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A *mer-lux* Transcriptional Fusion for Real-Time Examination of In Vivo Gene Expression Kinetics and Promoter Response to Altered Superhelicity

CHARLES W. CONDEE† AND ANNE O. SUMMERS*

Department of Microbiology, University of Georgia, Athens, Georgia 30605

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We constructed mercury resistance operon-luciferase (*mer-lux*) transcriptional fusion plasmids to evaluate in vivo gene expression rates of the *mer* structural gene promoter (P_{TPCAD}) of transposon Tn*21*. In vivo gene expression kinetics corresponded well with those previously determined in vitro, yielding an apparent $K_{0.5}$ for Hg(II)-stimulated induction by MerR of 9.3×10^{-8} M with the same ultrasensitive threshold effect seen in vitro. We also used the *mer-lux* fusions to elucidate subtle variations in promoter activity brought about by altered superhelicity. Binding of inducer [Hg(II)] to the transcriptional activator MerR is known to result in DNA distortion and transcriptional activation of the *mer* operon; it has recently been demonstrated that this distortion is a consequence of MerR-Hg(II)-induced local DNA unwinding to facilitate RNA polymerase open complex formation at P_{TPCAD}. Since negative supercoiling results in DNA unwinding similar to this MerR activation, we hypothesized that a global increase in plasmid supercoiling would facilitate MerR-mediated activation and compromise MerR-mediated repression, while removal of plasmid supercoils would compromise MerR's ability to induce transcription and facilitate its ability to repress transcription. Indeed, we found that increased negative supercoiling results in increased gene expression rates and decreased supercoiling results in reduced gene expression rates for the induced, repressed, and derepressed conditions of P_{TPCAD}. Thus, luciferase transcriptional fusions can detect subtle variations in initial rates of gene expression in a real-time, nondestructive assay.

Luciferase has been extremely useful as a reporter gene in determining the temporal and tissue-specific activation of genes in both prokaryotes and eukaryotes. Bacterial luciferase activity is easily detected both in vitro and in vivo, requires inexpensive substrates that are readily absorbed through cell membranes, and is more sensitive than other reporter enzyme systems (36). In addition, luciferase activity can be monitored continuously in real time without damage to the cell. Although these properties recommend luciferase as a reporter for examining initial rates of gene expression in vivo, all reported uses of it have been for much later times (i.e., greater than 1 h after induction). Since transcriptional regulation of the *mer* operons from Tn*21* and Tn*501* is relatively well defined, it is a good system with which to test the ability of bacterial luciferase to monitor early transcriptional events in vivo.

Mercury resistance (*mer*) is widespread in both gram-negative and gram-positive bacteria. The structural genes of the gram-negative Tn*21* *mer* operon (Fig. 1A) encode proteins that transport mercury [Hg(II)] into the cell (*merTPC*) and that reduce Hg(II) to the less toxic, volatile metallic Hg^0 (*merA*) (40). A fifth gene (*merD*) encoding a small, low-abundance protein (24) has recently been proposed to turn off structural gene transcription once Hg(II) has been reduced by mercuric reductase, *merA* (30).

In Tn*21* and Tn*501*, positive and negative transcriptional regulation of the *mer* operon is mediated by MerR expressed from the divergent *merR* promoter (P_R) (9, 17, 27, 31, 39). Dimeric MerR binds a single Hg(II) ion (31, 37) in a tricoordinate, thiolate metal bridge between the MerR subunits (15, 41). The *mer* regulatory region is composed of the divergent, overlapping P_{TPCAD} and P_R promoters (Fig. 1A and B) and the MerR binding site. The MerR binding site contains a palindrome of two 7-bp arms separated by 4 bp and is unusual in being located between the RNA polymerase (RNAP) recognition hexamers of the P_{TPCAD} promoter (Fig. 1B) (16, 17, 31). MerR bound to this region of dyad symmetry represses transcription of the structural genes in the absence of Hg(II), although it also fosters RNAP binding to P_{TPCAD} in the absence of Hg(II). MerR induces transcription of the structural genes in the presence of nanomolar Hg(II) (Fig. 1C) (9, 27, 31, 34, 37) but represses its own transcription from P_R regardless of whether Hg(II) is present (17, 18). Transcriptional runoff assays show that in vitro MerR is extremely sensitive to Hg(II) concentration, with an in vitro apparent K_m for Hg(II)-stimulated MerR-mediated induction of 1×10^{-8} to 5×10^{-8} M (31, 34).

The −10 (TAAGTT) and −35 (TTGACT) hexamers of P_{TPCAD} are homologous to the *Escherichia coli* σ^{70} consensus sequence (underlined bases indicate homology). Several bases within the σ^{70} consensus −10 (**TATAAT**) and −35 (**TTG**ACA) hexamers are known in other systems to be functionally more important for RNAP recognition (boldface type indicates the functionally most important bases) (29); all of these bases are conserved in the P_{TPCAD} hexamers. Apart from the extensive homology with the consensus σ^{70} promoter sequence, P_{TPCAD} has an unusually long 19-bp spacer. This overlong spacer makes P_{TPCAD} a relatively weak promoter in the absence of MerR and Hg(II) but is optimal for efficient MerR-mediated regulation (32).

In B-form DNA, the spatial difference between a 17-bp spacer and a 19-bp spacer is an ≈68° counterclockwise rotation of the −10 hexamer with respect to the −35 hexamer (5, 6) (Fig. 2). In the presence of MerR and Hg(II), a

* Corresponding author. Electronic mail address: summers@bscf.uga.edu.
† Present address: Envirogen, Inc., Lawrenceville, NJ 08648.

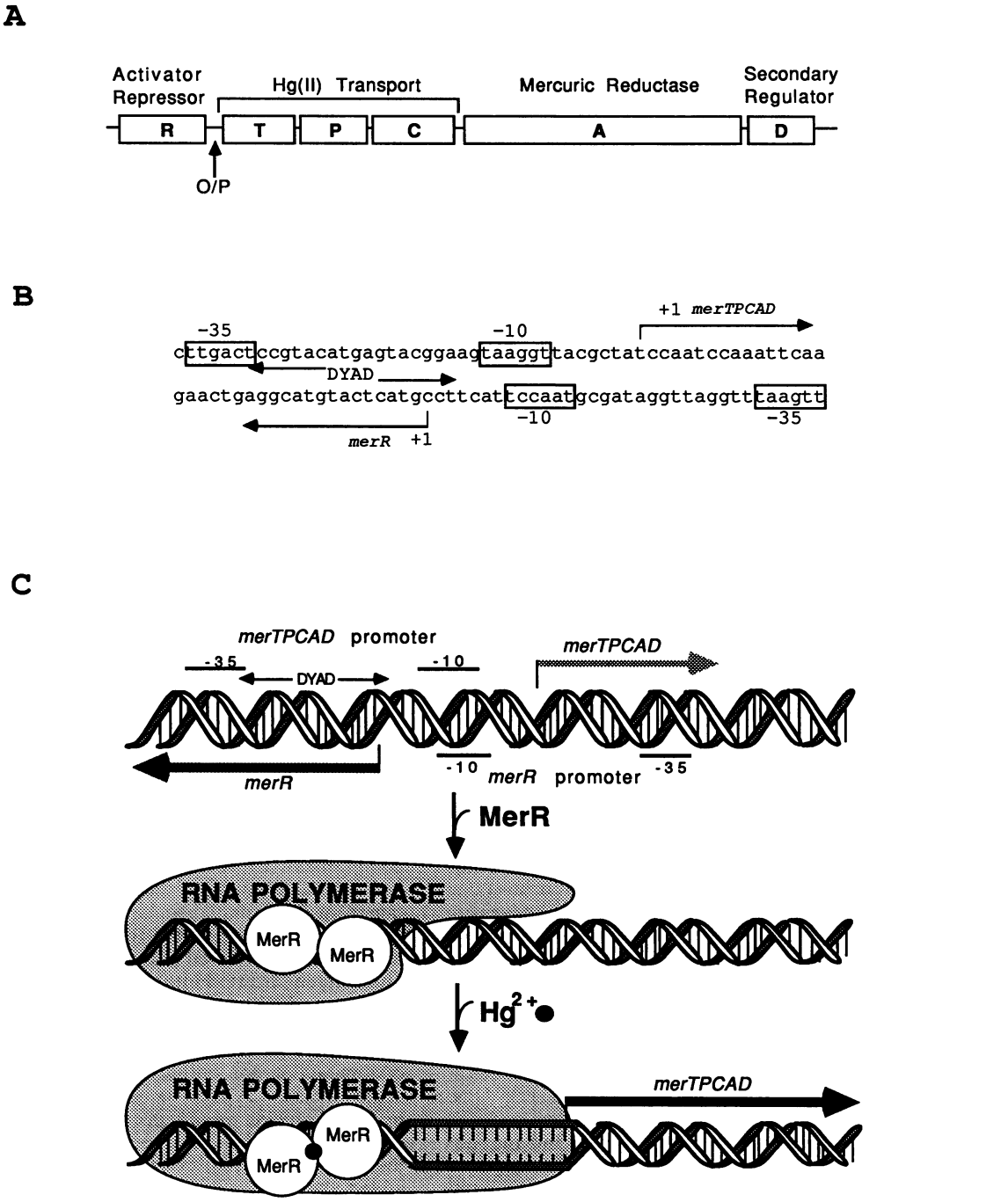

FIG. 1. (A) The 3.8-kb Tn*21* *mer* operon. R, activator/repressor; O/P, operator and promoter region; T, P, and C, the proteins involved in uptake of Hg(II); A, mercuric reductase; D, proposed transcriptional down-regulator (1). (B) Sequence of the *mer* O/P region. Boxed residues indicate RNAP recognition hexamers for *merTPCAD* (top strand) and *merR* (bottom strand). Arrows between the top and bottom sequences indicate the MerR binding dyad. Arrows above and below the sequence depict mRNA transcription for *merR* and *merTPCAD*. (C) Model for regulation of *mer*. Relative gene expression of P_{TPCAD} is depicted by the densities of the mRNA arrows. Top, the derepressed *mer* O/P. Center, repression by MerR. Bottom, induction by Hg(II) addition.

DNA structural distortion occurs within the 4 bp separating the dyadic binding site arms (11, 17). This distortion is a consequence of MerR-Hg(II)-induced DNA unwinding to facilitate RNA polymerase open complex formation at P_{TPCAD} (1).

Increased negative supercoiling (hereafter referred to simply as supercoiling) can overcome the deleterious effect of a long spacer on promoter activity by reorienting the RNAP hexamers via alterations of the DNA pitch and increasing the helix torsional energy (2). Since MerR-Hg(II) causes helix unwinding (1), global alterations in supercoiling should increase gene expression when supercoiling increases and

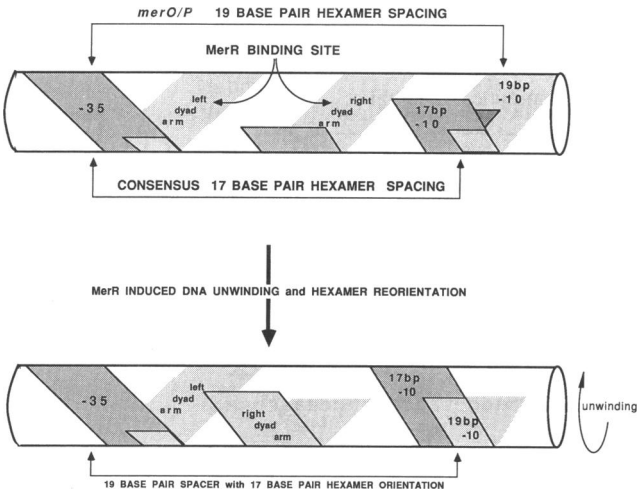

FIG. 2. Spatial orientation of the *merTPCAD* operator and promoter elements in B-form DNA. Top, MerR binding dyad and RNAP −10 recognition hexamer orientation with respect to the −35 hexamer for both the 19-bp *merTPCAD* and the 17-bp consensus spacers. The arrow-like brackets indicate the centers of the recognition hexamers for 17- (below) or 19-bp (above) spacers. Bottom, proposed hexamer reorientation brought about by MerR conformational changes.

reduce gene expression rates when supercoils are removed. We employed the *mer-lux* fusion plasmids to test the effect of altered superhelicity on P_{TPCAD} gene expression rates in vivo.

MATERIALS AND METHODS

Bacterial strain and plasmid constructions. *E. coli* K-12 (CB806) (*galK lacΔZ phoA8 thi* Str^r *rpsL recA56*) was used as a bacterial host strain for all experiments reported in this study. The parental plasmid (pDG106) used in constructing pCC306 is a pACYC177 derivative containing the entire Tn*21 mer* operon (12). To facilitate cloning of the luciferase genes downstream from P_{TPCAD}, a unique *Sca*I restriction site (located 79 bp downstream from the *merT* initiation codon) was linker tailed (23) by using nonphosphorylated *Apa*I linkers (no. 1079; New England Biolabs, Beverly, Mass.). The resulting plasmid (pCC303) was then linearized with *Bam*HI (3′ to the *merD* locus in pDG106), and the 5′ extension was filled in by Sequenase (version 1.0; U.S. Biochemical Corp., Cleveland, Ohio). Next, linearized and blunt-ended pCC303 was digested with *Apa*I, and the 4.2-kilobase pair (kb) fragment (containing the p15A origin of replication, the kanamycin resistance [Kan^r] locus, and *merR*-P_R-P_{TPCAD}-*merT*′) was eluted from an agarose gel (Fig. 3).

The *Vibrio harveyi* luciferase genes used in constructing the *mer-lux* fusions were provided by Alan Schauer, University of Texas, Austin, as an insert on pMLS1, a Bluescript derivative. An *Apa*I site was engineered into pMLS1 at a *Sal*I site (5′ to *luxA* in pMLS1). The resulting plasmid (pCC305) was digested with *Apa*I and *Pvu*II (3′ to *luxB*), and the 2.5-kb *luxAB* fragment was eluted from an agarose gel. Final construction of pCC306 (Fig. 3) was accomplished by ligation of the 4.2-kb pCC303 and 2.5-kb pCC305 fragments. The *luxAB* orientation was confirmed by restriction digestion.

The Δ*merR* plasmid pCC307, used to examine the derepressed expression, was constructed as described for pCC306, except that pDG121 (the Δ*merR* derivative of pDG106) was used as the parental plasmid (12).

Growth conditions and media. Frozen stock cultures were inoculated into 2 ml of Luria-Bertani broth, supplemented with 25 μg of kanamycin sulfate per ml, and incubated overnight at 37°C with aeration. Overnight cultures were diluted 1:100 into 125-ml Erlenmeyer flasks containing 25 ml of fresh 37°C kanamycin sulfate (25 μg/ml) and grown to mid-log phase (A_{620} = 1.4) at 37°C while being shaken (250 rpm). Cultures were maintained in exponential growth by regular 1:1 dilutions with 37°C media during each experiment in order to maintain a density of A_{620} = 1.4.

Luciferase reaction medium (LRM) consists of kanamycin sulfate (25 μg/ml) supplemented with 0.1% (vol/vol) decyl aldehyde (Sigma Chemical Co., St. Louis, Mo.). For induction, atomic absorption standard grade Hg(II), as the nitrate salt, $Hg(NO_3)_2$ (Aldrich Chemical Company, Inc., Milwaukee, Wis.), diluted with high-pressure liquid chromatography-grade water was added to the LRM. For assays on altered superhelicity, LRM contained the indicated coumermycin A_1 concentration and induction was with 1 μM $Hg(II)NO_3$. Coumermycin A_1 (Sigma) stock solutions (10 mg/ml in dimethyl sulfoxide) were stored at 4°C for up to 1 week.

Luciferase assays. Light (485 nm) generated by strains harboring a *lux* fusion plasmid was monitored by using an ATP-Photometer (model 2000; SAI Technologies Co., La Jolla, Calif.) linked to a strip-chart recorder (LKB 2210 one-channel recorder; LKB Instruments, Inc., Gaithersburg, Md.). The ATP-Photometer was standardized prior to assays by using ^{14}C and ^{3}H unquenched scintillation standards (Beckman Instruments, Inc., Fullerton, Calif.). The sample chamber of the photometer was maintained at a stable temperature of 35°C by circulation of heated air through the chamber.

For the luciferase assay, 995 μl (or 950 μl for the repressed condition) of LRM with or without 1 μM Hg(II) was dispensed into 20-ml glass scintillation vials which were maintained at 37°C until use. When cultures reached mid-log phase, 5 μl (or 50 μl for the repressed condition) of the culture was transferred to a vial containing LRM which was immediately inserted into the counting chamber of the photometer and light emission was monitored for 15 min. The photometer reports via a light-emitting diode the number of photons emitted (counts) during a 6-s interval, or it reports continuous photon output graphically on an attached strip-chart recorder. Typical 6-s counts for a strain with no fusion or for the fusion strains prior to addition of the luciferase substrate were the same, ca. 30 counts per 6-s reading. For the repressed strain, once the luciferase substrate was added, the average rate was ca. 1,900 counts per 6-s reading. The derepressed strain with luciferase substrate was ca. 20,000 counts per 6-s interval. For the induced strain with substrate, the counts detected changed as a function of time after induction (because of the increase in luciferase), ranging from ca. 2,000 counts per 6-s interval to more than 100,000 counts per interval late after induction. Rates were determined from the maximum slope of the line produced by the LKB strip-chart recorder; kinetic data are reported as the change in total photometer counts per second per 2×10^6 cells; assays on altered superhelicity are reported in the same manner by using 3×10^6 cells.

Coumermycin A_1 treatments. Cultures were grown to mid-log phase as described above, and a 5-ml aliquot was transferred to a separate 125-ml Erlenmeyer flask. Coumer-

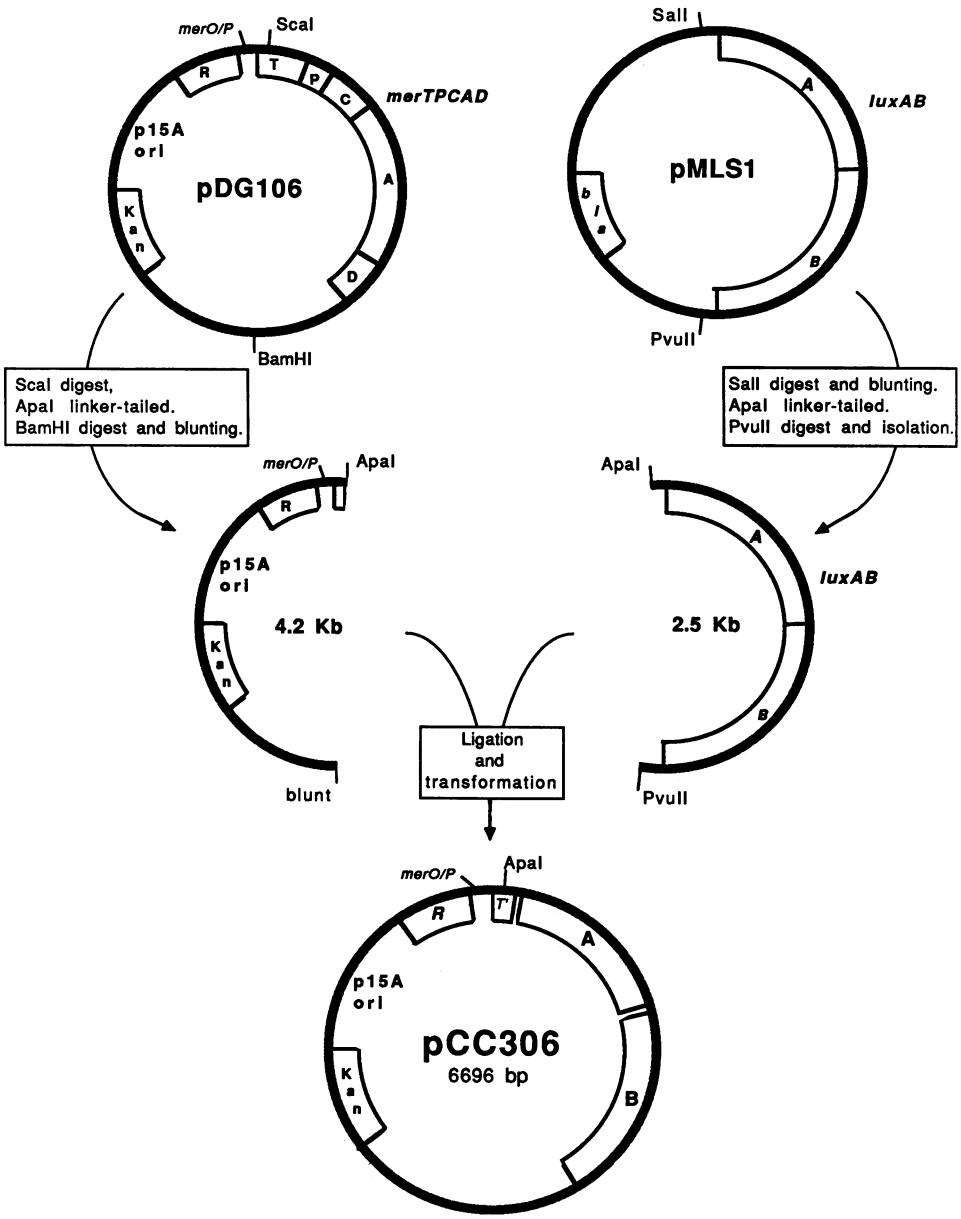

FIG. 3. Construction of the *mer-lux* reporter plasmid pCC306. Kan, kanamycin resistance; p15A ori, p15A origin of replication. The plasmid used for evaluating derepressed (Δ*merR*) P_{TPCAD} transcription (pCC307) was constructed in the same manner as pCC306; however, the parental plasmid (pDG121) used is the Δ*merR* derivative of pDG106 (12).

mycin A_1 was added at a final concentration of 2, 4, 6, 10, or 25 μg/ml. Cultures were incubated at 37°C with shaking for 15 min, an interval which results in maximum alterations of superhelicity (10). Controls were treated in the same manner with dimethyl sulfoxide alone. Log-phase cultures contained $\approx 4 \times 10^8$ CFU/ml, while controls and all cultures treated with coumermycin A_1 for 15 min contained $\approx 6 \times 10^8$ CFU/ml. Thus, the coumermycin A_1 treatment did not prevent cell division, possibly because exponential-phase cells are diploid. Visual inspection of linearized, serially diluted plasmids (prepared by the method of Birnboim and Doly [4]) showed no variation in plasmid DNA concentration between the coumermycin A_1-treated cells and nontreated controls. After coumermycin A_1 treatment, appropriate dilutions were made into LRM (containing the corresponding concentration of coumermycin) and luciferase was monitored as described above.

Chloroquine gels. Supercoiled plasmid DNA was isolated from coumermycin A_1-treated cultures by using the alkaline lysis method of Birnboim and Doly (4). Gels (15 by 15 by 0.4 cm) consisted of 1.2% (wt/vol) agarose (IBI Ultra-Pure Molecular Biology Grade agarose purchased from VRW Scientific, Inc., New Haven, Conn.) in 1× TPE buffer (45 mM Tris-phosphate and 0.87 mM EDTA, pH 7.2) and contained either 5 or 12 μg of chloroquine diphosphate (Sigma) per ml. In gels with 5 μg of chloroquine per ml, more negatively supercoiled plasmids run with faster mobility than those with lower negative supercoiling; in gels containing 12 μg of chloroquine per ml, the opposite is true (14, 21). Supercoiled plasmids were electrophoresed in the dark from 24 to 28 h at

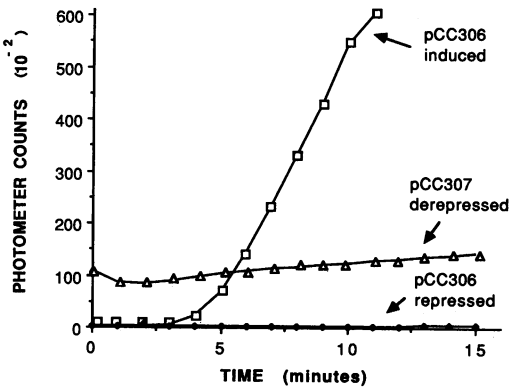

FIG. 4. Regulation of light production by the *mer-lux* fusions. P_{TPCAD} gene expression reported by 2×10^6 CFU of cells in the induced [pCC306 plus 1 μM Hg(II)], repressed (pCC306), and derepressed (pCC307) conditions. See Materials and Methods for details.

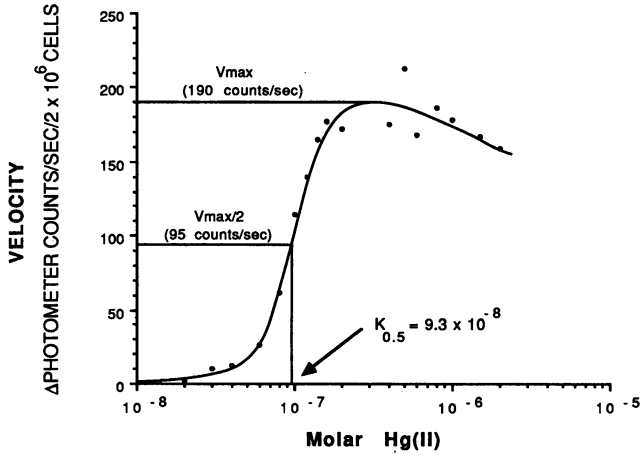

FIG. 5. Hg(II)-induced gene expression kinetics. Data are reported as the change in total photometer counts per second per 2×10^6 cells. The strain used was CB806(pCC306). See Materials and Methods for details.

1.5 V/cm either while the buffer was being recirculated to maintain a temperature of 24 to 26°C or at 4°C. After electrophoresis, gels were briefly rinsed with deionized H_2O, stained for 1 h in 1 μg of ethidium bromide per ml, destained in 1 mM $MgSO_4$ for 4 to 5 h, and photographed with UV illumination by using Polaroid Type 55 (positive-negative) film. For detection of topoisomers by Southern hybridization, DNA in the gels was denatured and transferred to nitrocellulose. The probe used was generated by using short, random primers (N_6; Sigma) to direct the incorporation by Klenow polymerase of α-^{35}S-dCTP into DNA fragments homologous to pCC306.

RESULTS

Behavior of the *mer-lux* fusions. The luciferase fusions had readily detectable activity under the induced, derepressed, and repressed conditions (Fig. 4) with relative activities comparable to those previously determined by using other reporters (9, 35). The induced condition showed a sigmoidal response (Fig. 4) with increased activity evident 2 to 3 min after induction with 1 μM Hg(II). The derepressed and repressed activities increased only slightly in 15 min.

Hg(II)-induced gene expression kinetics. The apparent K_m for Hg(II) induction in vitro by MerR from Tn*501* is 1×10^{-8} to 5×10^{-8} M (31, 34). Ralston and O'Halloran (34) also demonstrated that the in vitro induction kinetics exhibited what has been termed an "ultrasensitive threshold effect" (i.e., a large increase in activity over a narrow range of substrate [or inducer] concentrations) (13, 22). The luciferase kinetic data also produced a sigmoidal curve (Fig. 5) with an apparent $K_{0.5}$ for MerR induction of 9.3×10^{-8} M Hg(II), in good agreement with the in vitro observations (31, 34). The in vivo *mer-lux* fusion required a 4.2-fold increase in Hg(II) concentration (48 to 200 nM) to stimulate activity from 10 to 90% of the maximum, which compares well with the 5.1-fold increase required for similar stimulation in vitro. The decline in activity above 1 μM is due to Hg(II) toxicity; Hg(II) concentrations of ≥10 μM resulted in the complete elimination of light production (data not shown).

P_{TPCAD} response to altered superhelicity. Electrophoresis of supercoiled plasmid DNA in chloroquine-agarose gels separates topoisomers differing by one linking number. The plasmid topoisomer distribution allows comparison of superhelical densities in plasmid populations isolated from cells with different global levels of supercoiling (21, 33). Highly supercoiled plasmids have faster mobility in lower chloroquine concentrations (Fig. 6); less supercoiled plasmids run faster in higher chloroquine concentrations (Fig. 7). Coumermycin A_1 at concentrations of 4 and 6 μg/ml increased supercoiling (Fig. 6, lane 1 versus lanes 3 and 4), while higher drug concentrations decreased supercoiling (Fig. 6, lane 1 versus lanes 5 and 6). Similarly, as detected by autoradiography (Fig. 7), coumermycin A_1 at concentrations of 0.5 to 2 μg/ml increased plasmid superhelicity and concentrations of 4 to 25 μg of the drug per ml resulted in a loss of superhelicity. Thus, coumermycin A_1 had the expected effect on the supercoiled state of the plasmid.

Derepressed P_{TPCAD} activity increased 2.9-fold in response to increased supercoiling (compare control with treatments with 4 μg of coumermycin A_1 per ml) and decreased by 43% in response to decreased supercoiling (compare control with treatments with 25 μg of coumermycin A_1 per ml) (Fig. 8A). Similarly, induced P_{TPCAD} gene expression increased by 71% with increased supercoiling (compare control with treatments with 2 μg of coumermycin A_1 per ml) and decreased by 37% at lower superhelical densities (compare control and treatments with 25 μg of coumermycin A_1 per ml) (Fig. 8B). For repressed P_{TPCAD}, higher superhelical densities resulted in 29% higher activity (control versus treatments with 2 to 4 μg of coumermycin A_1 per ml); however, lower superhelical densities resulted in a 79% decrease in P_{TPCAD} activity (control versus treatments with 25 μg of coumermycin A_1 per ml) (Fig. 8C).

DISCUSSION

Kinetics of P_{TPCAD} gene expression in vivo. The *mer-lux* fusions provide an unusually sensitive reporter of early gene expression. With increased cells (10-fold) and higher Hg(II) concentrations (2 μM), light from the *mer-lux* fusion can be detected as early as 2.5 min after induction (data not shown). Assuming a transcription rate of 45 nucleotides per s and a translation rate of 15 amino acids per s, production of the first luciferase molecules would occur after ca. 1 min. Additional time will also be required for Hg(II) diffusion and amplification of luciferase to levels high enough to be detect-

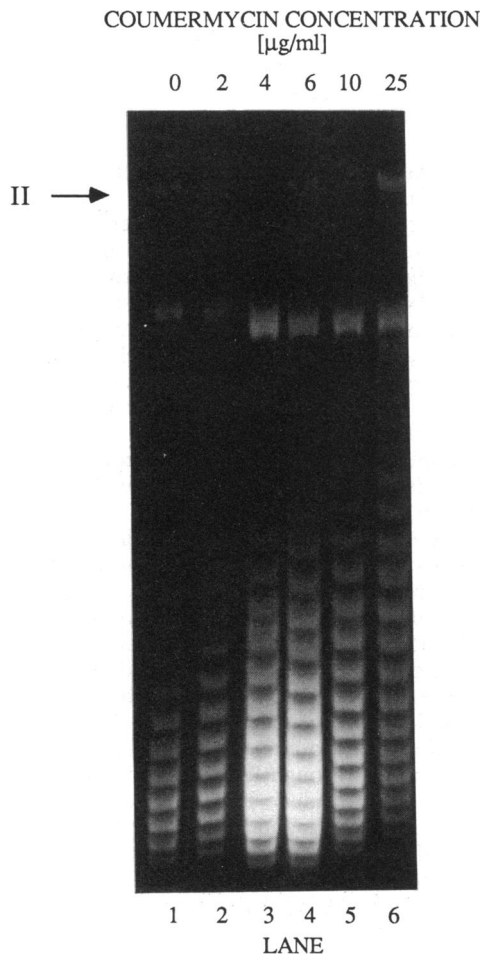

FIG. 6. Ethidium bromide-stained agarose gel containing 5 μg of chloroquine per ml. Supercoiled pCC306 topoisomers were electrophoresed in the dark for 24 h at 1.5 V/cm. At this chloroquine concentration, topoisomers with higher levels of supercoiling migrate faster. Lane 1, nontreated control plasmids; lanes 2 through 6, plasmids isolated from cultures treated with concentrations of coumermycin from 2 to 25 μg/ml, as indicated in the figure. II, form II DNA (i.e., circular, nicked DNA).

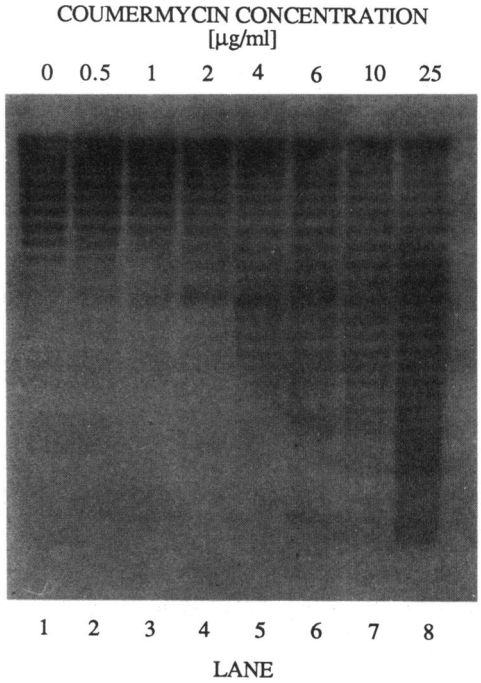

FIG. 7. Southern hybridization autoradiograph of a chloroquine gel containing pCC306 plasmid DNA isolated from cells treated for 15 min with various concentrations of coumermycin A_1 and controls (lane 1, dimethyl sulfoxide only). The gel was composed of 1.2% agarose with 12 μg of chloroquine diphosphate per ml in TPE buffer and was electrophoresed at 1.4 V/cm for 24 h. At this chloroquine concentration, highly supercoiled plasmids migrate more slowly.

able by the photometer. Thus, the assay is sufficiently sensitive to follow events very early after induction.

We found an apparent $K_{0.5}$ for Hg(II) gene expression of 9.3×10^{-8} M, a concentration only 2- to 10-fold higher than that reported in vitro [1×10^{-8} to 5×10^{-8} M Hg(II)] (31, 34). The in vitro transcription runoff and abortive initiation assays employed 1 mM dithiothreitol as a competitive Hg(II) liganding species (31, 34); however, the in vivo glutathione concentration in *E. coli* K-12 is 6 to 7 mM (3). Other potential Hg(II) ligands (e.g., sulfhydryl, carboxyl, and imino groups) in the cells and in the assay medium as well as membrane permeability barriers may also contribute to the observed differences between the in vivo and in vitro methods. Nonetheless, the correspondence is quite close, given these potential complicating factors.

Our data establish that the ultrasensitive threshold effect of Hg(II) on MerR-dependent gene expression is not an artifact of the in vitro assay system (34). In vivo, MerR is just as sensitive to minute changes in Hg(II) concentrations as it is in vitro, since a 4.2-fold increase in Hg(II) concentration (from 48 to 200 nM) stimulated activation from 10 to 90% of maximum (Fig. 5); this same degree of stimulation in vitro followed a 5.1-fold increase of Hg(II) concentration (6 to 32 nM) (34). Thus, the luciferase assay is capable of faithfully portraying gene expression kinetics in vivo.

Effect of global supercoiling changes on P_{TPCAD} expression. Several lines of evidence suggest that MerR undergoes a conformational change upon binding Hg(II). The DNA binding affinity of MerR decreases upon Hg(II) binding (MerR-DNA $K_d = 1.4 \times 10^{-10}$ M; MerR-Hg(II)-DNA $K_d = 4.2 \times 10^{-10}$ M [31]). DNA footprints showing chemical nuclease hyperreactivity of the 4 bp separating MerR's dyad arms indicate MerR-Hg(II)-dependent DNA distortions (11, 17). In addition, recent studies have demonstrated that MerR unwinds DNA ca. 33° concomitant with binding Hg(II) (1). Given these observations, we expected that global increases in supercoiling would facilitate MerR-Hg(II)-mediated induction and compromise MerR-mediated repression while decreased supercoiling would compromise MerR-Hg(II) induction and facilitate MerR-mediated repression. We also expected that increased supercoiling would stimulate derepressed P_{TPCAD} gene expression and that the removal of supercoils would reduce its activity.

The DNA gyrase-specific inhibitor coumermycin A_1 (7, 8) was used to alter superhelicity for three reasons.

(i) Unlike many topoisomerase inhibitors, coumermycin A_1 does not result in a covalent protein-DNA complex (7, 8, 38), and thus, direct blockage of RNAP by bound gyrase is not likely to occur.

(ii) Inhibition of gyrase supercoiling activity results from coumermycin A_1 blockage of the β-subunit's ATP binding

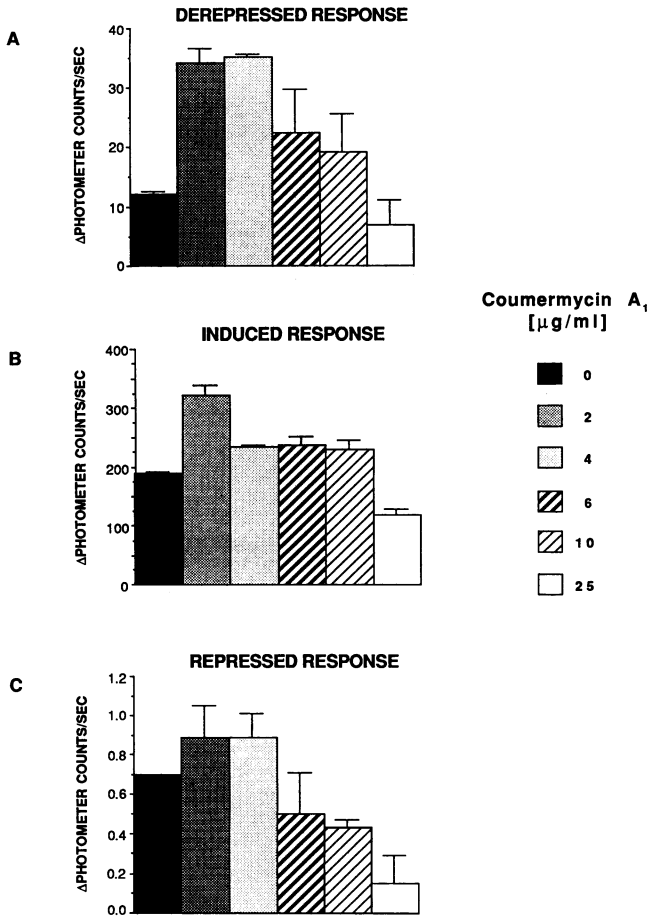

FIG. 8. P_{TPCAD} response to altered superhelicity expressed as change in photometer counts per second. (A) Derepressed P_{TPCAD} gene expression rates from pCC307. (B) Induced [1 μM Hg(II)] gene expression rates from pCC306. (C) Repressed gene expression rates from pCC306. Results are the means of two assays conducted on two separate days, with sample standard deviations calculated as $n-1$ weighted.

site; however, DNA gyrase still retains its ability to remove supercoils (7, 8), thus adding to the loss of supercoils effected by topoisomerase I.

(iii) Because of its effect on gyrase expression, coumermycin A_1 can be used at low concentrations to increase supercoiling and at high concentrations to decrease supercoiling (10, 28). Thus, one compound can be used to examine the entire range of responses.

Since transcription per se generates positive supercoils downstream of the transcriptional complex and negative supercoils in its wake (26), it is thought that DNA gyrase plays a role in preventing transcriptional termination brought about by excessive positive supercoiling ahead of the transcriptional complex. However, as our reporter is on a small, presumably unconstrained plasmid, the accumulation of positive supercoils ahead of the transcription complex will be compensated for by the accumulation of negative supercoils in its wake; thus, transcriptional interference due simply to excessive 3′ positive supercoiling should not occur.

We found derepressed P_{TPCAD} more responsive (2.9-fold) to increased superhelicity (Fig. 8A) than the wild-type lactose promoter (*lacP*) fused to luciferase (a 24% increase; data not shown); *lacP* has an 18-bp spacer and is expected to be less sensitive to increased supercoiling than promoters such as P_{TPCAD}, which have 19-bp spacers (2). The increased gene expression rate of derepressed P_{TPCAD} likely results from reorientation of the RNAP recognition hexamers through increased supercoiling concomitant with increased DNA pitch (2).

Hg(II)-induced gene expression rates increased with higher superhelicity and declined when superhelicity was decreased (Fig. 8B). Since unwinding requires energy, the increased helix torsional energy supplied by higher superhelicity would assist MerR-Hg(II)-mediated unwinding. Conversely, decreases in superhelicity and helix torsional energy would increase the energy barrier that MerR must overcome to unwind the DNA and therefore compromise its ability to induce transcription. Changes in helix spacing occurring with supercoiling alterations may also contribute to either increased or decreased stabilization of MerR-DNA or MerR-RNA polymerase contacts or both.

The hypothesis that MerR-Hg(II) mediates DNA untwisting is further supported by the response of repressed P_{TPCAD} to altered superhelicity (Fig. 8C). DNA molecules can relieve torsional stress arising from supercoiling via several mechanisms (5, 19, 20, 25, 33), and the in vivo situation is most likely a constant, simultaneous melding of these mechanisms via energy translations through the molecule to achieve the lowest free energy state. When acting as a repressor, MerR must overcome these supercoiling-induced energy translations through P_{TPCAD}, and thus, a global loss of supercoils would reduce the severity and frequency of these energy translations and ease the repression process for MerR. Indeed, MerR functioned as a slightly weaker repressor with higher-than-normal supercoiling, and with decreased supercoiling, MerR became an even stronger repressor.

While global changes in supercoiling affect many promoters, the mechanistic basis for these effects is not known. The *mer* operon is not unique in being affected by global changes in supercoiling; however, presently *mer* is unique in that there is unusually good evidence that the mechanism of activation involves an Hg(II)-induced MerR-dependent local DNA distortion, very likely an untwisting of the helix. It is not known at this level of detail how other promoters respond to supercoiling changes. Thus, it was reasonable to hypothesize for *mer* that, if local untwisting is the mechanism of activation, then global alterations of supercoiling will have a measurable effect on expression. That proved to be the case; thus, this accurate, noninvasive, rapid reporter assay may be added to the other tools available to explore questions about supercoiling in other promoters.

In summary, the *mer-lux* reporter plasmids are very sensitive real-time reporters with excellent kinetic fidelity for monitoring unperturbed and chemically perturbed transcription initiation. Our observations on the effects of altered superhelicity on these fusions are consistent with the hypothesis that MerR effects transcriptional control, at least in part, through DNA untwisting.

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