The Phage Mu Middle Promoter P_m Contains a Partial UP Element

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ABSTRACT There are three phases of transcription during lytic development of bacteriophage Mu: early, middle, and late. Transcription from the middle phase promoter P_m requires the activator protein Mor. In the presence of Mor, transcription from P_m is carried out by the Escherichia coli RNA polymerase holoenzyme containing σ70. A Mor dimer binds to two 5-bp inverted repeats within a 16-bp element centered at −43.5 in P_m, replacing the normal −35 element contacted by RNA polymerase (RNAP). In this study random and targeted mutagenesis of the sequence upstream (−88 to −52) of the Mor binding site was performed to determine whether P_m also contains an UP element for binding of the RNAP α subunit, thereby stimulating transcription. The results demonstrated that mutations upstream of −57 had no effect on P_m activity in vivo, assayed by expression of lacZ fused downstream of a wild-type or mutant P_m. Mutations at positions −57 through −52 led to decreased transcription from P_m, consistent with the presence of an UP element. In DNase I footprinting and gel mobility shift assays, paired mutations at positions −55 and −54 did not affect Mor binding but decreased the synergistic binding of Mor with histidine tagged α (His-α), indicating that His-α binds to P_m in a sequence- and/or structure-specific manner. Taken together, these results demonstrate that P_m has a strong proximal UP element subsite, but lacks a distal subsite.

In Escherichia coli K-12, promoters that are recognized by RNA polymerase (RNAP) containing the “housekeeping sigma factor” σ70 generally consist of a −35 hexamer, −10 hexamer, and a 17+/-1 bp spacer between them (Hawley and McClure 1982). Multiple bases of the consensus sequences for these hexamers are: −35 (TTGACA) and −10 (TATAAT), and increased promoter strength often correlates with a greater number of bases that match these hexamers. There are other well-defined interactions between RNAP and the spacer bases, and mutations in the spacer can decrease promoter activity at some promoters (Siebenlist et al. 1980; Auble et al. 1986).

The UP element, a very A/T-rich region located upstream of the −35 hexamer, is bound by the C-terminal domain of the α subunit (αCTD) of RNAP and increases rrnB P1 promoter activity up to 30-fold (Ross et al. 1993). At least one-third of stable RNA promoters and 4% of mRNA promoters may contain such UP elements (Estrem et al. 1999). In addition, the number of promoters with at least partial UP elements is even larger, because promoters with less AT-rich UP element-like sequences still exhibit UP-element function (Ross et al. 1998; Estrem et al. 1999). There are two subsites within the UP element: promoter proximal and distal; either can function independently, although the proximal subsite may exert a larger effect on promoter activity. The consensus sequence of the full UP element and its subsites (Figure 1) were identified by in vitro selection and contain mostly A and T tracts (Estrem et al. 1998, 1999). Some promoters contain another element, the extended −10 motif, which has bases T Gn located immediately upstream of the −10 hexamer. This sequence is recognized by σ70 (Keilty and Rosenberg 1987; Chan and Busby 1989) and drives transcription efficiently without a −35 hexamer or activator proteins.

Lytic development of phage Mu occurs with sequential synthesis of proteins: first those involved in replicative transposition, and later morphogenesis, DNA modification, and cell lysis (Marrs and Howe 1990). Phages often orchestrate their development by using phage-encoded activator proteins or sigma factors that are synthesized in and control different stages of gene expression. The Mu activator proteins Mor and C turn on middle and late transcription, respectively (Marrs...
and Howe 1990; Mathee and Howe 1990. The middle promoter, P_m, has a recognizable −10 hexamer but lacks a −35 hexamer and requires Mor to initiate middle transcription (Mathee and Howe 1990). Mor consists of 129 amino acid residues (14.7 kDa) and binds as a dimer to a dyad-symmetrical sequence element in P_m. This 16-bp element contains an inverted repeat of 5 bp each, separated with a G/C-rich hexanucleotide spacer (Artsimovitch and Howe 1996). The element contains an inverted repeat of 5 bp each, separated with a G/C-rich hexanucleotide spacer (Artsimovitch and Howe 1996). This goal of this study was to determine whether the region of P_m upstream of the Mor binding site contained an UP element and to identify the role of this region in P_m activation. We addressed these goals by making mutations in upstream regions of P_m and assaying their effects on promoter activity and binding of Mor and His-α to P_m DNA.

MATERIALS AND METHODS

Media, chemicals, and enzymes

Media used included M9 minimal medium with 0.2% casamino acids, Luria broth (LB), and MacConkey lactose plates with only half the

Table 1 Primers for P_m mutagenesis and sequencing

| Primer | Sequence | Coordinates | Comments |
|--------|----------|-------------|----------|
| JM1    | GAAATACCGCACTACGAGCCTACTCTCTNTTAATANNAGTA | top strand | To introduce mutations at -57 and -52 |
|        | −87 to −48 |            |          |
| JM2    | GAAATACCGCACTACGAGCCTACTCTCTNTTAATANNAGAG | top strand | To introduce mutations at −62, −59 and −57 |
|        | −87 to −46 |            |          |
| JM3    | GAAATACCGCACTACGAGCCTACTCTCTNNCNCGNTAATANNAGAG | top strand | To introduce mutations at −63, −61, −60 and −58 |
|        | −87 to −46 |            |          |
| JM4    | GAAATACCGCACTACGAGCCTACTCTCTNNCNCGNTAATANNAGAG | top strand | To introduce mutations at position |
|        | −87 to −57 |            | −69 to −62 |
| JM5    | GAAATACCGCACTACGAGCCTACTCTCTNTTAATANNAGTA | top strand | To introduce mutations at positions |
|        | −87 to −57 | −57 to −52 |          |
| JM7    | GTCTAGTCTTCTCTGTGTA | bottom strand | Downstream of BamHI, for sequencing |
| JM39   | GAAATACCGCACTACGAGCCTACTCTCTTAATNNANAC | top strand | To introduce mutations −56TA |
|        | −87 to −52 |            | (T to A) and −57GA G to A |
| JM40   | GAAATACCGCACTACGAGCCTACTCTCTTTSSACAGTA | top strand | To introduce mutations at −55 and −54 |
|        | −87 to −47 |            |          |
| JM41   | GAAATACCGCACTACGAGCCTACTCTCTCTTAATANNAGTA | top strand | To introduce mutations −68CA, −67CA, −66CA, −64CA, −55AC and −54AC |
|        | −87 to −49 |            |          |
| MEG4   | GGGCAATCCTATGCGACGGCTGAAATACGGCCAGT | top strand | for cloning with EcoRI site near 5’ end |
|        | −98 to −74 |            |          |
| MEG5   | GGGCAATCCTATGCGACGGCTGAAATACGGCCAGT | top strand | AT-rich from −88 to −79 with EcoRI |
|        | −98 to −74 |            | site near 5’ end |
| MEG6   | GGGCAATCCTATGCGACGGCTGAAATACGGCCAGT | top strand | GC-rich from −88 to −79 with EcoRI |
|        | −98 to −74 |            | site near 5’ end |
| MEG7   | GGGCAATCCTATGCGACGGCTGAAATACGGCCAGT | top strand | AT-rich from −78 to −69 with EcoRI |
|        | −98 to −63 |            | site near 5’ end |
| MEG8   | GGGCAATCCTATGCGACGGCTGAAATACGGCCAGT | top strand | GC-rich from −69 to −78 with EcoRI |
|        | −98 to −63 |            | site near 5’ end |
| MLK7   | CTTGGATCCTGACGGTATTCCATCACAG | bottom strand | For cloning with BamHI site near 5’ end |
|        | +10 to −9 |            |          |
| IR21   | TGGGGATCGGAATTACGT | top strand | Upstream of EcoRI, for sequencing |
|        |            |            | P_m mutants |
| IR22   | AACAGGGGCTCGTGGGATT | bottom strand | Downstream of BamHI, for sequencing |
|        |            |            | P_m mutants |

Notes:

a. Coordinates indicate the base locations in the P_m sequence relative to the transcription start site as +1.
b. N indicates an equal molar mixture of all four bases (A1T1G1C1).
c. When specific base changes were made, such as −56TA, the first base is the wild-type (T) and the second is the mutant base (A) that was substituted.
d. N indicates an equal molar mixture of G+C bases.
e. Restriction sites for cloning are underlined.
f. S indicates AT-rich primer synthesis corresponding to A2T2G1C1 or GC-rich primer synthesis corresponding to A1T1G2C2.
normal amount of lactose. Sources for ampicillin (Ap), chloramphenicol (Cm), isopropyl β-D-1-thiogalactopyranoside (IPTG), α-naphthyl β-galactoside, agarose, and radiolabeled [γ-32P]ATP (3000 Ci/mmol) are in the article by Mo and Howe (2014). Sources of enzymes used are in that same paper. Protein gel reagents were from Bio-Rad Laboratories. Oligonucleotides (Table 1) were synthesized by Integrated DNA Technologies, Inc. The N-terminal hexa-histidine tagged a subunit of RNAP(His-)

Bacterial strains

Strains used for phenotypic assays of Pm activity are derivatives of E. coli K-12 strain MH13312 [mcrA ΔproAB-lacZ uvrA endA hisD30 recA/F λ supE44 recA/F (pro+ lacI3 ΔlacZ58)] (Artsimovitch and Howe 1996). Strains MH13335, MH13339, and MH15001 are derivatives of MH13312 containing just pKM78 (MH13315) (Artsimovitch and Howe 1996), both pKM78 and pIA12 (MH13339) (Artsimovitch and Howe 1996), and both pKM78 and pPM1 (MH15001) (Mitchell and Howe, unpublished data). Strain RLGS3538 [F−ompT ΔhisS (rP + mE32) gal dcm ΔDE3/ plLysS/pHTT7f1-NH6] contains plasmid pHTT7f1-NH6 encoding His-α in an E. coli B strain BL21 background (Gaal et al. 1996). Strain MH10713 [ompT ΔhisS (rP + mE32) gal dcm ΔDE3/pLysS], the Howe lab version of strain BL21 with ΔDE3 and pLysS (Studier and Moffat 1986), was transformed with plasmid pKM90 (Mathee and Howe 1993) to create strain MH13231 (Ma and Howe 2004) used for overproducing Mor protein. The ΔDE3 in strains MH10713 and RLGS3538 contains the T7 RNA polymerase gene under the control of the IPTG-inducible PlacUV5 (Yanisch-Perron et al. 1985; Bachmann 1987).

Plasmids and plasmid construction

The promoter cloning plasmid, pIA12 (Figure 2), contains a HindIII-EcoRI-BamHI linker upstream from a promoter-less lacZ gene (Chiang and Howe 1993; Artsimovitch and Howe 1996). Plasmid pPM1 contains Pm sequence from −85 to +10 cloned into the EcoRI and BamHI sites in pIA12 (Chiang et al. 1993). Plasmid pKM78 (Figure 2) expresses Mor under the control of PlacUV5 (Mathee and Howe 1990). Plasmid pKM90 contains the mor gene downstream of the strong T7 promoter and ribosome binding site in pT7-7 (Tabor and Richardson 1985; Mathee and Howe 1993; Ma and Howe 2004). Plasmid pHTT7f1-NH6 contains an rpoA gene encoding the N-terminal His6-tagged α subunit of E. coli RNA polymerase (Tang et al. 1994).

Generation of Pm mutants

Multiple mutations were introduced into Pm using polymerase chain reaction (PCR) primers containing degeneracy in specific targetted positions (Table 1) (Chiang and Howe 1993; Chiang et al. 1993). One set of primers was designed to be degenerate, using all four (N) bases in equal molar amounts (A1T1G1C1) during oligonucleotide synthesis. For synthesis of a second set of primers, the N mixture was supplemented with extra A+T (A1T1G1C1) or G+C (A1T1G1C1) to produce primers that were AT-rich or GC-rich. Incorporation rates for all the primers were approximately 0.5/nucleotide or 0.25/nucleotide at each position.

Figure 3 Regional mutagenesis of Pm sequence upstream of the Mor binding site. The Pm promoter sequence is shown from −103 to +11 with respect to the transcription initiation site as +1. Dots above the sequence indicate 10-bp intervals that are numbered by their position in the promoter with respect to +1. The −10 sequence is in a box and +1 is indicated by the bent arrow. The bold lines above the sequence indicate the positions of the DNase I footprints for Mor and for the αCTD. The short inverted arrows above the sequence identify the locations of the 5-bp inverted repeats of the Mor binding site. The long thin arrows below the sequence show the Pm bases present in the primers used for mutagenesis. The bold lines below the sequence show the regions I-V that were mutagenized.
Two sequential PCR amplifications were performed. The first used wild-type (WT in Figures, Legends, and Tables) primer MLK7 and one degenerate primer (JM1-JM6 or M40 or MEG5-EG8) to create templates for the second PCR with wild-type primers MEG4 and MLK7, which contained EcoRI (MEG4) or BamHI (MLK7) restriction sites near their 5’ ends. The resulting PCR products were digested with EcoRI and BamHI and cloned into the EcoRI and BamHI sites in the promoter-less vector pIA12. The ligation mixtures were transformed into MH13335, which contains pKM78, with P$_\text{lacUV5-mor}$.

**Determination of MacConkey plate phenotypes**

Transformants were plated on LB plates containing Ap and Cm, and single colonies were picked and stabbed in parallel onto LB plates and MacConkey lactose plates with 0.01, 0.1, and 1 mM IPTG. After incubation at 32°C for 12 hr and/or 24 hr, the colony color was scored relative to that of MH15001 (WT P$_\text{m}$ positive control) and MH13339 (no P$_\text{m}$ negative control) on the same plate. Mutants that were red with less IPTG than that required for the wild-type were scored as Up, whereas mutants which required more IPTG than that needed for the wild-type to become red were scored as Down.

**In vivo transcription activation assays**

Strains grown overnight in LB with Ap and Cm were inoculated into 3 mL of M9 minimal medium with 0.2% casamino acids at a 1:100 dilution. The cells were grown at 32°C to an $OD_600$ of 0.3–0.5. Then, IPTG was added to a final concentration of 2 mM to induce Mor production, and the cultures were grown at 37°C for 1 hr. Uninduced control cultures were grown in parallel for all samples. Assays for β-galactosidase were carried out as described by Miller (1972) with minor modifications (Chiang and Howe 1993; Kumaraswami et al. 2004). The β-galactosidase activity was calculated in Miller units (Miller 1972) and normalized to the activity of a wild-type culture, which was assayed in parallel, and set to 1000 units. Enzymatic assays were performed in duplicate in each of three independent experiments, and the values were averaged to generate the activity reported.

**DNA binding assays**

Radiolabeled probes used in gel retardation and footprinting assays were prepared by PCR as described previously (Ma and Howe 2004). They consisted of 198-bp probes containing P$_\text{m}$ sequence from −98 to +10 and were generated by PCR using flanking vector primers IRI21 and IRI22, one of which was 32P-labeled. Plasmids containing wild-type (pMM1) or mutant P$_\text{m}$ sequences were used as templates. The probes were purified using a Qiagen spin purification kit and the DNA concentration estimated by comparison to a low-DNA-mass ladder.

Gel mobility shift assays for Mor binding were performed as described by Ma and Howe (2004) except that approximately half (∼0.38 nM) the prior amount of DNA probe was used. Mor protein was diluted into binding buffer on ice immediately before use, then added to the binding reaction. The mixture was incubated for 15 min at 30°C just before being subjected to native polyacrylamide gel electrophoresis. Gels were exposed to X-OMAT AR film (Kodak) without drying.

Gel mobility shift and DNase I footprinting assays using both Mor and N-terminal hexa-histidine-tagged α were performed as described previously (Ma and Howe 2004). The His-α protein was added after preincubation of the DNA probes with Mor for 10 min at 30°C, and incubation was continued for an additional 10 min at 30°C before polyacrylamide gel electrophoresis.

**RESULTS**

**Isolation of P$_\text{m}$ mutants**

The goal of this study was to determine whether P$_\text{m}$ contains an UP element. Our approach was to isolate and characterize P$_\text{m}$ mutants with base changes in the region upstream of the Mor binding site. The mutagenesis strategy was to divide the P$_\text{m}$ sequence upstream of the Mor binding site into multiple regions based on their GC-content, helix face, and proximity to the Mor binding site (Figure 3). Mutagenesis of each region was performed separately using degenerate oligonucleotide primers (Table 1). For regions I (−88 to −79) and II (−78 to −69), the primers were designed to change the targeted region to be more A/T- or G/C-rich. For regions III (−69 to −62), IV (−63 to −57), and V (−57 to −52), a random mixture of all four bases was used at targeted positions. PCRs were performed first with the degenerate oligonucleotide primers and an opposing wild-type primer (MLK7) to produce a template for a second PCR with overlapping wild-type primers (MEG4 and MLK7). This second PCR completed the promoter sequence and provided the EcoRI and BamHI restriction sites for cloning. The PCR products were cloned between the EcoRI and BamHI sites in the promoter-less lacZ fusion vector pIA12 (Figure 2) just upstream of the lacZ gene. The ligation mix was transformed into strain MH13335 containing the activator plasmid pKM78, which expresses Mor when induced by IPTG (Figure 2).

The transcription activities of the mutant promoters were examined by color development of each transformant on MacConkey lactose plates with different concentrations of IPTG, and scored as Up, Down, or wild-type relative to the color developed by the control strain (MH 15001) containing a plasmid with wild-type P$_\text{m}$ on the same plate.

Mutagenesis of P$_\text{m}$ sequences for regions I through IV resulted in transformant populations with a mixture of wild-type, Up, or Down...
Table 3: Promoter activity* of Pm upstream mutants with mutations in region V

| Strain | -57 | -56 | -55 | -54 | -53 | -52 | β-gal Activity* | SD |
|--------|-----|-----|-----|-----|-----|-----|-----------------|----|
| A      |     |     |     |     |     |     |                 |    |
| WT     | G   | T   | A   | A   | C   |     | 1000            |    |
| JM1-1  | C   | A   | A   |     |     |     | 536             | 51 |
| JM1-7  | A   | A   |     |     |     |     | 589             | 69 |
| JM1-14 | A   |     |     |     |     |     | 414             | 57 |
| JM1-2  | A   | T   |     |     |     |     | 550             | 116|
| JM1-3  | T   |     |     |     |     |     | 407             | 89 |
| JM1-5  | T   | T   |     |     |     |     | 642             | 73 |
| JM1-6  | T   |     |     |     |     |     | 434             | 65 |
| JM1-11 | C   | T   |     |     |     |     | 696             | 50 |
| JM1-16 | T   | G   |     |     |     |     | 856             | 63 |
| JM1-18 | G   |     |     |     |     |     | 892             | 69 |
| JM1-20 | C   | G   |     |     |     |     | 1003            | 87 |
| JM1-21 | A   | G   |     |     |     |     | 924             | 94 |
| JM1-25 | C   |     |     |     |     |     | 879             | 64 |
| B      |     |     |     |     |     |     |                 |    |
| WT     | G   | T   | A   | A   | C   |     | 1000            |    |
| JM6-103| G   |     |     |     |     |     | 1015            | 66 |
| JM6-12 | A   | G   | T   | T   |     |     | 416             | 82 |
| JM6-25 | C   | A   | G   | T   | C   |     | 473             | 54 |
| JM6-105| A   | G   | G   | T   | C   |     | 64              | 22 |
| JM6-104| T   | A   | G   | T   | C   |     | 67              | 16 |
| JM6-59 | A   | C   | C   |     |     |     | 247             | 78 |
| JM6-4  | A   | G   | G   |     |     |     | 538             | 77 |
| JM6-23 | T   | C   | G   |     |     |     | 421             | 80 |
| JM6-33 | G   | G   | G   |     |     |     | 338             | 74 |
| JM6-108| G   | G   | T   | G   |     |     | 245             | 96 |
| JM6-24 | T   | C   | G   | T   | G   |     | 494             | 62 |
| JM6-73 | A   | A   | G   | C   | T   |     | 273             | 66 |
| JM6-95 | C   | A   | G   | T   | C   |     | 287             | 53 |
| JM6-34 | A   | T   | C   |     |     |     | 390             | 50 |
| JM6-86 | T   | C   | C   |     |     |     | 456             | 117|
| JM6-70 | T   | C   | T   | G   |     |     | 358             | 119|
| JM6-1  | C   | G   | G   | T   | G   |     | 306             | 96 |
| JM6-26 | A   | T   | G   | T   | G   |     | 350             | 81 |
| JM6-10 | G   | G   | T   | G   |     |     | 415             | 42 |
| JM6-17 | T   | G   | T   | G   |     |     | 451             | 94 |
| JM6-5  | T   | T   | G   |     |     |     | 402             | 31 |
| JM6-87 | G   | G   | G   |     |     |     | 731             | 32 |
| JM6-22 | C   | G   | G   |     |     |     | 362             | 84 |
| JM6-14 | G   | G   | G   |     |     |     | 430             | 81 |
| JM6-35 | A   | C   | C   | G   | G   |     | 442             | 43 |
| JM6-88 | C   | G   | G   |     |     |     | 495             | 168|
| C      |     |     |     |     |     |     |                 |    |
| WT     | G   | T   | A   | A   | C   |     | 1000            |    |
| JM6-11 | T   | A   | T   | C   | G   |     | 367             | 141|
| JM6-36 | C   | T   | T   |     |     |     | 398             | 61 |
| JM6-80 | A   | A   | G   | C   | T   |     | 191             | 18 |
| JM6-16 | A   | T   | G   |     |     |     | 414             | 5   |
| JM6-21 | G   | T   | G   |     |     |     | 302             | 82 |
| JM6-2  | A   | G   | G   |     |     |     | 349             | 129|
| JM6-7  | G   | T   |     |     |     |     | 518             | 80 |
| JM6-15 | G   | T   |     |     |     |     | 542             | 114|
| JM6-90 | A   | G   | T   | T   |     |     | 593             | 107|
| JM6-106| G   | G   | T   | C   | T   |     | 39              | 11 |
| JM6-8  | T   | G   | G   | T   | A   |     | 356             | 118|
| JM6-6  | C   | G   | T   | T   | G   |     | 530             | 72 |
| JM6-93 | G   | T   | A   |     |     |     | 837             | 143|

* Promoter activity was assayed by determining β-galactosidase (β-gal) levels in liquid cultures after induction of Mor synthesis in cells containing the Pm-lacZ fusions. Assays were performed in duplicate in at least three experiments for each mutant in parallel with the wild-type (WT) strain MH15001. In each experiment the activities for the mutants were normalized relative to the closest

.. The results for strains from different experiments A, B, and C are separated by blank rows.

Analysis of Pm mutants by gel mobility shift and DNase I footprinting assays

Because mutations close to the Mor binding site (−51 to −36) could affect Pm activity by altering Mor binding, gel mobility shift assays were performed to examine Mor binding to mutant Pm DNA fragments. All of the mutants had substantial Mor binding as shown in the representative gels presented in Figure 4.

Previous DNase I footprinting analysis showed that Mor alone protected Pm positions from −56 to −33 from digestion by DNase I (Artsimovitch et al. 1996; Ma and Howe 1996; Ma and Howe 2004). Addition of RNA polymerase led to an extension of the footprint to +14 and produced a new footprint from −61 to −59 upstream of the Mor-binding site (Artsimovitch et al. 1996; Ma and Howe 2004). Addition of α or the αCTD instead of RNA polymerase resulted in the same −61 to −59 footprint, demonstrating that this footprint is caused by αCTD binding (Ma and Howe 2004). To

WT row above them, which was set to 1000. The activity shown here is the average of the results from those three experiments.

SD represents standard deviation.

The results for the representative mutants in all five regions by performing in vivo liquid β-galactosidase assays. Mutants with promoter activity in the range of 80–120% of the wild-type were grouped with the wild-type, because that level of variability could be found with independent assays of a single culture (Chiang and Howe 1993). The results were completely consistent with those from the MacConkey plate assays. Mutants with mutations in regions I through IV had wild-type activity. Mutants with mutations in region V had a range of β-galactosidase activities, most exhibiting only 10–50% of the wild-type activity.

Quantitative analysis of promoter activity was determined for representative mutants in all five regions by performing in vivo liquid β-galactosidase assays. Mutants with promoter activity in the range of 80–120% of the wild-type were grouped with the wild-type, because that level of variability could be found with independent assays of a single culture (Chiang and Howe 1993). The results were completely consistent with those from the MacConkey plate assays. Mutants with mutations in regions I through IV had wild-type activity. Mutants with mutations in region V had a range of β-galactosidase activities, most exhibiting only 10–50% of the wild-type activity.

Sequence and promoter activity of mutants with mutations in region V (−57 to −52) are shown in Table 3; note the grouping of bases at −52 and −53. These results demonstrated the following: (1) Substitutions of C at position −52 by A or T resulted in an approximately 50% reduction in Pm activity, whereas substitution of C by G did not significantly affect promoter activity. (2) In mutants with C or G at −52, all three possible substitutions of the base at position −57 had little or no effect on Pm activity. (3) Because mutants with wild-type bases at both −57 and −52 had greatly reduced activity, we conclude that one or more bases at positions −56 to −53 are important for promoter activity.
examine whether mutations in region V affected αCTD binding, we performed DNase I footprinting of the mutant promoters with Mor and RNA polymerase. Open complexes formed by Mor plus RNAP have a different footprint from those produced using α or the αCTD; therefore, these assays were done at 15°C to prevent open complex formation. In these experiments saturating or almost saturating concentrations of Mor protein (860 nM) were used, and each DNA probe assayed contained a Mor-only lane for comparison.

The footprinting patterns of Mor alone with wild-type or mutant DNA probes (Figure 5) were the same as observed previously (Artsimovitch et al. 1996; Ma and Howe 2004). Reactions with RNA polymerase alone produced little or no protection but did exhibit hypersensitive bands at positions −51 and −12, demonstrating the presence of unstable or weak interactions between RNAP and Pm DNA, as observed previously by Mo and Howe (2014). The upstream mutations had no effect on the −12 hypersensitivity, presumably because it reflects interaction of RNAP with the −10 hexamer. In contrast, none of the mutant probes showed the same high degree of hypersensitivity at band −51. There are two possible explanations: first, the interactions between RNAP and promoter DNA was affected by the mutations, leading to differences in the footprint patterns or second, the local structure of the DNA was changed by the mutations, affecting the accessibility of DNase I to the DNA, thereby altering the footprint pattern. When both Mor and RNA polymerase were present, the −61 to −59 region of Pm was protected. With increasing concentrations of RNA polymerase, this protection became more complete. At the same time, a very weak protection was observed extending downstream to −4. Protection to −4 is characteristic of a closed complex in which RNAP binding is unstable.

### Pm mutants containing mutations at selected sites

The aforementioned DNase I footprinting assays using Pm, region V mutants affected the degree of protection from −61 to −59 but did not indicate which bases were most important. To address this
question, we made several additional mutants containing specific targeted mutations. Because mutations of adenines at positions −41, −42, and −43 in the rrrB P1 UP element proximal subsite, corresponding to adenines at positions −53, −54, and −55 at Pm, led to dramatic decreases in promoter activity (Estrem et al. 1999; Ross et al. 2001), and to avoid mutating positions that might lead to decreased Mor binding (−52, −53), we made five new plasmids containing mutations upstream of −53.

Plasmid pJM7 contains mutations −57GA and −56TA, forming an A5 tract extending from positions −57 to −53, matching the preferred binding sequence of the αCTD. Plasmids pJM8, pJM9, and pJM10 all contain mutations at positions −55 and −54, where both As were changed to G or C, mutations expected to disrupt αCTD-DNA interactions. In addition to mutations at −55 and −54, plasmid pJM11 contains mutations of Cs to As at positions located one helical turn upstream from the present αCTD-binding site and expected to create a good distal αCTD-binding site.

Activity assay in vivo
Plasmids pJM7-pJM11 were transformed into strain MH13335, and liquid β-galactosidase assays were performed to quantitate the activities of the mutant promoters. A summary of the results is shown in Table 4, which also contains data for several of the earlier plasmids with multiple mutations for comparison. Mutation of bases at positions −56 and −57 in pJM7 to form an A5 tract did not significantly increase or decrease promoter activity, suggesting that the wild-type Pm sequence is already strong. The three mutants (pJM8, 9, 10) containing G or C mutations at both positions −55 and −54 in the native A3 tract showed substantial reductions in Pm activity to 22%, 46%, and 19% of wild-type, respectively. The β-galactosidase activities expressed from previous mutants JM6-73, JM6-80, and JM6-95 are similar to that from pJM8, suggesting that the two bases −55AG and −54AC are the most important in reducing Pm activity. The mutants JM6-1, JM6-22, and JM6-25 share mutations −55AG and −54AC with pJM9 and had similar promoter activities, with only modest effects from their additional mutations. In contrast, the mutant pJM7 with substitutions −57GA and −56TA had an activity similar to WT, and the same two substitutions in mutants JM6-73 and JM6-80 were not able to compensate for the deleterious effects of their additional mutations. The mutant pJM11 containing mutations that moved the αCTD-binding site one helical turn upstream had the largest defect, making only 15% of wild-type activity, implying that the location of the αCTD-binding site in Pm is important, possibly because the αCTD requires direct interaction with Mor.

DNase I footprinting and gel mobility shift assays with promoter DNA from plasmids pJM7 to pJM11

The mutations in pJM7-pJM11 were designed not to affect Mor binding. As expected, gel mobility shift assays with these mutants showed wild-type levels of Mor binding (data not shown), allowing DNase I footprinting assays to be carried out to examine His-α binding without different levels of Mor binding as a confounding variable. The DNase I footprinting pattern of the pJM7 probe, containing mutations −57GA and −56TA and making almost wild-type levels of β-galactosidase was very similar to that of the wild-type probe (Figure 6A); a slight footprint at position −59 and a more pronounced footprint at −61 were generated when Mor and His-α were both added. The pJM8 and pJM10 probes containing −55AG and −54AC mutations (pJM8) or −55AG and −54AC mutations (pJM10) showed little or no footprint at −59 and −61, indicating a substantial reduction in His-α binding (Figure 6B). These results are consistent with the substantially lower β-galactosidase activities of these mutant plasmids. The probe pJM9 containing −55AG and −54AC mutations showed a His-α binding ability intermediate between that of the wild-type probe and that of pJM8 and pJM10, consistent with its in vivo transactivation assay which was slightly less than half of the wild-type β-galactosidase activity. When pJM11 was assayed, it had no −61 band, presumably due to the multiple mutations it carries, and showed no changes in band pattern beyond those caused by Mor binding, providing no evidence for or against His-α interaction with the DNA.

To examine the effects of the pJM7-pJM11 mutations on synergistic binding of His-α and Mor to Pm, we carried out gel mobility shift assays with purified His-α and Mor (Figure 7). When wild-type probes were used, no shift was observed for His-α alone even at a high concentration of His-α (2.7 µM), and only one-third to one-half of the probe was shifted with Mor alone. In contrast, when both proteins were added, the wild-type DNA probes were almost completely found in a supershifted species corresponding to a Mor-His-α-DNA ternary complex, even at a low concentration of His-α (1.3 µM). When mutant probes were used, different shifting behaviors were observed. Probe containing mutations at positions −57 and −56 (pJM7) gave a shift similar to the wild-type probe, consistent with its wild-type β-galactosidase activity. When probes containing −55AG and −54AC mutations
(pJM10 and pJM11) were used, only a binary complex of Mor and probe was observed, even at a greater concentration of His-α (2.7 μM), suggesting that these mutations disrupted interactions between the αCTD and DNA, which is consistent with their very low β-galactosidase activities. The probe containing −55AG and −54AC mutations (pJM8) showed reduced synergistic binding, relative to the wild-type probe. At the lower concentration of His-α (1.3 μM), there was no supershifted complex, and when more His-α (2.7 μM) was used, only a small fraction of the probe was seen in a supershifted complex. The probe containing −55AG and −54AG mutations (pJM9) showed a shifting ability intermediate between the wild-type probe and the pJM10 and pJM11 probes containing A to C substitutions at these positions. For pJM9, at 1.3 μM His-α, both binary and ternary complexes were observed and when a higher concentration of His-α (2.7 μM) was used, more than half of the probe was seen in the supershifted species.

**DISCUSSION**

This study demonstrated that the DNA sequence in the region immediately upstream of the Mor-binding site in Pm had a substantial influence on promoter activity and α DNA binding, both characteristics of an UP element. When mutations designed to destroy an UP element were used, i.e., substitutions of −55A and −54A by cytosines (pJM10, Figure 7), there was a more than 80% decrease in Pm activity and extremely decreased binding of α. Interactions between α (i.e., the αCTD) and DNA can be sequence-dependent or sequence-independent (Ross et al. 1993; Busby and Ebright 1999; Gourse et al. 2000). At promoters containing strong UP elements, for example, the rrnB P1 promoter, α binds to DNA in the absence of activators, and mutation of these high-affinity DNA sequences reduces αCTD binding. At other promoters, α binds to DNA only in the presence of activators. At these promoters, the interaction between α and DNA is only partially sequence-dependent, and the binding site for α is usually located adjacent to the activators (Flatow et al. 1996; Czarniecki et al. 1997). At Pm, α binds just upstream of the Mor-binding site, as it does at the consensus promoter CC (−41.5), the best-characterized Class II CAP-dependent promoter (Savery et al. 1998). When this α-DNA interaction was disrupted by mutations −55AC and −54AC, α binding to Pm was undetectable even in the presence of Mor (pJM10 in Figure 7). When
Thus, interactions between Mor and α are mutually stabilized by the interactions between α and the promoter DNA. Full activation of Pm most likely requires Mor-α interactions as well as Mor-DNA and α-DNA interactions.

These results led us to conclude that the bases immediately upstream of the Mor binding site constitute a promoter-proximal subsite of an UP element, but these sequences lack a promoter-distal subsite.

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Figure 7 Gel retardation analysis of His-α and Mor binding to WT and mutant Pm DNA. Radiolabeled DNA probes (~0.4 nM) containing Pm sequence –98 to +10 were incubated with 136 nM Mor or 2.7 μM (+) His-α for 10 min at 30°C. When both proteins were used, Mor was incubated with the probe for 10 min, His-α was added to a final concentration of 1.3 μM (+) or 2.7 μM (+ +), and a second incubation for 10 min at 30°C was performed. Binding mixtures were then loaded onto 4% acrylamide native gels containing 5% glycerol and 0.5x Tris/Borate/EDTA (TBE) buffer and subjected to electrophoresis in 0.5x TBE buffer containing 2% glycerol at 3°C. The name of each mutant plasmid template and the mutations in each is given above each bracket. The positions of free probe, Mor-DNA binary complex, and Mor-His-α-DNA ternary complexes are indicated with F, B, and T, respectively. Note the unusual behavior of the ternary complex: the greater the His-α concentration, the slower the mobility of the complex. This behavior is characteristic of α whether or not it is tagged. In fact, when multiple concentrations are used, each increased concentration yields a slower band (Savery et al. 1998). An explanation for this behavior has not been determined.
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