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Overexpression and DNA-Binding Properties of the *mer*-Encoded Regulatory Protein from Plasmid NR1 (Tn*21*)

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In plasmid NR1 the expression of genes involved in mercury resistance (Tn*21*) is regulated by the *trans*-acting product of the *merR* gene. An in vivo T7 RNA polymerase-promoter overexpression system was used to detect a protein of approximately 16,000 daltons encoded by the *merR* reading frame. Overexpressed MerR constituted about 5% of labeled proteins. An in vitro MerR–*mer-op* (*mer-op* is the *mer* operator and promoter region) gel electrophoresis binding assay established that the binding site for MerR was located between the putative −35 and −10 sequences of the promoter for the *mer* structural genes. A nonsense mutation in the carboxyl half of MerR resulted in the loss of biological function and the loss of in vitro *mer-op* binding properties.

The expression of *mer* genes in Tn*21* is under control of the *trans*-acting *merR* product, which regulates the structural genes both negatively and positively (3, 12, 25). When cells are exposed to ionic mercury [Hg(II)], the structural genes (*merTPCA*) are expressed, whereas in the absence of mercury, *merR* acts as a repressor and prevents transcription of *merTPCA*.

Extensive homology of the *mer* operons from the IncFII plasmid NR1 (1, 25) and from Tn*501* (25) has been shown in DNA sequence analyses. The *merR* region of the *mer* operon of Tn*21* (1, 25) encodes three open reading frames (ORFs), ORF 1, ORF 2, and ORF 3 (Fig. 1). The two smaller (6,457- and 13,139-dalton) polypeptides predicted from the ORFs are oriented in the same direction as the *mer* structural genes. In Tn*21* and Tn*501* (1, 25), the largest ORF (ORF 3), which should encode a protein of 16,000 daltons, is oriented divergently from *merTPCA*.

Previous reports (10, 22, 26) suggest that ORF 3 encodes the *merR* gene product. Linker insertions into the *Hin*cII site, which lies within ORF 2 and ORF 3, destroy the activator function of *merR* (26). The smallest reading frame (ORF 1) can thus be eliminated as a *merR* candidate with respect to positive regulation of *merTPCA*. Transcriptional (ORF 2 and ORF 3) and translational (ORF 3) *lacZ* fusions in *merR* from Tn*21* (10) and transcriptional *lac* fusions in ORF 1, ORF 2, and ORF 3 of *merR* from Tn*501* (22) indicate that ORF 3 is expressed as a protein product. S1 nuclease mapping in Tn*501* reveals a single transcript corresponding to ORF 3 of *merR*, whereas no detectable transcripts exist for ORF 1 or ORF 2 (22). *merR* transcription is repressed by wild-type *merR* in *trans* in *merR* (ORF 3)-*lac* fusions (10, 26). Therefore, in addition to its effects on *merTPCA*, *merR* synthesis is subject to negative autoregulation (10, 22, 26).

While there are many similarities in the putative *cis*- and *trans*-acting regulatory elements in these two systems (1, 11, 25), there are also some important differences. There are nine changes in the amino acid sequences of the proteins themselves (32). Though the putative DNA-binding sites are identical, there are differences in their nearby flanking regions, notably in the 5′ end of the structural gene transcript (25, 32). Moreover, the operons differ in that Tn*21* contains an additional membrane protein (MerC) which has been implicated, along with MerR, in efficient expression of the monocopy operon (3). Furthermore, while Hg(II)-inducible transcription is known to proceed beyond the *mer* operon into the transposition genes of Tn*501* (31), in the Tn*21* operon Hg(II)-inducible transcription terminates with high efficiency immediately after the putative *merD* ORF (B. D. Gambill and A. O. Summers, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, H149, p. 164). Finally, Tn*21* is representative of a highly conserved *hgr* locus apparently limited in its natural occurrence to members of the family *Enterobacteriaceae*. In contrast, Tn*501* is a rare representative of a poorly conserved class of MerC-lacking *hgr* loci found in nonfermentative gram-negative bacteria (M. P. Gilbert and A. O. Summers, submitted for publication). Although recombinant DNA work has demonstrated that each *mer* operon can function in both types of hosts in the laboratory (14, 22), regulation of gene expression in each locus will likely play a role in niche specificity.

To date, MerR from Tn*21* has not been isolated, though MerR (ORF 3) from Tn*501* has recently been purified and characterized as a specific DNA-binding protein (27). In this study, a T7 RNA polymerase-promoter system was used to identify and overexpress *merR* as a protein product and to demonstrate specific DNA-binding properties of MerR. The behavior of mutants constructed in vitro confirmed the role of this protein in *mer* regulation in Tn*21*.

Expression of *merR* with a T7 RNA polymerase-promoter overexpression system. (i) Constructions of a *merR* overexpression plasmid and a *merR* frameshift mutant in this plasmid. A 665-base-pair (bp) *Nco*I-*Sca*I fragment carrying the *merR* gene from pDB7 (Fig. 1) (1) was cloned into the *Sma*I site of pT7-3 (Fig. 2A) (35). The orientation of the fragment in pDG124 (ORF 1 and ORF 2) and pDG125 (ORF 3) was determined by a *Pvu*II-*Hin*cII double digest. Both pDG124 and pDG125 complemented pBS1139 (*merR* mutant) (3), as determined by zone-of-inhibition assays which have been described previously (17, 18). To introduce a TGA terminator codon into the *merR* frame, pDG125 containing ORF 3 downstream from the T7 promoter was digested with *Bss*HII. The resulting staggered ends were filled in with DNA polymerase I (Klenow fragment). A 12-bp *Eco*RI linker was inserted by the method of Lathe et al. (21). This construction resulted in a terminator mutant (pAH026) adding 11 nonspecific amino acids downstream from the *Bss*HII

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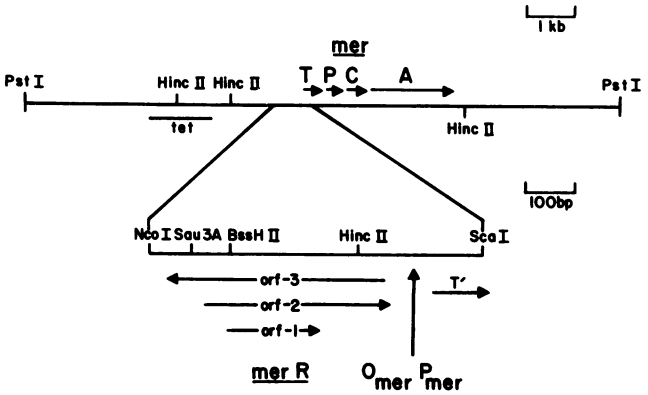

FIG. 1. Map of the *mer* region of pDB7. $O_{mer}P_{mer}$, *mer-op*.

site before termination at a TGA codon in frame with ORF 3. The nonsense mutant described above did not complement pBS1139 (*merR* mutant), indicating loss of *merR* positive regulatory function with respect to *merTPCA* structural genes.

(ii) In vivo *merR* overexpression. The T7 RNA polymerase-promoter overexpression system (35) was used to visualize the *merR* product because it allows controlled and nearly exclusive expression of cloned genes with a unique T7 promoter and the selective inhibition of host RNA polymerase by rifampin (5). The system consists of a plasmid expressing the T7 RNA polymerase under lambda p_L control (pGP1-2) (35) and a second vector (pT7-3) with the T7 promoter preceding a polylinker site.

The 665-bp *ScaI-NcoI* fragment contains the entire *merR* gene, the putative transcriptional regulatory site of the operon, and the first 30 amino acid residues of *merT* (1). The fragment was cloned downstream from the T7 promoter in both directions such that either ORF 1 and ORF 2 or ORF 3 would be transcribed by the T7 RNA polymerase. With heat induction, high levels of T7 RNA polymerase are synthesized (35), and transcription by T7 RNA polymerase is initiated only at the T7 promoter on the pT7-3 vector.

Expression of β-lactamase, which is also dependent on transcription from the T7 promoter, serves as an internal control to assess the efficiency of the expression system. The *bla* product appears as two bands, the 29,000-dalton precursor (34) and the 27,000-dalton mature enzyme. A third band corresponds to a translational segment reading counterclockwise on the pT7 vector (35).

Cells (JM101) (24) containing pGP1-2/pT7-3, pGP1-2/pDG124, and pGP1-2/pDG125 were grown at 30°C in L broth with 50 μg of ampicillin and 40 μg of kanamycin per ml. At 40 to 50 Klett units, 0.2 ml of cells was harvested and washed in M9 medium (7). After centrifugation, the cells were suspended in 1 ml of M9 medium plus 20 μg of thiamine per ml and a 0.01% concentration of 18 amino acids (minus cysteine and methionine) and were starved at 30°C for 1 to 1.5 h (uninduced). For induction, the temperature was shifted to 42°C for 15 min (induced), and rifampin was then added to a final concentration of 200 μg/ml (induced plus rifampin). Cells were incubated for an additional 15 min at 42°C, and the temperature was shifted to 30°C for 20 min before the cells were pulse-labeled for 5 min with 15 μCi of [^{35}S]cysteine (Amersham Corp., Chicago, Ill.). After they were harvested, the cells were suspended in 50 μl of Na_2PO_4 buffer (pH 7.2), and phenylmethylsulfonyl fluoride was added to a final concentration of 2.5 mM. Samples were diluted with an equal volume of 2× sample buffer (20), heated for 5 min at 100°C, and immediately loaded onto a 15% polyacrylamide gel. One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis was done by the method of Thomas and Kornberg (36). After gels were run overnight, they were stained and destained by the method of Dempsey et al. (9) and treated with En^{3}Hance (New England Nuclear Corp., Boston, Mass.). Gels were dried under heat and vacuum on filter paper (Whatman, Inc., Clifton, N.J.) and exposed to X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) at −70°C (23) for 24 to 48 h.

Only pDG125 synthesized an approximately 16,000-dalton protein (Fig. 2B, lane I). However, the *merR* signal was weak compared with that of the internal control β-lactamase. The *merR*-ORF 3 frame contains four cysteine residues (1), whereas the mature β-lactamase has three cysteines (33). Densitometric analysis of gels prepared by using several dilutions of labeled protein showed that the expression level of the labeled 16,000-dalton *merR* protein was about 10% of β-lactamase (data not shown). The intensity of the 16,000-dalton protein signal was not enhanced by supplying a wild-type *mer* operon (uninduced or mercury induced) in *trans* (data not shown).

MerR-DNA-binding assay with crude lysate. (i) Preparation of *mer-op* DNA fragments (the *mer* operator and promoter regions). Fragments carrying the *mer* transcriptional regula-

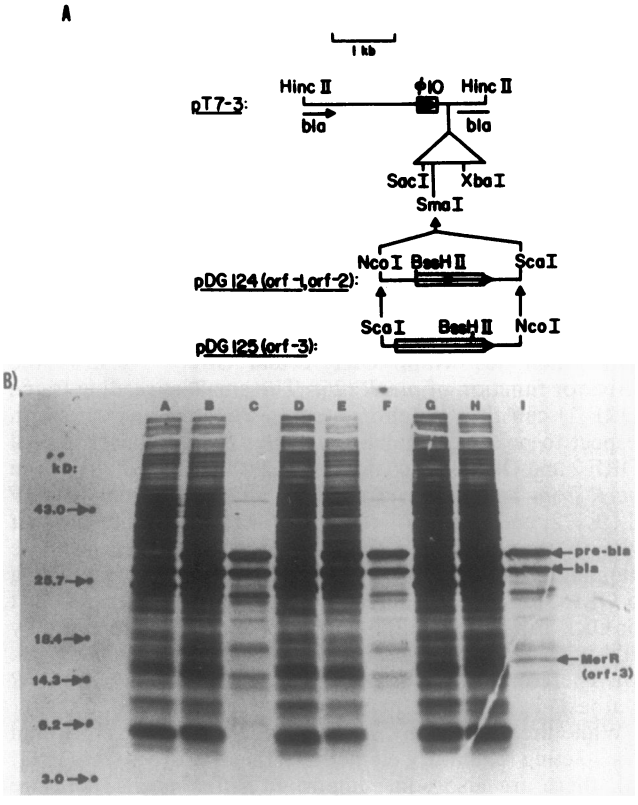

FIG. 2. (A) Maps of pT7-3 and its derivatives (φ10 is the T7 promoter). kb, Kilobase. (B) Overexpression of *merR*: pGP1-2/pT7-3 (lanes A, B, and C), pGP1-2/pDG124 (lanes D, E, and F), pGP1-2/pDG125 (lanes G, H, and I). Lanes A, D, and G, uninduced; lanes B, E, and H, induced; lanes C, F, and I, induced plus rifampin (see text for definitions of uninduced, induced, and induced plus rifampin). Sizes of protein markers (ovalbumin, α-chymotrypsinogen, β-lactoglobulin, lysozyme, bovine trypsin inhibitor, and insulin) are indicated on the left in kilodaltons (kd).

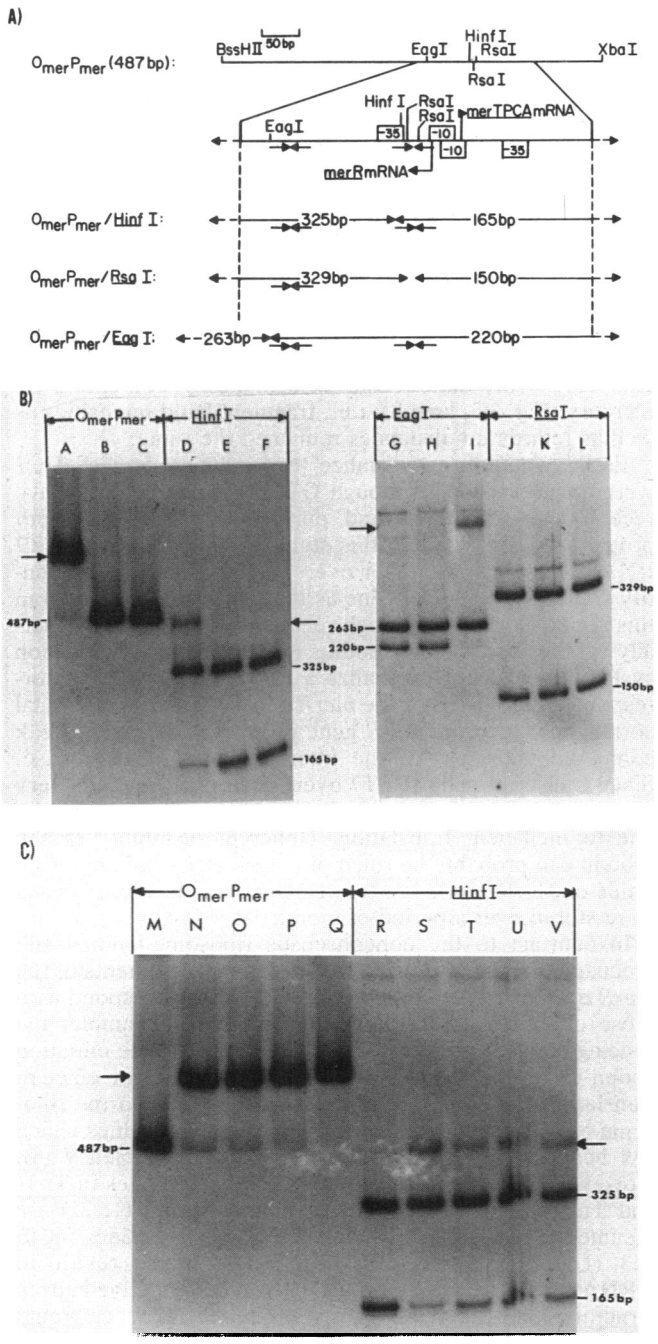

FIG. 3. (A) *mer-op* ($O_{mer}P_{mer}$) fragment derived from pDG124 and fragments resulting from digestion with *Hin*fI, *Rsa*I, and *Eag*I. Putative −10 and −35 regions on each strand are boxed. Regions of dyad symmetry are indicated with single arrows. Putative *merR* and *merTPCA* transcription start points in Tn*21* are designated with arrows. (B) MerR binding to the 487-bp *mer-op* ($O_{mer}P_{mer}$) fragment (lanes A, B, and C) digested with *Hin*fI (lanes D, E, and F), *Eag*I (lanes G, H, and I), and *Rsa*I (lanes J, K, and L). Fragments were separated on a 12% polyacrylamide gel. Lanes: C, F, G, and J, no-lysate controls; B, E, H, and K, 0.7 μg of lysate protein of a strain without the *merR* insert; A, D, I, and L, 0.7 μg of MerR-lysate protein. (C) Mercury effect on MerR binding to *mer-op* ($O_{mer}P_{mer}$) DNA (12% polyacrylamide gel). MerR-binding assays were done as described in the text. Lanes M to Q contain the 487-bp *mer-op* ($O_{mer}P_{mer}$) fragment, whereas lanes R to V contain the same fragment digested with *Hin*fI. Lanes M and R are DNA controls with 10^{-5} M $HgCl_2$. Lanes N and T, O and U, and P and V contain 10^{-5}, 10^{-6}, and 10^{-7} M mercuric chloride, respectively. Lanes Q and S are MerR-lysate controls without mercury.

tory region between ORF 3 and the structural genes were isolated by digesting 1 μg of either pDG124 or pDG125 plasmid DNA with *Xba*I-*Bss*HII (pDG124) or with *Bss*HII-*Sac*I (pDG125). The putative *mer* regulatory region is located on a 487-bp fragment from the pDG124 double digest as well as on a 483-bp fragment from the pDG125 *Bss*HII-*Sac*I digest (Fig. 3A). The resulting fragments were labeled with 10 μCi of [^{32}P]dCTP (Amersham Corp., Chicago, Ill.) by using DNA polymerase I (Klenow fragment) and the appropriate unlabeled nucleotides. After the labeled DNA was electrophoresed on a 1.4% ultrapure agarose gel, the band corresponding to the 487- or 483-bp fragment was excised, and the DNA was electroeluted. After phenol extraction and ethanol precipitation, the labeled DNA was suspended in TE buffer (10 mM Tris hydrochloride [pH 7.4], 1 mM EDTA) or in 10 mM Tris hydrochloride (pH 7.4). The 487-bp fragment from pDG124 (labeled at the *Xba*I and *Bss*HII ends) was digested with either *Eag*I, *Hin*fI, or *Rsa*I. To restore the region of dyad symmetry (see below), the staggered ends in the *Hin*fI digest were filled in with DNA polymerase (Klenow fragment), as described above. The resulting fragments from each digest were phenol extracted, ethanol precipitated, and suspended in 10 mM Tris hydrochloride (pH 7.4).

(ii) Preparation of MerR-containing lysates. A strain containing both plasmids (pGP1-2/pDG125) was grown to a density of 130 to 140 Klett units. The temperature was raised to 42°C, and incubation was continued for 25 min. Rifampin was added to a final concentration of 100 μg/ml, and the cultures were shifted to 37°C for 2 h before they were harvested. Cell pellets were suspended in 10 mM Tris hydrochloride (pH 7.4)–1 mM EDTA–2.5 mM phenylmethylsulfonyl fluoride and were broken at 12,000 lb/in^2 with a French pressure cell. Cell membranes were removed by centrifugation at 35,000 × *g*, and glycerol was added to the supernatant for a final concentration of 40%. Lysates were frozen at −70°C and could be used for several months without loss of binding activity.

(iii) Gel electrophoresis binding assay. Unless stated otherwise, MerR-DNA complexes were formed in 0.010 to 0.015 ml containing 10 mM Tris hydrochloride (pH 7.4), 1 mM EDTA, 50 mM KCl, 0.1 mM dithiothreitol, 0.5 μg of calf thymus DNA, and 1 ng of labeled *mer-op* DNA. In assays with *mer-op* fragments derived from pDG124 (Fig. 3B and C) and the fragments resulting from digestion with *Hin*fI, *Rsa*I, and *Eag*I, EDTA was omitted. Crude lysate was added to achieve concentrations varying from 0.7 to 0.007 μg of protein per assay. The tubes were gently mixed to avoid shearing, which might destroy repressor-DNA complexes (13), and incubated at 22°C for 15 min. Assays containing mercury (complexed with a twofold-higher concentration of glutathione) were done by preincubating MerR lysates and mercury for 10 min at 22°C (Fig. 3C). After preincubation, calf thymus and labeled *mer-op* DNA were added, and the assays were incubated for an additional 15 min at 22°C.

After incubation, 1 μl of dye mix (50% glycerol, 0.1% bromophenol blue) was added to each assay, which was immediately loaded onto a polyacrylamide gel (acrylamide-bisacrylamide [29.2:0.8, wt/wt] in Tris borate EDTA) overlaid with 20 μl of binding buffer containing 100 mM KCl (15). After electrophoresis at room temperature, the gels were

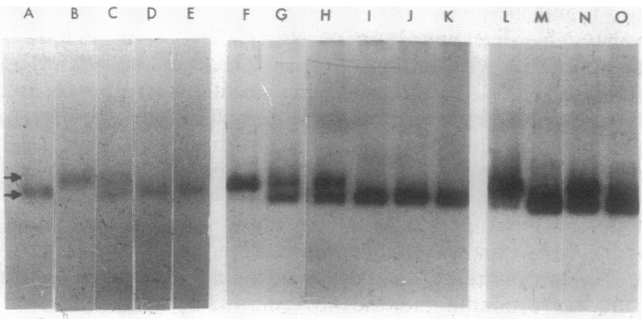

FIG. 4. DNA-binding properties of wild-type MerR and *merR* frameshift mutant pAH026. MerR–*mer-op* (483-bp) complexes were separated on a 5% polyacrylamide gel. Arrows indicate locations of free and complexed DNA. Lanes: A, DNA only; B, C, and D, 0.7, 0.07, and 0.007 μg, respectively, of MerR-lysate protein; E, 0.7 μg of lysate protein of a strain without the *merR* insert (pGP1-2/pT7-3); F, H, and J, 0.7, 0.07, and 0.007 μg, respectively, of MerR-lysate protein. Lanes G, I, and K are the same as F, H, and J except that in each assay, 0.5 μg of unlabeled *mer-op* DNA was included (pDG125 was digested with *Bss*HII-*Sac*I). Lanes: L and N, 0.7 and 0.07 μg, respectively, of MerR-lysate protein; M and O, 0.7 and 0.07 μg, respectively, of lysate protein of the *merR* frameshift mutant pAH026.

dried on filter paper (Whatman) at 60°C under vacuum and exposed to X-Omat AR film (Kodak) at −20°C (23) for 16 to 24 h.

(iv) MerR binding to *mer-op*. Lysates containing overexpressed MerR were prepared from double plasmid strains containing *merR*-ORF 3 (pGP1-2/pDG125) as well as from negative controls (pGP1-2/pT7-3) and the frameshift mutant (pGP1-2/pAH026). Lysates of JM101(pGP1-2/pDG125) formed a MerR protein-DNA complex (Fig. 4, lanes B and C) with a different electrophoretic mobility than the 483-bp free *mer-op* DNA (Fig. 4, lane A). In contrast, lysates of strains without the *merR* insert (pT7-3) (Fig. 4, lane E) or with the *merR* nonsense mutant pAH026 (Fig. 4, lanes M and O) did not retard the mobility of the *mer-op* DNA compared with that of the no-lysate control (Fig. 4, lane A). Maximal fragment retardation was achieved with 0.7 μg of MerR lysate protein per assay. No further mobility alteration was seen with lysate protein concentrations as high as 3.5 μg per assay (data not shown). At a lysate concentration of 0.07 μg of protein per assay, the inclusion of 500-fold-excess unlabeled pDG125 DNA containing the *mer-op* region abolished the retardation of the labeled *mer-op* fragment, presumably by titrating out MerR in the lysate (Fig. 4, lane I), though the fortuitous occurrence of a MerR-binding site on some other fragment in pDG125 cannot be ruled out.

(v) MerR-binding site on *mer-op*. To determine the binding site for MerR, a 487-bp *mer-op* fragment was digested with *Hin*fI, *Rsa*I, and *Eag*I (Fig. 3). The approximate MerR-binding site(s) can be deduced by using fragments from the digests described above which carry overlapping portions of the *mer* regulatory region. A MerR-containing lysate retarded the 165-bp *mer-op*–*Hin*fI fragment (Fig. 3B, lane D), which carries a region of dyad symmetry located 10 bp upstream from the *merR* initiation codon. When this symmetrical sequence was cleaved by digestion with *Rsa*I, no MerR-specific retardation of the resulting two fragments was observed (Fig. 3B, lane L). Thus, the in vitro MerR-binding site is located in a region of dyad symmetry between the putative −35 and −10 sequences of the *merTPCA* promoter. A 220-bp *mer-op* fragment that contains this symmetrical sequence as well as a second dyad region located within the *merR* reading frame was also retarded by MerR lysate (Fig. 3B, lane I).

A 487-bp *mer-op* fragment as well as *Hin*fI-digested *mer-op* DNA were used to detect a possible shift in relative occupancy by MerR upon the addition of mercury. The addition of mercury to the assays containing the 487-bp *mer-op* fragment (Fig. 3C, lanes N to P) did not change binding patterns for MerR–*mer-op* with respect to the control without mercury (Fig. 3C, lane Q). The *Hin*fI-digested *mer-op* fragments carry one or the other region of dyad symmetry on a 325- or 165-bp fragment (Fig. 3A). Neither concentration of mercuric chloride had an effect on *mer-op*–*Hin*fI fragment retardation (Fig. 3C, lanes T to V) compared with that in the control assay containing no mercury (Fig. 3C, lane S); i.e., fragment band intensities as well as retardation distances remained the same.

Earlier attempts to visualize the *merR* product of Tn*21* were unsuccessful (2), though O'Halloran and Walsh have recently overexpressed and purified the *mer* regulatory protein of Tn*501* (27). In this study, we used a coupled T7 RNA polymerase-promoter overexpression system to identify ORF 3 of the *merR* gene as the only protein product in this region (Fig. 2). Although regulatory proteins are generally synthesized in low amounts (4), the T7 overexpression system allowed us to visualize a 16,000-dalton protein corresponding to ORF 3 of the *merR* gene; however, the signal corresponding to the *merR* gene product is relatively weak compared with that of the internal control β-lactamase. Since transcription in the T7 overexpression system is very efficient (35), poor expression of the *merR* product may be due to inefficient translation. (Inherent instability of the protein can probably be ruled out since DNA-binding properties of MerR crude lysates remained stable when lysates were stored over a period of months at −70°C.)

In contrast to the nonconsensus ribosome-binding site preceding the *merR* reading frame (1), other elements of the *merR* translational recognition sequence do correspond with those of efficiently translated systems. For example, the spacing between the ribosome-binding site and the initiation codon (nine nucleotides) of *merR* is favorable for efficient translation (16), and the region immediately 3′ to the ribosome-binding site lacks the excess of G or C residues which has been associated with low translational efficiency (8). However, if identical transcriptional initiation sites in Tn*21* and Tn*501* (which are identical in this region) (1, 25) are assumed, a region of dyad symmetry from nucleotides 560 to 543 (UCCGUAC-UCAU-GUACGGA) might result in mRNA capable of base pairing (Fig. 5). This putative hairpin structure 5′ to the *merR* ribosome-binding sequence would include three nucleotides of the ribosome-binding site (GGA at nucleotides 545 to 543) and could also form in the T7-*merR* overexpression vector (pDG125). It has been shown that if the ribosome-binding site is part of mRNA capable of forming stable hairpin structures, translational efficiency is decreased (6, 30). The potential secondary structure in the *merR* message might inhibit ribosome binding to the mRNA, which in turn would result in translational inefficiency. As has been demonstrated with the *trp* repressor (19), translational inefficiency resulting in low cellular levels of the repressor can play an important role in the expression of an operon. Whether this has a role in *mer* expression is being investigated.

The predicted secondary structure of the *merR* protein in Tn*21* as well as in Tn*501* (32) indicates several possible

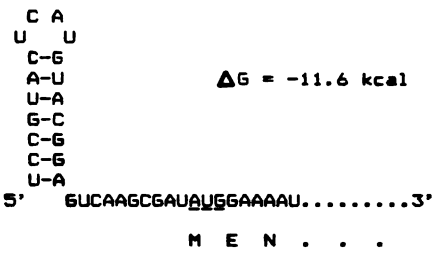

FIG. 5. Potential hairpin structure in the *merR* mRNA of Tn*21* and Tn*501*. The ribosome-binding site is indicated in boldface letters, and the initiation codon is underlined. The Tn*501* message start is identified by Lund et al. (22). The free energy value (ΔG) was estimated by the method of Saenger (29).

regions for a helix-turn-helix supersecondary structure associated with specific DNA-binding properties in other proteins (28). A nonsense mutant in the COOH-terminal half of *merR* (pAH026) does not alter the sequence of the predicted helix-turn-helix structures (32) but lacks three of four cysteine residues which might be involved in dimerization of MerR. Other DNA-binding proteins (28) have been shown to bind symmetrical operator sequences as a dimer or tetramer. Thus, if disulfides are involved in dimerization and if dimerization is required for MerR binding to *mer-op*, the truncated *merR* monomer might be nonfunctional (Fig. 4) because of failure to dimerize.

Our deduction of the *merR* binding region (Fig. 3) is consistent with the binding and protection pattern of MerR from Tn*501* (27). Tn*501* MerR protects a sequence between positions +21 and −6 with respect to the *merR* transcription start. Since promoter regions of *merR* and *merTPCA* genes overlap opposing transcripts (Fig. 3A), MerR binding of this palindromic sequence might sterically prevent RNA polymerase transcription of *merR* and *merTPCA*. The location of the MerR-binding site is therefore consistent with MerR negative autoregulation as well as negative regulation of the structural genes. With respect to positive regulation of the *mer* operon, a shift in relative occupancy or dissociation of MerR from the *mer-op* region would be expected to allow induction of *mer* structural genes; however, the limits of our in vitro binding assay did not allow us to see such a shift. Inclusion of mercuric chloride at biologically relevant concentrations did not have any effect on MerR binding to *mer-op* fragments (Fig. 3C). A similar observation has been reported for MerR binding to the *mer* regulatory region in Tn*501* (27). We are developing a kinetic approach to detect binding alterations of MerR in the *mer-op* region.

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