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Escherichia coli OxyR protein represses the unmethylated bacteriophage Mu *mom* operon without blocking binding of the transcriptional activator C

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ABSTRACT

Transcription of the bacteriophage Mu *mom* operon requires transactivation by the phage-encoded C protein. DNase I footprinting showed that in the absence of C, *Escherichia coli* RNA polymerase Eσ^{70} (RNAP) binds to the *mom* promoter (P_{mom}) region at a site, P2 (from –64 to –11 with respect to the transcription start site), on the top (non-transcribed) strand. This is slightly upstream from, but overlapping P1 (–49 to +16), the functional binding site for rightward transcription. Host DNA-[N^6-adenine] methyltransferase (Dam) methylation of three GATCs immediately upstream of the C binding site is required to prevent binding of the *E.coli* OxyR protein, which represses *mom* transcription in *dam*$^-$ strains. OxyR, known to induce DNA bending, is normally in a reduced conformation *in vivo*, but is converted to an oxidized state under standard *in vitro* conditions. Using DNase I footprinting, we provide evidence supporting the proposal that the oxidized and reduced forms of OxyR interact differently with their target DNA sequences *in vitro*. A mutant form, OxyR-C199S, was shown to be able to repress *mom* expression *in vivo* in a *dam*$^-$ host. *In vitro* DNase I footprinting showed that OxyR-C199S protected P_{mom} from –104 to –46 on the top strand and produced a protection pattern characteristic of reduced wild-type OxyR. Prebinding of OxyR-C199S completely blocked RNAP binding to P2 (in the absence of C), whereas it only slightly decreased binding of C to its target site (–55 to –28, as defined by DNase I footprinting). In contrast, OxyR-C199S strongly inhibited C-activated recruitment of RNAP to P1. These results indicate that OxyR repression is mediated subsequent to binding by C. Mutations have been isolated that relieve the dependence on C activation and have the same transcription start site as the C-activated wild-type promoter. One such mutant, *tin7*, has a single base change at –14, which changes a T_6 run to T_3GT_2. OxyR-C199S partially inhibited RNAP binding to the *tin7* promoter *in vitro*, even though the OxyR and RNAP-P1 binding sites probably do not overlap, and *in vivo* expression of *tin7* was reduced 5- to 10-fold in *dam*$^-$ cells. These results suggest that OxyR can repress *tin7*.

INTRODUCTION

Among the bacteriophages, Mu is unique. Besides its remarkable property of functioning as a transposable element (1–4), Mu employs two unusual strategies to extend its host range. One involves expression of alternative sets of tail fibers that specify different adsorptive capabilities on various host cells; this is accomplished by DNA inversion of the phage G region, which encodes these proteins (5,6). The other strategy involves an unusual type of DNA modification function, encoded by the phage *mom* gene. 'Momification' is an unusual sequence-specific modification of adenines that protects the phage DNA against a variety of host-controlled restriction/modification systems (7–9). However, untimely Mom production is cytotoxic (10,11). Therefore, in order to establish and maintain the lysogenic pathway, Mu has evolved an intricate set of transcriptional and translational controls to regulate *mom* expression (12).

The *mom* operon is at the rightmost end of the bacteriophage Mu genome and is comprised of two overlapping genes, *com* and *mom* (13); the *com* gene product is a sequence-specific mRNA binding protein that appears to activate *mom* translation by melting a stem–loop structure to expose the translational start signals (14,15). Transcription of *mom* by *Escherichia coli* RNA polymerase Eσ^{70} (RNAP) is subject to a complex regulation scheme; it requires transactivation by the phage-encoded C protein (16,17), as well as function of the host DNA-[N^6-adenine] methyltransferase (Dam) (18–21). C is also required for the transcription of three other late operons, which are involved in phage morphogenesis and cell lysis during lytic development (22,23). Of the four late promoters (P_{lys}, P_I, P_P and P_{mom}), P_{mom} has the most conserved –35 hexamer sequence, but is at best poorly homologous to the *E.coli* consensus RNAP promoter sequence, TTGACA-$X_{16–18}$-TATAAT (24). Analysis of the *mom* promoter sequence reveals that it has identities of 3/6 and 4/6 respectively with the canonical *E.coli* –35 and –10 hexamers, but the three matches in the –35 hexamer are not at so-called 'invariant' positions (25). Furthermore, the spacer between the two hexamers is a suboptimal 19 (instead of 17) bp. Thus, it is no surprise that RNAP cannot by itself initiate transcription at the *mom* promoter. The precise mechanism by which C activates transcription is currently unclear. C binds P_{mom} from –55 to –28, as defined by DNase I footprinting (26,28); chemical footprinting with MPE·Fe(II) narrows the boundaries from –53 to –35 (16). C binding promotes RNAP binding at a site, P1, functional in

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rightward transcription, and precludes RNAP binding at another site, P2, which is slightly upstream and overlapping P1 (27,28; unpublished results). Whether or not C is also required at some later step in transcription initiation is unknown. Recent *in vitro* studies indicate that RNAP bound at P2 produces a low level of short transcripts in the leftward direction (28), adding one more dimension to the complexity of *mom* regulatory control.

Mutations have been isolated that relieve the dependence on C activation, without altering the transcription start site (27). One such mutant, *tin7*, has a single base change at –14, which changes a T_6 run (in the top strand) to T_3GT_2. Not only does this substitution disrupt any intrinsic bending due to the T_6 run, but it also creates a TG at –15, –14, which has been shown to relieve dependence upon accessory factors for several other *E.coli* promoters (29).

Interestingly, Dam methylation of the adenine residues in three closely spaced GATCs located immediately upstream of the proposed C recognition site is also required for *mom* transcription (18–21). This was unexpected because of the cases known where DNA methylation negatively regulates gene expression in eukaryotes. Later studies showed that Dam methylation blocks binding of another host protein, OxyR, which represses *mom* transcription in *dam*$^-$ strains (30). Recent experiments indicate that OxyR modulates *mom* expression even in *dam*$^+$ cells (unpublished observations). The *E.coli* OxyR protein is a redox-sensitive transcriptional regulator that, under oxidative stress, induces the expression of a set of antioxidant defense genes. OxyR is 305 amino acids in length and belongs to the LysR family, whose members share a conserved helix–turn–helix motif involved in DNA binding (31,32). OxyR represses its own transcription during growth in the absence of oxidative stress (31). Detailed studies indicated that increased expression of OxyR-activated genes by treatment with low doses of hydrogen peroxide (H_2O_2) is a consequence of an induced conformational change in the protein and that the reduced and oxidized forms of OxyR have different DNA binding properties and protection patterns (33,34). During growth in the absence of oxidative stress intracellular OxyR is in the reduced conformation (33).

In this paper, we show that both reduced and oxidized forms of OxyR *in vitro* bind P_{mom} at a distance further upstream relative to its binding in the *oxyR* promoter (31), suggesting that the mechanisms of repression of the two promoters might be different. Our results on *in vitro* DNase I footprinting of P_{mom} support the notion (34) that the reduced and oxidized forms of OxyR make different contacts in DNA binding. In order to carry out an *in vitro* investigation of P_{mom} binding by reduced OxyR, C and RNAP, it was necessary to obviate the requirement for high dithiothreitol (DTT) concentrations. Therefore, we took advantage of the existence of a mutant, OxyR-C199S, which appears to be 'locked' in a reduced conformation (34,35). We observed that, like the wild-type OxyR *in vivo*, OxyR-C199S was able to repress *mom* transcription in *dam*$^-$ cells. Furthermore, *in vitro* OxyR-C199S gave a DNase I footprint identical to that of reduced wild-type OxyR. Although OxyR-C199S only slightly reduced C binding to its target site *in vitro*, prebinding of OxyR-C199S prevented both RNAP binding at P2 as well as C-activated RNAP binding at P1. These results indicate that OxyR-mediated repression of P_{mom} transcription is mediated subsequent to binding by C.

In the absence of C, OxyR-C199S partially inhibited RNAP binding to the *tin7* promoter *in vitro*, even though the OxyR and RNAP P1 binding sites probably do not overlap (5′- and 3′-boundaries defined by DNase I protection extend further than the actual contacts), and *in vivo* expression of *tin7* was reduced in *dam*$^-$ cells. These results suggest that OxyR may exert repression of *tin7* through its ability to bend DNA. Finally, the mechanism involved in the regulation of *mom* transcription initiation is discussed.

MATERIALS AND METHODS

Bacterial strains and plasmids

Escherichia coli K12 strains TA4484 *oxyR*Δ*3* (pMC7, pGSO68) (35,36) and GSO9 were kindly provided by Dr G. Storz; GSO9 carries an *oxyR*::*kan* insertion, with the *kan* promoter in the same direction as the *oxyR* promoter. Plasmid pGSO68, a pKK177-3 derivative, contains a mutant *oxyR-C199S* gene with a modified Shine–Dalgarno sequence and under control of the *tac* promoter (35). *Escherichia coli* DH5αF′ *ϕ80dlacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17*($r_K^-m_K^+$) *deoR thi-1 supE44* λ$^-$ *gyrA96 relA1*, carrying the wild-type OxyR overproducing plasmid pJL*momR*Δ*15*, was a generous gift from R. Kahmann (30). *Escherichia coli* JM83 *ara* Δ(*pro-lac*) *rpsL thi ϕ80dlacZ*Δ*M15* was from Bethesda Research Laboratories (BRL). *Escherichia coli* GM1853 *dam3 dcm6* Δ(*pro-lac*) *thi* and GM2972 *thr1 leuB6 thi1 supE44 lacY1 proA2 galK2 ara14 xyl5 mtl1 rpsL31 his4 tsx33 dam13*::*Tn9 mutH34* were from M. G. Marinus (37).

Plasmid pLW4 (~8.8 kb) has been described previously (27); it contains the P_{mom} region (from –136 to +79) and a 5′ portion of the proximal *com* gene fused in-frame to the *E.coli lacZ* gene. A Com–LacZ fusion protein with β-galactosidase activity is produced by pLW4, but only if transactivated by C. Plasmid pLW4-*tin7* differs from pLW4 in that it has a T→G transversion at –14 within a run of six thymines on the top strand (27); it produces β-galactosidase activity constitutively.

Construction of plasmids

Escherichia coli TA4484 *oxyR*Δ*3* (pMC7, pGSO68) was grown in LB + ampicillin (100 μg/ml) in order to promote segregational loss of plasmid pMC7, which confers tetracycline resistance (36). Plasmid DNA was prepared from 1.5 ml of overnight cells by the alkaline lysis method (38) and used to transform competent *E.coli* DH5αF′ cells. An Amp^r Tet^s transformant was characterized by restriction nuclease digestion and confirmed to carry only pGSO68. Plasmid DNA was cleaved with *Hin*dIII and the single-stranded tails filled-in with the *E.coli* Klenow fragment (PolIK). After digestion with *Eco*RI, the ~1.5 kb fragment containing the *oxyR-C199S* gene was recovered from a low melting temperature agarose gel and then cloned into the *Eco*RI and *Sca*I sites of pACYC184 (39) under the *cat* gene promoter. This construct (~5.3 kb) was designated pROR184.

Plasmid pMLC322 was created by replacing the ~1.2 kb *Sca*I–*Bsu*36I fragment from pMLF-2 (17) with the corresponding ~2.1 kb fragment from pTLG1(+) (28). The resulting Amp^r plasmid (~11.2 kb) carries the pBR322 replication origin, which makes it compatible with pACYC184 derivatives; it also contains the Mu *C* gene (under the *bla* promoter), as well as a *com–lacZ* translational fusion under control of P_{mom} (27).

Plasmid pMLC322Δ*mom* was constructed by removal of a 220 bp *Eco*RI–*Bam*HI *mom* promoter fragment, followed by fill-in with PolIK and blunt-end ligation.

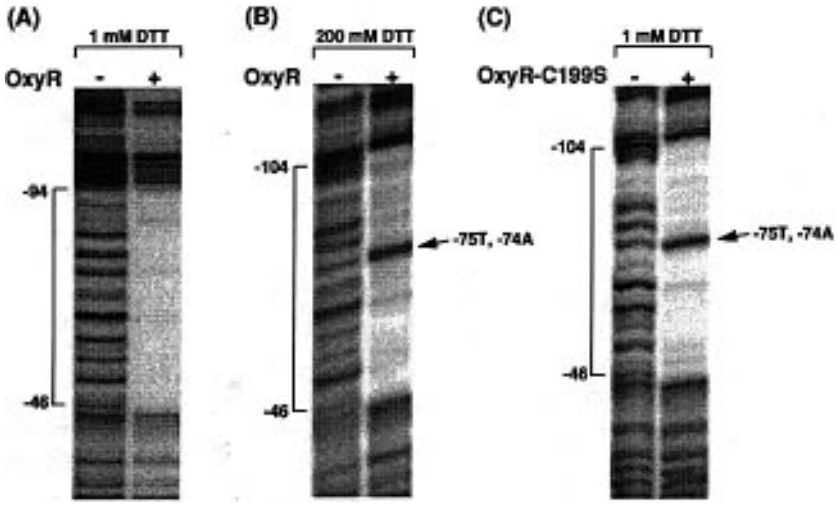

Figure 1. *In vitro* DNase I footprinting analysis of the unmethylated wild-type P_{mom} promoter. Plasmid pLW4 DNA carrying P_{mom} was isolated from a *dam*$^-$ host and incubated for 20 min at 22°C with or without either OxyR or OxyR-C199S in binding buffer containing 1 or 200 mM DTT, as indicated. After DNase I cleavage, primer extension with the ^{32}P-end-labeled *lac* primer complementary to the top strand and subsequent electrophoresis on 6% polyacrylamide–urea sequencing gels was used to map the DNA regions protected by protein binding. The final concentrations of DNA and proteins used were: pLW4, 9 nM; wild-type OxyR and OxyR-C199S (in tetrameric equivalents), 360 nM. The brackets denote the positions of the footprints with respect to the*mom* transcriptional start site and the arrows indicate the DNase I hypersensitive sites.

Construction of strains

Phage P1*vir* GM2972 *dam*13::Tn*9* was used to transduce *E.coli* JM83, essentially by the method of Miller (40). The *dam*$^-$ phenotype of chloramphenicol-resistant transductants was screened by *Mbo*I digestion of plasmids transformed into and isolated from these strains and by poor growth phenotype on 2-aminopurine (41). JM83 *dam*13::Tn*9* was subsequently used to generate JM83 *dam*13::Tn*9* Δ*oxyR*::*kan* by P1*vir* GSO9Δ*oxyR*::*kan* transduction. The Δ*oxyR* genotype was confirmed by the loss of OxyR repression, as evidenced by increased expression of β-galactosidase activity from pMLC322 in JM83 *dam*13::Tn*9* Δ*oxyR*::*kan* relative to the JM83 *dam*13::Tn*9* parent.

β-Galactosidase assays

For qualitative screening, cells were streaked on MacConkey lactose plates (Difco Laboratories) supplemented with appropriate antibiotics and grown for 12–16 h at 37°C. For quantitative liquid culture assays, overnight cells grown in LB plus appropriate antibiotics were diluted 1:100 into fresh medium and allowed to grow to log phase at 37°C. Aliquots were assayed in triplicate as described by Miller (40).

Overexpression and purification of proteins

C protein was previously purified as described (25,26). Wild-type OxyR protein was overproduced from the heat-inducible phage λ P_L-P_R tandem promoters in pJL*momR*Δ*15* as described (30). OxyR-C199S protein was overexpressed from the *tac* fusion promoter in pGSO68 (35) by IPTG induction. After sonication, the wild-type and mutant OxyR forms were purified by the method of Bölker and Kahmann (30). To further purify and concentrate the proteins, we diluted the final fractions to [NaCl] = 0.1 M and applied them to 2 ml SP Sepharose ion-exchange columns and eluted at 0.5 M NaCl. Protein concentration was determined according to the BioRad protein microassay using BSA as standard and adjusted to 1 mg/ml. Both proteins were at least 90% pure as estimated by 15% SDS–PAGE and silver staining.

DNase I footprinting

Unmethylated supercoiled plasmid DNA (pLW4 or pLW4-*tin7*) was isolated from a *dam*$^-$ host (GM1853) by the alkaline lysis method and purified by CsCl/EtBr gradient ultracentrifugation (38). One microgram of plasmid DNA (~0.18 pmol) was incubated in a total volume of 20 µl binding buffer (25 mM Tris–HCl, pH 7.9, 30 mM KCl, 6 mM $MgCl_2$, 50 µg/ml BSA, 4% glycerol and 1 mM DTT) at room temperature (22°C) with various combinations of proteins added in different orders, as described in the figures. One microliter of 0.2 U/µl DNase I (Pharmacia Biotech) was added and, after 45 s, the digestion was terminated by addition of 20 µl stop buffer (0.1 M Tris–HCl, pH 7.5, 25 mM EDTA and 0.5% SDS). The sample was then brought to 125 µl with H_2O and extracted successively with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1) and then precipitated in 0.2 M NaCl plus 2 vol 95% ethanol in the presence of carrier yeast tRNA.

Primer extension was performed essentially as previously described (42). Briefly, DNase I-treated DNA was suspended in 31 µl H_2O and incubated with 5 µl (3×10^5 c.p.m.) ^{32}P-end-labeled *lac* primer (NEB), which was complementary to the top strand of P_{mom}. Following alkali denaturation with 4 µl 0.1 M NaOH at 80°C for 2 min, the DNA was placed on ice. The primer was annealed at 50°C for 3 min after adding 5 µl TMD buffer (0.5 M Tris–HCl, pH 7.2, 0.1 M $MgCl_2$ and 2 mM DTT). The annealed primer was extended for 10 min at 45°C following addition of 5 µl of all four dNTPs (5 mM each) and 1 µl 1 U/µl PolIK. The reactions were terminated with 5 µl 0.1 M EDTA and precipitated with 110 µl 95% ethanol. After centrifugation, pellets were

suspended in 10 µl 0.5× loading dye and applied to a 6% polyacrylamide–urea denaturing gel; sequencing reactions with untreated DNA were run in parallel lanes.

RESULTS

DNase I footprinting of P_{mom} with the oxidized and reduced forms of OxyR

Using random mutagenesis with bacterial transposon Tn*5*, Bölker and Kahmann (30) identified OxyR as the repressor of *mom* transcription in *dam*⁻ cells. They also showed that (oxidized) OxyR protected a region of P_{mom} *in vitro* against MPE-Fe(II) cleavage from –92 to –50 on the top strand. While both oxidized and reduced forms bound to the *oxyR* promoter, they made strikingly different contacts with the DNA (34). In order to investigate how the two redox states of OxyR might affect its binding patterns on unmethylated P_{mom}, we carried out a DNase I footprinting analysis using supercoiled plasmid pLW4 DNA as the substrate.

As shown in Figure 1A, oxidized OxyR (1 mM DTT) gave a DNase I footprint from –94 to –46 on the top strand. These boundary values are in good agreement with MPE-Fe(II) cleavage data above (30), although DNase I footprinting generally gives broader protection compared with chemical agents. Under reducing conditions (200 mM DTT) this protection was extended ~10 bp upstream and DNase I hypersensitive sites at –75T and –74A became apparent (Fig. 1B). This is consistent with the notion that oxidized OxyR binds to four successive major grooves on one face of the DNA helix, while the reduced OxyR binds to two pairs of adjacent major grooves separated by one helical turn (34). The presence of DNase I hypersensitive sites suggests that reduced OxyR can bend P_{mom} DNA, as it does the *oxyR* promoter.

It was proposed that substitution of serine for cysteine at position 199 locks OxyR in the reduced conformation, e.g. OxyR-C199S gave a DNase I protection pattern at the *oxyR* promoter (even in 1 mM DTT) characteristic of reduced wild-type OxyR (34,43). Therefore, we extended our analysis to the mutant OxyR-C199S protein. As seen in Figure 1B and C, the DNase I footprint of OxyR-C199S was identical to that of reduced wild-type OxyR, consistent with its being locked in the reduced conformation. Thus, OxyR-C199S could be useful for *in vitro* studies (see below) because it obviated the need for high DTT concentrations, which might provoke structural or functional changes in C or RNAP.

OxyR-C199S represses *mom* expression *in vivo*

Intracellular wild-type OxyR is in a reduced conformation during normal bacterial growth, i.e. when oxidative stress is not applied (33). To test whether OxyR-C199S can repress *mom* transcription *in vivo*, we constructed a JM83 *dam*⁻ Δ*oxyR* derivative (see Materials and Methods). This strain was transformed with plasmid pMLC322, a pBR322 derivative containing a *com–lacZ* translational fusion under control of the *mom* promoter, as well as the *C* gene under the *bla* promoter. A second plasmid, vector pACYC184 (Fig. 2, strain B) or pROR184 (which has the *oxyR-C199S* gene under the *cat* promoter in vector pACYC184) (strain C), was then introduced by transformation. As a negative control, JM83 *dam*⁻ Δ*oxyR* was transformed with pMLC322Δ*mom* (containing a deletion of the *mom* promoter fragment) and vector pACYC184 (strain A). After 12–16 h

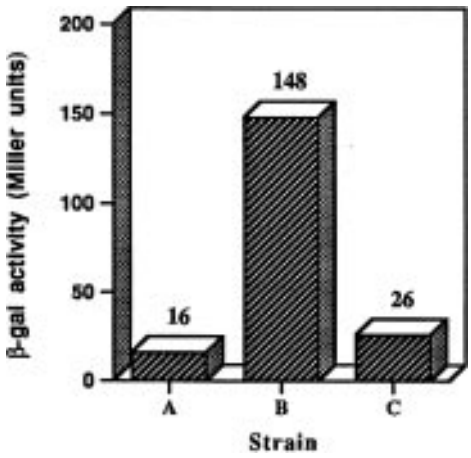

Figure 2. Production of β-galactosidase activity in *E.coli* JM83 *dam*⁻ Δ*oxyR* transformed with different plasmids. Strain A contained vector pACYC184 and pMLC322Δ*mom* (a derivative of pMLC322 that has a deletion of the *mom* promoter); strain B contained vector pACYC184 and pMLC322 (a pBR322 derivative containing the *C* gene under the *bla* promoter and a *com–lacZ* translational fusion under control of the *mom* promoter); strain C contained pMLC322 and pROR184 (a pACYC184 derivative containing the *oxyR-C199S* gene under the *cat* gene promoter). The value of β-galactosidase activity (in Miller units) for each strain represents the average from three independent assays, with a standard deviation < 10%.

growth on MacConkey lactose plates at 37°C, strain B gave dark red colonies, while strain C colonies were white or slightly pink. This indicates that *com–lacZ* expression was strongly reduced in the presence of OxyR-C199S. Quantitative β-galactosidase activity assays were also carried out in liquid culture. As shown on Figure 2, OxyR-C199S-producing cells exhibited a 13-fold reduction in P_{mom}-directed production of the Com–LacZ fusion. This inhibition was much lower when a higher copy number plasmid was used in place of pMLC322 (data not shown), suggesting that OxyR-C199S may be limiting in this system. Nevertheless, we can conclude that OxyR-C199S repressed transcription of P_{mom} in *dam*⁻ cells.

OxyR-C199S does not prevent C binding to the wild-type P_{mom} promoter

Initially, gel retardation assays were used to examine whether OxyR-C199S affected the binding of C to P_{mom}. Increasing amounts of C were added to mixtures of OxyR-C199S preincubated with an unmethylated ^{32}P-end-labeled DNA fragment containing P_{mom}. We observed that formation of the OxyR-C199S–DNA binary complex was gradually replaced by a supershifted band, indicative of an OxyR-C199S–DNA–C ternary complex (data not shown). However, significant amounts of radioactivity were always seen between the two bands, suggesting that the ternary complex dissociated during electrophoresis.

DNA supercoiling can play a role in the transcription initiation process, including binding of RNAP or regulatory proteins to promoter sequences (44). We have previously observed that *mom* transcription from linearized templates is reduced compared with transcription from supercoiled minicircle DNA (26,28). Therefore, we undertook a DNase I footprinting analysis with supercoiled plasmid DNA. To determine the effect of prebound OxyR-C199S on C binding, supercoiled P_{mom} DNA was preincubated with a saturating amount of OxyR-C199S; densitometric measurements

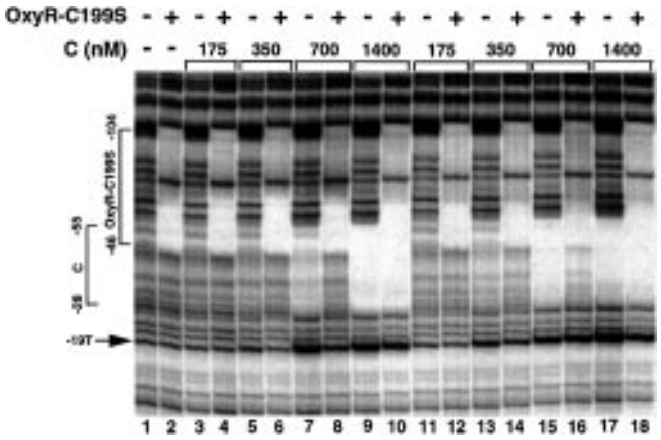

Figure 3. Effect of OxyR-C199S on the binding of C to wild-type P_{mom}. Increasing amounts of C (in dimeric equivalents) were added to unmethylated pLW4 DNA (9 nM) at 22°C in the absence or presence of 360 nM OxyR-C199S that had been preincubated for 20 min (lanes 3–10); conversely, OxyR-C199S (360 nM) was added 20 min after prebinding with increasing amounts of C (lanes 11–18). Twenty minutes was sufficient time to equilibrate binding of either protein. Treatment with DNase I was started 20 min after addition of the last protein and the samples were analysed as in Figure 1.

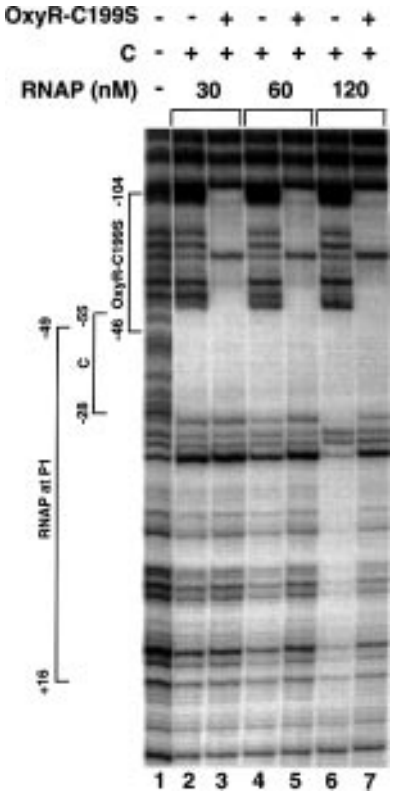

Figure 4. OxyR-C199S inhibition of C-activated RNAP binding to the P1 site in wild-type P_{mom}. Unmethylated pLW4 DNA (9 nM) was incubated at 22°C for 20 min in the absence or presence of OxyR-C199S (360 nM). C was added (1.40 µM) and the incubation continued for 20 min, when increasing amounts of RNAP were added, as indicated. After an additional 20 min incubation, the samples were treated with DNase I and analysed as in Figure 1.

showed that >95% of the P_{mom} DNA in each lane was protected. Various concentrations of C were then added and each sample was run on a sequencing gel alongside a reaction that contained the same amount of C but without OxyR-C199S (Fig. 3). In the absence of OxyR-C199S, the C footprint was barely detectable at 350 nM (in dimeric equivalents). However, when the C concentration was increased 4-fold, the target sequence was completely protected in both the absence (lane 9) and presence (lane 10) of OxyR-C199S, showing that OxyR-C199S only weakly inhibited C binding. Furthermore, this pattern is consistent with a strong cooperativity in C binding (S. Hattman, X. Song, T.L. Cabot and W. Sun, submitted for publication). The order of protein additions was reversed in order to examine whether OxyR-C199S could influence C prebound to P_{mom} (Fig. 3, lanes 11–18). As expected, OxyR-C199S added to the preformed C–DNA complex reduced C binding to about the same extent as when it was incubated with the DNA prior to C addition.

OxyR-C199S inhibition of C-activated RNAP binding to P1

Clearly, the weak *in vitro* reduction of C binding due to OxyR-C199S does not suffice to account for *mom* repression seen *in vivo*, since transcription of the *mom* gene in a dam^- strain is at least 20-fold lower than in a dam^+ strain (18). This prompted us to address what effect OxyR-C199S exerts on *E.coli* RNAP binding (under conditions where C binding is not affected). Plasmid pLW4 DNA was incubated with or without OxyR-C199S for 20 min prior to the addition of C; the protein concentrations used produced complete protection of both their respective target sequences, regardless of the presence of the other protein (Fig. 3, lanes 9 and 10). After binding of C, varying amounts of RNAP were added and DNase I digestions were carried out 20 min later. As seen in Figure 4 (lanes 4 and 6), in the absence of OxyR-C199S, a C-dependent RNAP-protected region from –49 to +16 (corresponding to the P1 site) was clearly evident at concentrations of 60 nM RNAP or higher. This protection was strongly diminished when OxyR-C199S was present (lanes 5 and 7). These results indicate that OxyR repression inhibited C-activated RNAP binding subsequent to binding by C.

We also investigated the effect of OxyR on RNAP binding at P2. In the absence of C, RNAP binds to P2 (from –64 to –11), upstream of and overlapping P1. *In vitro* both wild-type OxyR and OxyR-C199S completely blocked P2 binding and they were also able to displace RNAP prebound at P2 (data not shown). The exclusion of RNAP P2 binding by OxyR is probably due to steric hindrance, since their target sites overlap extensively (see Fig. 6 in Discussion).

OxyR-C199S inhibition of RNAP binding to P1 in *tin7-P_{mom}*

The foregoing data demonstrated that OxyR-C199S bound in the wild-type P_{mom} region interfered with both C-activated RNAP binding at P1 and with RNAP binding at P2. Unlike the wild-type promoter, however, in the absence of C, RNAP binds *tin7*-P_{mom} predominantly at P1 (24); although C still stimulates transcription from *tin7*-P_{mom} *in vivo* and *in vitro* (27,28). It is interesting to note that the *tin7* promoter has a –14T→G transversion within a T_6 run, which abolishes any intrinsic bending potential; free *tin7* promoter DNA also exhibits increased nuclease accessibility between –10 and –17, as previously reported (27) and also observed here (Fig. 5A and B, lane 1). In some, as yet unknown,

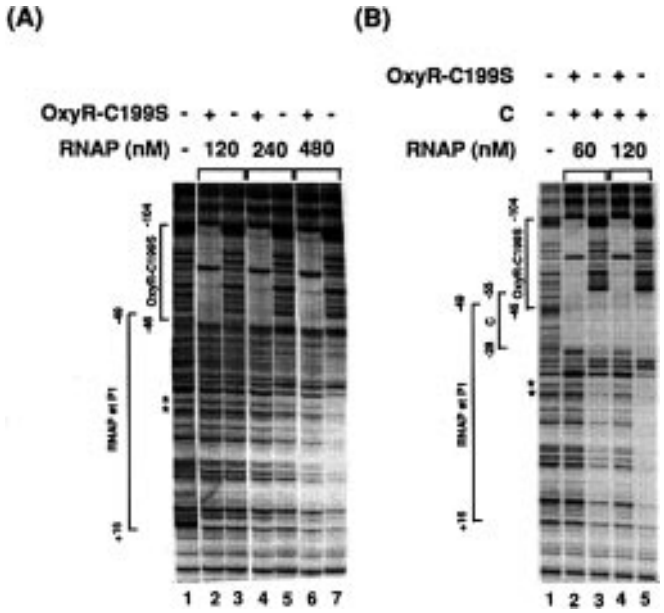

Figure 5. OxyR-C199S inhibition of RNAP binding to the mutant *tin7*-P_{mom}. (**A**) Unmethylated pLW4-*tin7* DNA (9 nM) was incubated with or without OxyR-C199S (360 nM) at 22°C for 20 min, when various amounts of RNAP were added. After 20 min, DNase I digestion and *lac* primer extension were performed as in Figure 1. (**B**) DNase I footprinting analysis with the unmethylated pLW4-*tin7* DNA was carried out as described in Figure 4. The DNase I hypersensitive sites present on *tin7* but not wild-type naked DNA are indicated by asterisks.

fashion the structure of *tin7*-P_{mom} is different from that of wild-type P_{mom}. Thus, it was of interest to determine whether OxyR-C199S could inhibit C-activated and direct RNAP binding to P1 in *tin7*-P_{mom}. The results are shown in Figure 5. In the absence of C, OxyR-C199S reduced direct RNAP binding to P1 ~4-fold, as estimated by a densitometric analysis (Fig. 5A, lanes 6 and 7). This correlates fairly well with the 5- to 10-fold reduced expression of *tin7*-P_{mom} in *dam*⁻ cells (data not shown). In the absence of OxyR-C199S, addition of C substantially increased RNAP affinity for P1. For example, in the presence of C, 120 nM RNAP produced a higher degree of protection than 480 nM RNAP in the absence of C (compare Fig. 5A, lanes 3 and 7, with Fig. 5B, lanes 3 and 5), consistent with the known C-mediated transcriptional stimulation of *tin7*-P_{mom}. The presence of OxyR-C199S, however, reduced this protection ~9-fold (Fig. 5B). These results indicate that OxyR-C199S inhibited both direct and C-activated RNAP binding to P1.

DISCUSSION

In the work described here, we show that OxyR gave distinctly different DNase I footprints on an unmethylated *mom* promoter under oxidizing (1 mM DTT) versus reducing (200 mM DTT) conditions *in vitro* and that OxyR-C199S behaved like wild-type OxyR in its reduced state (33). Because *in vivo* experiments showed that OxyR-C199S acted as the *mom* repressor in a *dam*⁻ host, we were able to use OxyR-C199S *in vitro* at 1 mM DTT in place of wild-type OxyR at 200 mM DTT. Thus, by DNase I footprinting, we demonstrated that while OxyR-C199S has only a weak inhibitory effect on C binding, it blocked C-activated RNAP binding at the functional P1 site of P_{mom}, but the molecular mechanism remains to be elucidated. The DNase I footprint of OxyR-C199S extends 10 bp into the region protected by C (Fig. 6), making it possible that the two proteins contact each other. The slight decrease in DNA affinity for C can be explained by either steric hindrance or DNA structure distortion (e.g. bending). Studies with the partially C-independent *tin7-mom* promoter indicate that OxyR-C199S inhibited RNAP binding directly, as well as indirectly through its influence on C-activation.

Significantly, binding of C to the wild-type *mom* promoter gave rise to a pronounced DNase I hypersensitive site in the spacer region at –19T, well downstream of the C footprint (Fig. 3), suggesting some C-induced DNA conformational change. In this regard, we propose that C binding to P_{mom} may modify the spacer to a three-dimensional structure productive for RNAP placement and that *tin7* DNA, by eliminating a misoriented DNA bend embedded in the spacer and also by creating –15T, –14G, compensates to some degree for the divergence of the –10 and –35 sequences from the consensus *E.coli* hexamers. Moreover, the

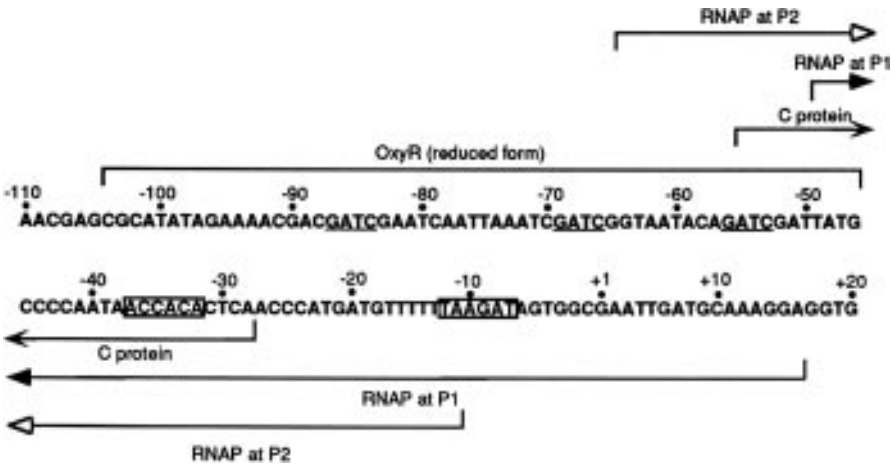

Figure 6. Summary of the protein binding sites on the top strand of the P_{mom} promoter. The regions protected by reduced OxyR, C and RNAP (at both P1 and P2) against DNase I cleavage are indicated; the three Dam methylation sites (GATC) important in regulation of *mom* transcription are underlined; the –35 and –10 hexamers are enclosed in rectangles. The +1 denotes the start of *mom* transcription.

tin7- and the wild-type *mom* promoters have a similar affinity for RNAP in the presence of C (at both 60 and 120 nM RNAP, as determined from Figs 4 and 5B), suggesting that the *tin7* mutation and C binding might produce similar functional topologies of the promoter. It should be noted that the *tin7* and the wild-type promoters have the same transcription start site. It is not known whether OxyR-C199S makes contact with RNAP at the *tin7* promoter and, if so, whether this interaction accounts for the direct inhibitory effect on RNAP binding. Considering the fact that the DNase I footprints of OxyR-C199S and RNAP overlap by at most 4 bp (Fig. 6) and the marked conformational alteration caused by OxyR-C199S binding, it is more likely that decreased RNAP binding occurs as a result of a DNA conformational change.

In the absence of C, RNAP binds to the P2 site slightly upstream of and overlapping P1, protecting a region against DNase I cleavage from –64 to –11 (27). Both OxyR-C199S and wild-type OxyR completely block P2 binding, presumably by steric exclusion, and they can also displace RNAP already bound at P2 (data not shown). The same steric hindrance mechanism is probably operative in self-repression of *oxyR* transcription, because OxyR binds to its own promoter in a region spanning the –10 hexamer to the +1 site (31,33). All findings taken together, we favor the notion that OxyR acts as a repressor primarily by blocking C activation of RNAP binding at P1. Whether the redox states of OxyR are relevant to repression of P_{mom} transcription awaits further study. OxyR is proposed to bind on one face of its target DNA sequences as a dimer of dimers (34). Oxidation of this protein rearranges its contacts with the upstream half of the *oxyR* site, while contacts with the downstream half-site remain unchanged. Based on the close similarity of its footprints on P_{mom} and P_{oxyR} (under reducing or oxidizing conditions), it appears that OxyR interacts with the two promoters in essentially the same fashion. C binds to P_{mom} just 3′ of and overlapping the downstream OxyR half-site, suggesting the likelihood that C–OxyR interactions, if any, are not affected by OxyR redox state. On the other hand, whether and how the redox-dependent OxyR conformational change and the DNA bend directed by reduced, but not oxidized, OxyR affect *mom* transcription deserve further investigation.

Intriguingly, the –35 elements of activatable promoters often deviate from consensus, leading to the hypothesis that one role of the activators in these cases is to provide a contact point for RNAP (44). In the *mom* system, the DNase I footprints of C and RNAP overlap extensively around the –35 region (Fig. 6), suggesting that RNAP may be recruited to the promoter by interaction with C bound at its target site. In agreement with this view, RNAP binding to the *tin7* promoter is further stimulated by C. C-induced DNA curvature and protein–protein contacts are potentially two aspects of C function. The fact that the four Mu late promoters, despite their different DNA sequences, have the same requirement for C activation supports the notion that direct C–RNAP communication plays a critical part in RNAP promoter recognition and transcription initiation. With this consideration, an efficient mechanism of OxyR repression would be to force C out of register with respect to RNAP, either indirectly by DNA bending or by direct interaction with C (or both), so that specific C–RNAP contact cannot be achieved. It will be worthwhile to look for C mutations that can (partially) circumvent the repression effect by OxyR (or OxyR-C199S). If we can then obtain suppressor mutants of OxyR that can restore repression, support for direct protein–protein interaction will be strengthened and we may also be able to define the contact domains.

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