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Escherichia coli OxyR modulation of bacteriophage Mu *mom* expression in *dam*$^+$ cells can be attributed to its ability to bind hemimethylated P_{mom} promoter DNA

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ABSTRACT

Transcription of the bacteriophage Mu *mom* operon is strongly repressed by the host OxyR protein in *dam*$^-$ but not *dam*$^+$ cells. In this work we show that the extent of *mom* modification is sensitive to the relative levels of the Dam and OxyR proteins and OxyR appears to modulate the level of *mom* expression even in *dam*$^+$ cells. *In vitro* studies demonstrated that OxyR is capable of binding hemimethylated P_{mom}, although its affinity is reduced slightly compared with unmethylated DNA. Thus, OxyR modulation of *mom* expression in *dam*$^+$ cells can be attributed to its ability to bind hemimethylated P_{mom} DNA, the product of DNA replication.

INTRODUCTION

The bacteriophage Mu *mom* gene encodes an unusual DNA modification function (1–4) that protects the viral DNA against a variety of host controlled restriction systems (5). Transcription initiation from P_{mom} by *Escherichia coli* σ^{70} RNA polymerase requires transactivation by a phage-encoded protein, C (6–8), which is also required for transcription of three other late operons (9,10). Surprisingly, activity of the host DNA-[N^6-adenine] methyltransferase (Dam), which methylates adenine in the sequence GATC (11,12) is also required for *mom* expression (1,3,13–15), since transcription of the bacteriophage Mu *mom* operon is strongly repressed in *dam*$^-$ hosts (13,14). The role of Dam is to methylate three closely spaced sites located just upstream of the C recognition site (3,16,17). This methylation prevents binding of another host protein, OxyR, the repressor of *mom* transcription (18). OxyR, a member of the LysR family, is a redox-sensitive transcriptional regulator that under oxidative stress induces expression of a set of antioxidant defense genes (19). Consistent with Dam-dependent expression of *mom* is the fact that OxyR binds to unmethylated, but not fully methylated, P_{mom} DNA *in vitro* (18,20). Since most of the naturally occurring enteric strains that Mu infects are *dam*$^+$, it has been something of an enigma as to why OxyR should play a regulatory role in *dam*$^-$ hosts.

Another perplexing observation has been that the degree of *mom* modification of Mu progeny phage DNA varies as a function of the mode of virus propagation, namely phage grown via an exogenous infection and lytic cycle is modified to a much lower extent than growth following thermal induction of a Mu lysogen (15). This lower degree of *mom* modification can be measured either directly by DNA base analysis or indirectly by biological assay of the efficiency of plating (EOP) on restricting versus non-restricting hosts; for the latter, the phage P1 system has been particularly useful, since *mom* modification protects against P1 restriction. For example, the EOP on K12(P1) versus K12 is 0.01–0.02 for Mu grown lytically in *dam*$^+$ cells; this value is 0.4–0.7 for phage grown following prophage induction (1). In contrast, phage grown in *dam*$^-$ cells by either method have an EOP of only 10^{-4}–10^{-3}.

It is also known that in general bacteriophage virion DNA methylation is usually incomplete, presumably due to packaging of the phage DNA prior to saturation of all potential modification sites, whereas intracellular DNAs are generally fully modified (21). Thus, it was suspected that the difference in *mom* modification levels for Mu grown via the lytic cycle versus prophage induction could be attributed to some difference in P_{mom} methylation during these two growth cycles. For example, the temperature shift-up to induce the prophage might affect the methylation pattern by either increasing the rate of DNA methylation or access to sites. Support for the notion of an effect of DNA methylation came from an observation that *mom* modification of lytically grown Mu increased to the same level as following prophage induction if the host harbored a plasmid expressing a cloned *dam*$^+$ gene (3). This suggested that higher intracellular levels of Dam increased the extent of *mom* expression. Interestingly, the requirement for Dam was obviated when the three methylation target GATC sites were deleted or mutated (3,16,17). This led to the prediction that in *dam*$^-$ cells a host-encoded protein binds in this region and represses *mom* transcription (22). This hypothesis was confirmed when it was shown that the *E.coli* OxyR protein is the repressor; e.g. in *dam*$^-$ cells an *oxyR*$^-$ mutation suppressed the Dam requirement for *mom* expression (18).

The first hint that OxyR might also influence *mom* expression in *dam*$^+$ cells came from the following observations. The EOP of Mu progeny phage produced after prophage induction in a *dam*$^+$*oxyR*$^+$ host was 0.5; it was increased to 1.0 if induction was in a *dam*$^+$*oxyR*$^-$ host (18). In contrast, the EOP was lowered to 8×10^{-3} if the cells harbored a plasmid expressing a cloned *oxyR*$^+$

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gene. Because of the high EOP baseline for induced prophage, a 2-fold increase was not a convincing demonstration that OxyR reduces *mom* expression in *dam*$^+$ cells. We have used a more sensitive biological assay involving phage grown by exogenous infection to demonstrate OxyR modulation of *mom* gene expression in *dam*$^+$ cells and we also show directly that OxyR is able to bind hemimethylated P_{mom} DNA *in vitro*. Thus, OxyR-mediated *mom* regulation operative in *dam*$^+$ cells can be attributed to the ability of the protein to bind hemimethylated P_{mom} DNA, the product of DNA replication.

MATERIALS AND METHODS

Strains and plasmids

Both *oxyR*$^-$ and *dam*$^-$ derivatives of *E.coli* strain JM83 were prepared by P1*vir* transduction (20) from appropriate *oxyR*::*kan* and *dam*::Tn*9* *Cm*^{R} donors respectively. Kanamycin- and chloramphenicol-resistant transductant clones were picked and screened for the OxyR$^-$ phenotype by growth sensitivity in the presence of 1 mM H_2O_2. The Dam$^-$ phenotype was screened by sensitivity to cleavage of cellular DNA by the restriction endonuclease *Dpn*II (New England Biolabs, NEB).

Production of P_{mom} DNA in different methylation states

Plasmid pLW4 contains the P_{mom} promoter region on an ~220 bp *Bam*HI–*Eco*RI fragment cloned into pNM480 (23,24). Plasmid pLW4 DNA was isolated from *E.coli* strain JM83 *dam*$^-$ (20) and digested with *Bam*HI and *Eco*RI and the P_{mom} fragment purified from a low melting point (LMP) agarose gel. The DNA was digested with *Dpn*II, dephosphorylated with calf intestinal alkaline phosphatase (Gibco BRL) and end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. Plasmid pLW6 contains the P_{mom} promoter region on the same ~220 bp *Bam*HI–*Eco*RI fragment, but cloned into plasmid pGC2. Plasmid pLW6 DNA was isolated from a *dam*$^+$ host and methylated to saturation with *Eco*Dam methyltransferase (NEB) *in vitro*.

To generate hemimethylated DNA for restriction nuclease digestion (Fig. 1), 0.1 µg (18 fmol) pLW4 *dam*$^-$ DNA was linearized with *Bam*HI and ^{32}P-end-labeled by filling in with Sequenase (United States Biochemical, USB). Following deproteinization with phenol/chloroform, the DNA was mixed with a 100-fold excess (1.8 pmol) of unlabeled, *Bam*HI-digested pLW6 *dam*$^+$ DNA and digested with *Eco*RI. The *Bam*HI–*Eco*RI P_{mom} fragments (labeled on the top strand from pLW4) were isolated from an LMP agarose gel and dissolved in 8 µl TE buffer, pH 7.9, in a 500 µl microfuge tube. After applying a top layer of 10 µl mineral oil (ultrapure for use in PCR; USB) the DNA was denatured by boiling for 10 min, vortexed briefly and put on ice. Two microliters of 5× Sequenase buffer (USB; 5× = 200 mM Tris–HCl, pH 7.5, 100 mM $MgCl_2$ and 250 mM NaCl) were added and the DNA annealed by slow cooling from 65°C to between 35 and 30°C. The same method was used to generate P_{mom} hybrid DNA with ^{32}P-labeling on the bottom strand, except that the plasmids were first linearized with *Eco*RI and then digested with *Bam*HI.

The hemimethylated P_{mom} DNA used in binding studies (Fig. 2) was produced by denaturation and annealing of labeled (in one strand) fragments from fully methylated pLW6 with a 100-fold excess of unlabeled, unmethylated, but otherwise identical fragments. Unmethylated P_{mom} DNA, labeled on one strand, was mixed with a 100-fold excess of unlabeled fragments; half the sample was taken for denaturation and annealing. By using labeled methylated rather than unmethylated fragments to prepare the hemimethylated duplexes, we avoided formation of retarded complexes derived from undenatured labeled fragments.

Hemimethylation was monitored by resistance to cleavage with *Dpn*II, which cleaves only unmethylated GATC. As a control for DNA digestibility after heat denaturation and annealing, digestion was also performed with *Sau*3AI, which is indifferent to adenine methylation in GATC. All samples were then electrophoresed on a non-denaturing 16% polyacrylamide gel.

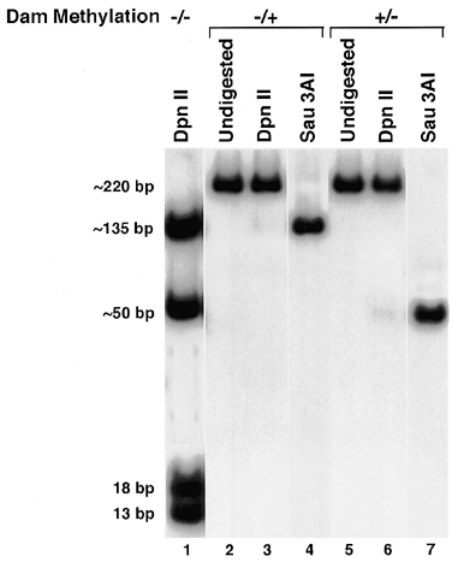

Figure 1. Assay of Dam methylation at the three GATC sites in the *mom* promoter region. P_{mom} was on an ~220 bp *Eco*RI–*Bam*HI fragment described previously (23,24). Lane 1, plasmid pLW4 DNA was isolated from JM83 *dam*$^-$ cells, digested with *Dpn*II and ^{32}P-end-labeled with T4 kinase; lanes 2–7, heat-denatured and annealed DNA samples, ^{32}P-end-labeled on the unmethylated strand, were digested as indicated. The methylation states of the labeled DNA duplexes are denoted by two symbols; for example –/+ indicates that the DNA was unmethylated on the top strand and methylated on the bottom strand.

In vitro binding of OxyR to P_{mom} DNA

Intracellular OxyR is in a reduced conformation during growth in the absence of oxidative stress (25), however, *in vitro* it is in an altered conformation and requires high concentrations of dithiothreitol (DTT) to avoid oxidation. Previously we found that a mutant form, OxyR-C199S, which appears to be 'locked' in a reduced conformation (26), is able to repress *mom* expression both *in vivo* and *in vitro* (20), therefore, we used purified OxyR-C199S to study *in vitro* binding to variously methylated forms of P_{mom}. Labeled P_{mom} DNA (300 c.p.m.) was incubated with serial dilutions of purified OxyR-C199S (20) at room temperature (22°C) for 45 min in a total of 22 µl binding buffer [25 mM Tris–HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 5% (v/v) glycerol, 50 µg/ml double-stranded poly(dI–dC), 50 µg/ml BSA and 1 mM DTT]. As a control, unmethylated DNA without denaturation and annealing treatment was analyzed in a separate binding assay. Samples were electrophoresed on a non-denaturing 6% polyacrylamide gel in low ionic strength electrophoresis

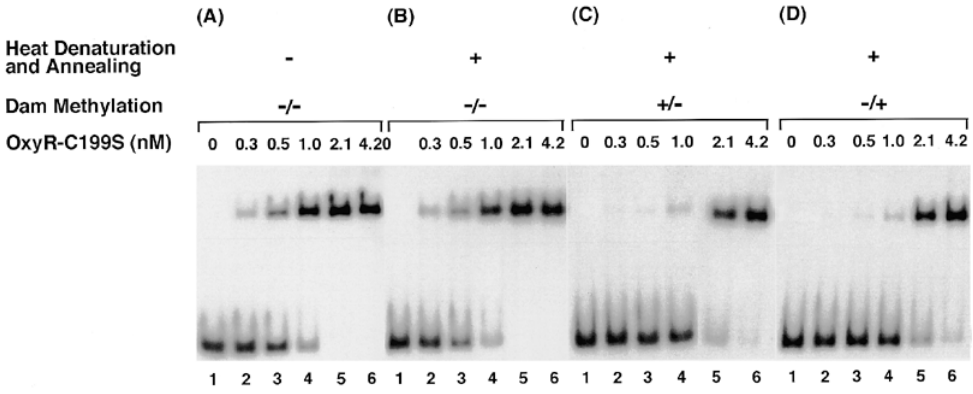

Figure 2. Binding of OxyR-C199S to P_{mom} DNA *in vitro*. DNA samples, with or without heat denaturation and annealing as indicated, were incubated with serially diluted OxyR-C199S and electrophoresed on a non-denaturing 6% polyacrylamide gel. See Materials and Methods for details on labeling and production of the hemimethylated species.

buffer (27) at 5 V/cm for 5 h at 22°C. The gel was dried and autoradiographed.

RESULTS AND DISCUSSION

Modulation of *mom* expression in *dam*$^+$ cells

In order to have a more sensitive assay for OxyR modulation of *mom* expression in *dam*$^+$ cells, we took advantage of the fact that a relatively low level of *mom* modification is produced during Mu lytic infection (1). Thus, phage Mu*cts62* was grown lytically by plating it on various host strains and the progeny phage from pooled plaques were serially diluted and plated on K704 (P1) and K704. As shown in Table 1, lytically grown phage from both *dam*$^+$*oxyR*$^-$ and *dam*$^-$*oxyR*$^-$ hosts were efficiently modified; i.e. they had a relative EOP within the range 0.4–0.6, which is a value typical for progeny from prophage induction in *dam*$^+$ cells. Thus, Mu grown in a *dam*$^+$*oxyR*$^+$ or *dam*$^-$*oxyR*$^+$ host had a 40- or 600-fold lower EOP than Mu grown in the corresponding *oxyR*$^-$ variant. Therefore, loss of OxyR activity by mutation, which abolishes repression in *dam*$^-$ cells, also elevated *mom* modification of Mu phage grown lytically in *dam*$^+$ cells (about the same high level EOP was observed in both *dam*$^-$ and *dam*$^+$ cells deficient in OxyR). It should be noted here that phage λcI_{857} grown lytically in each of these four strains exhibited a low EOP (10^{-4}) on K704(P1); this rules out the possibility that P1 modification genes were left behind during the P1*vir* transductions creating the mutant strains (data not shown).

Table 1. Relative EOPs of phage Mu grown lytically on various host strains

Phage grown on host[a]	Relative EOP[b]
dam$^+$ *oxyR*$^+$	0.01
dam$^+$ *oxyR*$^-$	0.4
dam$^-$ *oxyR*$^+$	0.001
dam$^-$ *oxyR*$^-$	0.6

[a]Phage Mu*cts62* was plated on various derivatives of *E.coli* K12 strain JM83 (see text).
[b]Relative EOP is defined as the titer on K704(P1)/titer on K704.

As noted in the Introduction, increasing the intracellular level of OxyR decreased *mom* modification of induced prophage in *dam*$^+$ cells by 50- to 100-fold (18). We have repeated those experiments and found at least a 10^3-fold inhibition of *mom* expression (unpublished observation); this may be due to differences in the hosts and/or OxyR plasmids used. The results are consistent with the notion that OxyR modulates *mom* expression in *dam*$^+$ cells. Taken together with the fact that *mom* modification is enhanced by expression of a cloned *dam*$^+$ gene (3), it appears that the intracellular Dam MTase/OxyR ratio is important in determining the degree of *mom* expression. However, it is not clear whether an increased OxyR level decreases *dam* expression or whether OxyR is actually capable of binding hemimethylated P_{mom} DNA. The former possibility was ruled out by (methylation-sensitive) restriction nuclease analysis of DNA from the plasmid harboring a cloned, expressed *oxyR* gene (data not shown). Experiments to test OxyR binding to hemimethylated P_{mom} DNA are described below.

Monitoring hemimethylation of artificial P_{mom} duplexes

The role of Dam methylation in regulating *mom* transcription suggested that binding of OxyR to P_{mom} depends on the presence and methylation state of the three GATC sites within an upstream OxyR recognition sequence (17). This has indeed been shown, as (oxidized) OxyR can footprint *dam*$^-$ but not *dam*$^+$ P_{mom} DNA *in vitro* (18). In our studies the P_{mom} fragment isolated from *dam*$^-$ cells could be completely digested by *Dpn*II (Fig. 1, lane 1), indicating that all three GATC sites were unmethylated (28). When a P_{mom}-containing high copy number plasmid (pLW6) was grown in *dam*$^+$ cells methylation of the GATC sites was not quite complete (data not shown). Therefore, *dam*$^+$ P_{mom} DNA was methylated to completion *in vitro* with the GATC-specific *Eco*Dam methyltransferase. Hemimethylated P_{mom} DNA duplexes were then prepared as described in Materials and Methods. These duplexes were resistant to *Dpn*II digestion (lanes 3 and 6), showing that the parental strands derived from pLW6 had been fully methylated. An extremely weak band was also observed in both lanes; these are attributed to the products of self-annealing by the unmethylated DNA (which should occur with 1% probability). Digestion with *Sau*3AI was complete and no unexpected bands

were detected (lanes 4 and 7), indicating that the DNA had been completely denatured and annealed according to their complementarity and that they were not noticeably damaged by the heat treatment.

OxyR binds to hemimethylated DNA *in vitro*

The results of *in vitro* binding of OxyR-C199S to P_{mom} DNA, analyzed by gel retardation, are shown in Figure 2; it should be noted here that binding of the (oxidized) wild-type OxyR gave virtually identical results (data not shown). Similar binding profiles were obtained with unmethylated DNA before and after heat denaturation/annealing (Fig. 2A and B), showing further that the strategy to produce hemimethylated DNA yielded intact strands in the annealed duplexes. Binding of hemimethylated DNA [with methylation on the top (+/–) or the bottom (–/+) strand] was barely detectable at 0.5 nM and definitely present at 1.0 nM OxyR-C199S (Fig. 2C and D, lanes 3 and 4). In contrast, at the lower concentration 37% of unmethylated DNA was bound (Fig. 2B, lane 3), indicating that OxyR-C199S–P_{mom} binding was reduced by virtue of hemimethylation on either strand (+/– DNA gave slightly better binding than –/+ DNA). However, substantial binding with both hemimethylated DNAs (>70%) was achieved when the protein concentration was raised by only 4-fold to 2.1 nM (Fig. 2C and D, lanes 5); comparable levels of unmethylated DNA binding required roughly half the concentration of OxyR-C199S (Fig. 2B, lane 4). Separate analyses showed that OxyR-C199S binding was very weak when both DNA strands were methylated (data not shown).

These results clearly demonstrate that *in vitro* OxyR can bind hemimethylated P_{mom} DNA with only a slight reduction in affinity compared with unmethylated DNA. This suggests that *in vivo* binding of OxyR to hemimethylated P_{mom} DNA (generated by DNA replication) is responsible for the observed modulation of *mom* expression in *dam*$^+$ cells. Moreover, the intracellular Dam MTase/OxyR ratio seems to be important in determining the degree of *mom* expression. This competition between DNA methylation and DNA binding by a regulatory protein may be a model for the control of methylation-sensitive genes in higher eukaryotes.

We also tested the possibility that the procedure of thermal induction of Mu prophage at 42°C might inactivate OxyR or increase the Dam methylation rate (and lead to GATC methylation saturation in P_{mom}); either process would favor expression of the *mom* modification. However, Mu grown lytically at 42°C in *oxyR*$^+$*dam*$^+$ cells showed the same EOP on K704(P1) as phage grown lytically at 37°C (data not shown). This ruled out a convenient explanation for the difference between lytic infection and prophage induction in the extent of *mom* modification of progeny phage. Nevertheless, it would be worthwhile to probe the methylation status of the P_{mom} GATC sites in prophage (before and after thermal induction) and lytically grown phage DNAs to determine whether there is, in fact, any difference between them.

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