 DNA which had cleaved with *Bam*HI (to release the *lac* fragments) and with *Hin*dIII (to cut the vector fragment to reduce the number of undesired recombinants). The DNA was ligated and transformed into strain DU5003 selecting for Tc^r colonies on MacConkey-lactose agar. Red Lac^+ colonies which were Tc^r Ap^s Ka^s were picked, and the structure of the plasmids was analyzed with restriction enzymes. Those which were shown to be composed of the two pDU1205 *Bam*HI fragments plus the 7.0- or 6.8-kb *lac* fragment from pMC903 or pMC931 (Fig. 2, lanes F and G) were then cleaved with *Bgl*II and *Pst*I to determine the orientation of the inserted fragment (Fig. 2, lanes J through L). In the case of recombinants formed with the *lac* fragment of pMC931 (putative translational fusions), all the Lac^+ plasmids tested (six of six) had the inserted fragment in the same orientation. This was shown by the small (1.2 kb) junction fragment formed by cleavage with *Pst*I and *Bgl*II (Fig. 2, lane K) which is composed of the 1.1-kb *Pst*I-*Bam*HI fragment of pDU1205 fused to the *lac* promoter-distal 0.1-kb *Bgl*II-*Bam*HI fragment (3) of the insert. Five transcriptional fusions formed with pMC903 had their insert in this orientation, forming a small *Pst*I-*Bgl*II junction fragment of the same size as the translational fusions (Fig. 2, lane L), whereas one was in the opposite orientation, with *Pst*I-*Bgl*II fragments of 8.8 and 5.9 kb (Fig. 2, lane J).

RESULTS

Construction of *merR-lac* fusions. The pBR322-derived plasmid pDU1102 was manipulated in vitro to convert the *Hin*cII site in *merR* (12) into a *Bam*HI site by insertion of a decameric linker sequence. The structure of the insertion was verified by DNA sequence analysis. It caused a *merR* mutation which demonstrates again that the *merR* gene spans the *Hin*cII site.

TABLE 1. Plasmids

Plasmid	Phenotype[a]	*mer* genotype	Comments	Source or reference
pDU1003	Tc^r Hg^r (Ap^s)	pBR322*mer*$^+$	*mer* operon of R100 cloned between the *Pst*I and *Eco*RI sites of pBR322, causing an Ap^s mutation	12
pDU1102	Tc^r Hg^{ss}	pDU1003 Δ*merA*	Deletion of 4.5-kb *Eco*RI fragment of pDU1103	This study
pDU1205	Tc^r Hg^s	pDU1003 *merR* Δ*merA*	Linker insertion mutation at the *Hin*cII site in *merR* gene of pDU1102	This study
pDU1355	Tc^r Hg^s Lac^+	pDU1205 *merR lacZ*	7-kb *Bam*HI fragment of pMC903 in *Bam*HI site in *merR* gene of pDU1205	This study
pDU1356	Tc^r Hg^s Lac^+	pDU1205 Φ(*mer'-lacZ*)	6.8-kb *Bam*HI fragment of pMC931 in *Bam*HI site in *merR* gene of pDU1205	This study
pDU1357	Tc^r Hg^s Lac^+	pDU1205 *mer lacZ*	7-kb *Bam*HI fragment of pMC903 in *Bam*HI site in *merR* gene of pDU1205 (opposite orientation to pDU1355)	This study
pDU1125	Cm^r Hg^r	pACYC184 *mer*$^+$	*mer*$^+$ *Hin*dIII fragment cloned from R100-1 into pACYC184	12
pDU1126	Cm^r Hg^s Ap^r	pDU1125 *merR*	*merR*::Tn*801* mutation cloned from pDU3321 into pACYC184	12
pDU3321	Cm^r Sm^r Su^r Hg^r Ap^r Tra^+	*mer*::Tn*801*	*merR*::Tn*801* mutant of pDU202, a Tc^s variant of R100-1	5
pDU3316	Cm^r Sm^r Su^r Hg^{ss} Ap^r Tra^+	*merA*::Tn*801*	*merA*::Tn*801* mutant of pDU202, a Tc^s variant R100-1	5
pMC903	Ap^r Ka^r		With 7-kb *Bam*HI fragment with promoterless *lac* operon	3
pMC931	Ap^r Ka^r		With 6.8-kb *Bam*HI fragment suitable for constructing gene fusions	3

[a] Abbreviations: Tc, tetracycline; Cm,chloramphenicol; Ap, ampicillin; Ka, kanamycin; Sm, streptomycin; Su, sulphonamides; Hg, mercuric ions; Tra^+, proficient in conjugational transfer; r, resistant, s, sensitive, ss, hypersensitive.

Two types of *merR-lac* fusions were constructed. First, a transcriptional (operon) fusion was generated by inserting the 7-kb *Bam*HI *lac* fragment from pMC903 into the *Bam*HI site of pDU1205. This fragment lacks a promoter but carries a ribosome-binding site and translational initiation signal for *lacZ* (3). Six Lac^+ clones were examined further. The *lac* operon fragment was found in both possible orientations at the *Bam*HI site which suggests that promoters are active in both directions in this region.

Second, a gene (translational) fusion was formed by in-

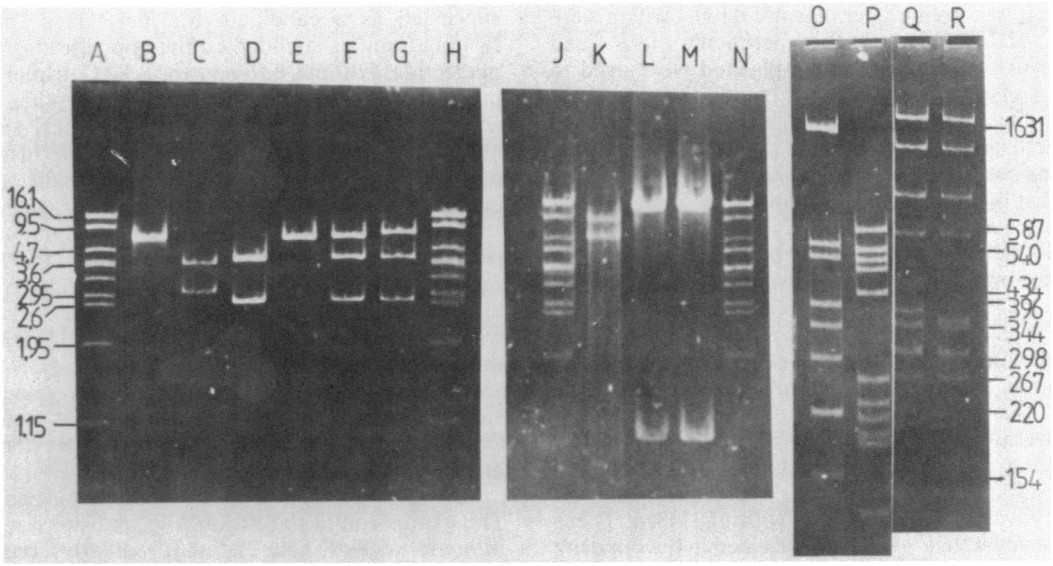

FIG. 2. Restriction endonuclease cleavage of *mer* and *mer-lac* plasmids. Plasmid DNA was cleaved with restriction enzymes and fractionated on 1% agarose gels (lanes A through N) or 5% polyacrylamide gels (lanes O through R). Bacteriophage lambda DNA cleaved with *Eco*RI and *Bam*HI provided size markers for agarose gels (lanes A, H, J, and N), while pBR322 DNA cleaved with *Hin*fI (lane O) or *Hae*III (lane P) provided markers for polyacrylamide gels. pDU1102 was cut with *Bam*HI (lane B) and *Hin*cIII (lane C). pDU1205 was cut with *Bam*HI (lane D) and *Hin*cII (lane E). pDU1356 was cut with *Bam*HI (lane F). pDU1355 was cut with *Bam*HI (lane G). pDU1357 was cut with *Bgl*II and *Pst*I (lane K). pDU1356 was cut with *Bgl*II and *Pst*I (lane L). pDU1355 was cut with *Bgl*II and *Pst*I (lane M). pDU1205 was cut with *Taq*I (lane Q). pDU1102 was cut with *Taq*I (lane R). Numbers to the right of the figure are in bp and those to the left of the figure are in kbp.

TABLE 2. Regulation of expression of β-galactosidase specified by *mer-lac* fusions

Complementing plasmid[a]	β-Galactosidase activity (units)[b] conferred by *mer-lac* plasmids					
	pDU1355 (*mer*R-*lacZ*)		pDU1356 φ(*merR'-lacZ*)		pDU1357 (*mer-lacZ*)[c]	
	Uninduced	Induced	Uninduced	Induced	Uninduced	Induced
pDU3321 *merR*	1,650	1,855	451	486	617	562
pDU3316 *merR*$^+$	541	468	163	161	393	443
pDU1126 *merR*	2,573	2,473	555	597	586	608
pDU1125 *merR*$^+$	570	534	59	66	601	638

[a] pDU3321 and pDU3316 are derivatives of plasmid pDU202 (R100-1 Tc^s) and hence have a low copy number. pDU1125 and pDU1126 are derived from pACYC184 and have a high copy number.
[b] Units of β-galactosidase have been defined previously by Miller (9).
[c] pDU1357 has the *lac* fragment inserted in *merR* in the same orientation as the *merTCAD* genes, whereas in pDU1355 the insert is in the opposite orientation.

serting the 6.8-kb *Bam*HI *lac* fragment from pMC931 into pDU1205. This sequence has a *lacZ* gene which lacks a promoter, a ribosome-binding site, and the codons for the first seven amino acids of β-galactosidase. For the enzyme to be expressed all these functions must be supplied by another gene which is fused in frame to *lacZ*. Six putative *merR-lacZ* gene fusions (i.e., Lac$^+$) were isolated, each having the *lac* fragment oriented in the opposite direction to the *merTCAD* genes (Fig. 1 and 2). This indicates that a divergently transcribed gene is being actively expressed in the cloned *mer* region of R100.

Complementation tests with *merR-lac* fusions. Results of earlier work with a *merR*::Mu *d lac* transcriptional fusion of plasmid R100-1 showed that *merR* transcription was repressed by a functional *merR*$^+$ gene in *trans* (12) and suggested that *merR* is autogenously regulated. To determine whether the expression of the in vitro-constructed *merR-lac* fusions is regulated in a similar fashion, complementation tests were performed with two sets of *mer* plasmids. (i) Derivatives of the multicopy plasmid pACYC184 (which is compatible with pBR322) having a insert of the entire *mer* operon of R100-1 or a similar construct carrying the *merR*::Tn*801* insertion mutation from pDU3321. (ii) Mutants of the low-copy-number plasmid R100-1 which were *merR*$^+$ *merA* (pDU3321). In both experiments *merR*$^+$ and *merR* variants of the complementing plasmid were used to eliminate any nonspecific effects that the additional plasmid might have on the expression of β-galactosidase.

The β-galactosidase activities of the double plasmid-carrying strains were measured both in uninduced cultures and in those that had been induced with Hg^{2+} (Table 2). The β-galactosidase activity of the divergently oriented transcriptional and the translational fusion plasmids was strongly repressed by the multicopy *merR*$^+$ plasmid pDU1125 but not by the *merR* element pDU1126. Futhermore, the repressed expression of β-galactosidase in the *merR-lacZ*/*merR*$^+$ cells could not be elevated by induction. This is a similar result to that obtained with the R100-1 *merR*::Mu *d lac* fusion (12). A similar effect was observed in the complementation tests with the lower-copy-number pDU3316 plasmid, but the level of repression was not as marked as that in the experiment with the pACYC184 *merR*$^+$ element. Presumably less repressor activity was available in tests with pDU3316. These data strongly suggest that *merR* is transcribed in the opposite orientation to that of *merTCAD* and that the coding sequence traverses the *Hin*cII site.

In contrast, the transcriptional fusion with the *lac* fragment in the same orientation as the *merTCAD* genes expressed a somewhat lower level of β-galactosidase than pDU1355. This activity was not repressed by a complementing *merR*$^+$ plasmid.

DISCUSSION

Results of several studies have indicated that the *Hin*cII site in the promoter-proximal part of the R100 *mer* operon is located in the *merR* gene. (i) An in vitro-constructed deletion mutation which removed all of the *mer* sequences to the right of the *Hin*cII site was *merR* (12). (ii) *merR*::Tn*5* insertion mutations mapped on both sides of the *Hin*cII site (12). (iii) The insertion of a 10-bp linker sequence into the *Hin*cII site described here also inactivated *merR*. Thus the suggestion of Misra et al. (10) that a 180-bp open reading frame (orf1, Fig. 1) located in this region corresponds to *merR* is unlikely to be correct. This open reading frame reads in the same direction as *merTCAD* but stops before the *Hin*cII site.

Examination of the DNA sequence of the R100 *merR* region (1; T. K. Misra and S. Silver, personal communication) has shown that there are three open reading frames (Fig. 1). orf1 was originally thought to be *merR* (10). It begins at residue 210 and could code for a polypeptide of 60 amino acids. However, it does not cross the *Hin*cII site known to be in *merR*. The reading frame beginning at nucleotide 173 (orf2) could encode a polypeptide of 120 residues (orf2) and does span *Hin*cII. It has also been suggested as a candidate for *merR* (1). The third open reading frame is encoded on the opposite strand. It starts at nucleotide 526 and extends to a TAG triplet beginning at nucleotide 94. It could specify a polypeptide of 144 amino acids and also spans the important *Hin*cII site. Consideration of the changes that occurred on insertion of the decameric linker sequence at the *Hin*cII site and the subsequent insertion of the 6.8-kb *Bam*HI *lac* fragment of pMC931 suggested that an in-phase gene fusion would occur between orf3 and *lacZ*. This was borne out by the high level of expression of β-galactosidase by pDU1356. orf2 could not form an in-phase gene fusion with the pMC931 *lac* fragment, which explains the failure to isolate Lac$^+$ derivatives with the fragment in the other orientation.

The active expression of the fusion β-galactosidase also suggests that orf3 is transcribed and translated in wild-type cells and provides strong evidence that the divergently oriented reading frame is *merR*.

This is strengthened by two additional observations. (i) The expression of the fusion β-galactosidase was repressed in *merR*$^+$ cells (Table 2). (ii) A similarly constructed transcriptional fusion with the 7-kb fragment of pMC903 gave high expression of β-galactosidase which was also repressible (Table 2). The different levels of β-galactosidase activity expressed by the gene and operon fusions is most likely due to the different structures of the proteins. In an operon fusion the enzyme has the same structure as the wild-type protein, whereas a hybrid polypeptide is formed in a gene

fusion. Thus the *mer* operon seems to be organized in a similar fashion to other operons such as *ara* (13), *tet* (2, 8), and Tn*3 tnp* (7), in which the regulatory gene is subject to autogenous regulation. However, this conclusion is seemingly contradicted by the properties of a deletion isolated by Barrineau et al. (1) which removes the codons for the 15 carboxy-terminal amino acid residues of orf3 but remains *merR*$^+$. The most likely explanation for this is that the truncated polypeptide retains its functional properties.

Another question that remains to be answered concerns the functions of the products (if any) of orf1 and orf2. Results of this study show that transcription occurs in the orientation of these open reading frames. This activity was not repressible by a *merR*$^+$ element and could emanate from a bona fide *mer* promoter, from a promoter located in the remnant of IS*1*, or from a vector promoter. It is possible that regulation of the *mer* operon is complex, with the inducer function being provided by the orf3 protein and the repressor activity being supplied by either orf1 or orf2.

By analogy with the known starting point for divergent transcription through the *merR* region of the closely related Tn*501* element (residue 554; P. A. Lund and N. L. Brown, manuscript in preparation) the most likely candidate for the −35 and −10 sequences of the *merR* promoter can be identified as the TTGGCC sequence between residues 594 and 589 and the TAGCGT sequence between residues 569 and 564. The promoter overlaps the likely *mer* operon promoter and regions of dyad symmetry which may be regulatory sites. Additional work is needed to identify these sequences unequivocally.

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ADDENDUM IN PROOF

In Tn*501* the repressor and inducer activity of *merR* resides in the same open reading frame, orf3 (P. A. Lund, S. J. Ford, and N. L. Brown, submitted for publication).

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