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Intrinsic DNA Distortion of the Bacteriophage Mu *mom*P1 Promoter Is a Negative Regulator of Its Transcription

A NOVEL MODE OF REGULATION OF TOXIC GENE EXPRESSION*

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The *mom*P1 promoter of the bacteriophage Mu *mom* operon is an example of a weak promoter. It contains a 19-base pair suboptimal spacer between the −35 (ACCACA) and −10 (TAGAAT) hexamers. *Escherichia coli* RNA polymerase is unable to bind to *mom*P1 on its own. DNA distortion caused by the presence of a run of six T nucleotides overlapping the 5′ end of the −10 element might prevent RNA polymerase from binding to *mom*P1. To investigate the influence of the T_6 run on *mom*P1 expression, defined substitution mutations were introduced by site-directed mutagenesis. *In vitro* probing experiments with copper phenanthroline ($(OP)_2Cu$) and DNase I revealed distinct differences in cleavage patterns among the various mutants; in addition, compared with the wild type, the mutants showed an increase (variable) in *mom*P1 promoter activity *in vivo*. Promoter strength analyses were in agreement with the ability of these mutants to form open complexes as well as to produce *mom*P1-specific transcripts. No significant role is attributed to the overlapping and divergently organized promoter, *mom*P2, in the expression of *mom*P1 activity, as determined by promoter disruption analysis. These data support the view that an intrinsic DNA distortion in the spacer region of *mom*P1 acts in *cis* as a negative element in *mom* operon transcription. This is a novel mechanism of regulation of toxic gene expression.

Optimal activity of bacterial promoters depends on the precise and controlled interactions between the promoter with regulatory proteins and RNA polymerase (RNAP).[1] In many instances, promoter activity is modulated by protein-induced changes in DNA structure such as DNA distortion, looping, bending, and unwinding (1–3). DNA structural distortions are known to influence promoter activity (4, 5). Certain oligo (A/T) tracts exhibit unusual curvature (6) and play an important role in the regulation of transcription initiation (7–11). A different role is attributed to the A tract when it is positioned upstream and in phase with the promoter elements. A number of *Escherichia coli* promoters have A+T-rich tracts (also known as UP elements) upstream to the −35 hexamer of σ^{70} promoters (12, 13). UP elements, when present, are integral components of promoters, because they interact with the carboxy terminal domain of the RNAP α-subunit (12). *In vitro* studies showed that the *E. coli* RNAP (σ^{70}) holoenzyme alone is sufficient for transcriptional activity from several such promoters (12, 14, 15).

The regulatory region of the *mom* operon of bacteriophage Mu, which controls a unique DNA modification function (see Ref. 16 for a recent review), exhibits several interesting features. The promoter, *mom*P1, which directs the transcription of *com-mom* dicistronic mRNA, is a typical example of a weak promoter with a poor −35 (ACCACA) element and a suboptimal spacing of 19 bp between the two consensus elements (Fig. 1*a*). The spacer region of the promoter contains a run of six T nucleotides from −12 to −17. RNAP does not bind to *mom*P1 by itself (17). Instead it binds an overlapping, divergent promoter region, *mom*P2, which brings about "leftward" transcription (18). The stretch of six A nucleotides complementary to the T_6 run appears to be part of an UP element for leftward transcription from *mom*P2 (18).

The regulation of *mom* operon expression occurs at both the transcriptional and translational levels (16). The Mu C protein, a "middle" gene product, is an obligatory transcriptional activator of the *mom*P1 promoter (19, 20), as well as for the other three late promoters (21, 22). C protein binding to a site located at −28 to −57 in the *mom*P1 region (23, 24) brings about an asymmetric distortion and unwinding of the DNA (25–27).

Mutants have been isolated that relieve the dependence of *mom*P1 on C activation. In one such (partially) C-independent mutant, *tin7*, there is a single-base change (T to G at position −14) that disrupts the T_6 run in the *mom*P1 spacer region (17). Few explanations could account for the increased promoter activity of *tin7* mutant: 1) T tract-mediated intrinsic DNA curvature is lost because of disruption of the T_6 run (to T_3GT_2), resulting in RNAP binding to *mom*P1; 2) because P1 and P2 are overlapping divergent promoters, weakening of the UP element of *mom*P2 (18) may facilitate the binding and activity of RNAP at *mom*P1; and 3) the T(−14) to G change converts *mom*P1 to an extended −10 promoter (28). The present study is an attempt to delineate the role of the T_6 run in *mom*P1 promoter activity.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, Primers, Enzymes, and Chemicals—E. coli DH10B was used for generating the different plasmid constructs. *E. coli* LL306 Δ(*pro-lac*) was from L. Lindahl (29). Plasmid pVN184 (+), a C protein-producing construct, has been described earlier (17). The primers used in this study to generate site-directed mutants or synthetic duplexes are available upon request. Restriction and modifying en-

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[1] The abbreviations used are: RNAP, RNA polymerase; bp, base pairs; WT, wild type.

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zymes were purchased from Stratagene, New England Biolabs, and Roche Molecular Biochemicals and were used according to the suppliers' recommendations. DNase I was from Worthington, and *E. coli* DNA polymerase (PolIk) was from New England Biolabs. Superscript reverse transcriptase was purchased from Life Technologies, Inc. Chemicals and other reagents were purchased from Life Technologies, Inc. and Sigma. Primers were synthesized by Bangalore Genei (Pvt.) Ltd. (Bangalore, India), Life Technologies, Inc., and the University of Rochester Core Lab Facility. [γ-^{32}P]ATP (6000 Ci/mmol) was purchased from New England Nuclear. Most of the standard procedures were carried out as described by Sambrook *et al*. (30).

Construction of momP1 and momP2 Mutants—The mutants used in this study were generated by site-directed mutagenesis using either pLW4 (17) or pUW4 (31) as the template DNA. pUW4 was used as template for the polymerase chain reaction-based mutagenesis methods. The mutants, pT2G, pT3G, and pT2GT3G (Fig. 1*b*), were generated by using the Stratagene QuickChange™ site-directed mutagenesis method involving a pair of mutagenic oligonucleotides and *Pfu*I DNA polymerase. The mutant pT3C was generated by using a Promega Gene Editor site-directed mutagenesis kit. pT1C, pT5C, pT4C, pWT-P2, p*tin7*-P2, pT2GT3G-P2, and pG21C were generated by using the modified mega primer method. In this method, a mega primer was first generated using the pUC reverse primer and the mutagenic oligonucleotide (as described in Ref. 32). The mega primer was then used in the Stratagene QuickChange™ site-directed mutagenesis method. All of the mutants generated in the pUW4 background were subcloned into pLW4 using E*co*RI and B*am*HI restriction enzymes to generate the promoter mutants as *lacZ* transcriptional fusions. All of the mutants generated were confirmed by carrying out Sanger's dideoxy method of sequencing (30).

The promoter expression plasmid pLC1 (22) was generously provided by Dr. M. M. Howe; it contains an *Eco*RI-*Sma*I-*Bam*HI linker upstream of a promoterless *lacZ* gene. Plasmid pLO1 was created by cloning the smaller *Pst*I-*Bam*HI fragment from pLC1 into pRSGC3 *Sma*I (a derivative of phagemid pGC1; Ref. 33). A synthetic duplex containing either *mom*P1 or *mom*P2 was generated by annealing pairs of appropriate synthetic oligodeoxynucleotides that had appropriately located 5′ *Eco*RI and 5′ *Bam*HI single-strand overhangs. Plasmids pLO1/P1 and pLO1/P2 were constructed by ligating the synthetic duplexes into the *Eco*RI and *Bam*HI sites, respectively, of pLO1 and were used for generating site-directed mutations in *momP1* and *momP2,* respectively. After DNA sequencing confirmed the nature of each mutation, the *mom* promoter-containing *Pst*I-*Bam*HI fragment was cloned into the corresponding sites in pLC1 for promoter expression analyses (additional details of the plasmid and mutant constructions are available upon request).

Promoter Strength Analysis—Isolated colonies of *E. coli* DH10B cells harboring either a promoter mutant plasmid alone or with plasmid pVN184 were inoculated into LB broth containing 100 μg/ml of ampicillin (for mutant promoter plasmids alone) or ampicillin and 25 μg/ml chloramphenicol (both plasmids present); the cultures were incubated at 37 °C for ~16 h with vigorous shaking. The overnight cultures were diluted 100-fold into 3 ml of fresh medium in duplicate tubes and incubated at 37 °C till the cultures reached an A_{600} of 0.3–0.7. The samples were then placed on ice. β-Galactosidase activity in SDS-$CHCl_3$-treated cells was determined as described by Miller (34). In the experiments with plasmid pLC1 constructs, β-galactosidase assays were carried out with exponential cultures grown from isolated colonies. The values in the tables are the averages from at least two separate experiments, and replicate assays were done on each culture. The variation was 10–20% around the mean value.

DNase I and (OP)$_2$Cu Cleavage Reactions—2.0 μg (~0.36 pmol) of negatively supercoiled DNA was incubated with DNase I (final concentration, 0.1 ng/μl) in presence of buffer (20 mM Tris-HCl, pH 7.2, 1 mM EDTA, 5 mM $MgCl_2$, and 50 mM NaCl) in a total reaction volume of 20 μl. After 30 s at 22 °C, the reaction was terminated by addition of 20 μl of stop buffer (0.1 M Tris-HCl, pH 7.5, 25 mM EDTA, and 0.5% SDS). The sample volume was made to be 400 μl with water and extracted successively with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1) and then precipitated with 2.5 volumes of 100% (v/v) ethanol in the presence of glycogen as a carrier. Primer extension protocol is adapted from Gralla (35). The extension reactions were performed with end-labeled *mom* forward and reverse primers as previously described (27).

For the (OP)$_2$Cu cleavage reaction, 2.0 μg of negatively supercoiled DNA was incubated with a 10-μl sample of 4 mM 1,10-phenanthroline, 0.3 mM $CuSO_4$, and 10 μl of 58 mM 3-mercaptopropionic acid on ice for 1 min. Reactions were quenched by adding 7.0 μl of 100 mM 2,9-dimethyl-1,10-phenanthroline; the samples were then deproteinized by phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1) extractions, and the DNA was precipitated with 2.5 volumes of 100% (v/v) ethanol. The DNA was used for primer extension with end-labeled *mom* forward and *mom* reverse primers.

In Vivo KMnO$_4$ Footprinting Reaction—*In vivo* $KMnO_4$ footprinting reaction was carried out as described by Sasse-Dwight and Gralla (36). *E. coli* DH10B cells harboring a *mom*P1 promoter mutant plasmid alone or along with pVN184 were grown to A_{600} 0.6 in 4.0 ml of LB broth. The cultures were treated with 200 μg/ml rifampicin for 20 min. The samples were then incubated with 30 mM $KMnO_4$ for 2 min. Reactions were stopped by transferring the cultures to prechilled tubes. The cells were harvested, and plasmid DNA was isolated. Primer extension reactions were carried out as described above.

Total RNA Isolation and Primer Extension—Total RNA was isolated from *E. coli* DH10B cells harboring the various promoter mutant plasmids using the hot acid phenol method. Primer extension was carried out as per the manufacturer's protocol (Life Technologies, Inc.) using superscript reverse transcriptase and end-labeled *mom* forward (for *mom*P2 transcript detection) and reverse (for *mom*P1 transcript detection) primers. An end-labeled primer annealing 150 bases downstream of the ampicillin transcription +1 start site was used to normalize the levels of transcripts produced in the different mutant promoter constructs. Scanning of the autoradiographs was carried out using a Bio-Rad GS710 Calibrated Imaging Densitometer. Quantification was done using Quantity One software.

RESULTS

DNA Structure Analysis of T$_6$ Run Mutants—The variation in helical structure of the DNA depends on base sequence. Specific sequences contribute to alterations in groove width and DNA curvature (37). In addition to their use in probing DNA-protein interactions, nucleases are often used to detect distortions in DNA. Cleavage reaction of orthophenanthroline cuprous complex ((OP)$_2$Cu) depends on the local DNA structure rather than the base sequences as demonstrated previously by Spassky *et al.* (38). We used (OP)$_2$Cu to probe possible structural or conformational differences between the wild type (WT) and the *tin7* mutant in the region of the T$_6$ tract in *mom*P1 (Fig. 1). Negatively supercoiled plasmid DNA harboring the WT or *tin7* mutant *mom*P1 promoter was subjected to *in vitro* single hit cleavage, and the sensitivity pattern was assessed by primer extension analysis (Experimental Procedures). The results of a typical (OP)$_2$Cu footprinting are shown in Fig. 2 (*a* and *b*). The sensitivity patterns of both the top and the bottom strands in the region containing the T$_6$ run were different for the two promoters. Several hypersensitive sites are seen in the WT that are not reactive in *tin7*. For example, at −14T (top strand) and at −15A, −16A, −17A, and −18C (bottom strand), the WT was cleaved more often by (OP)$_2$Cu. In contrast, *tin7* DNA was relatively refractory to cleavage by (OP)$_2$Cu at these residues, whereas −10A in the top strand was hypersensitive. We also probed the promoter structure by using DNase I as a footprinting agent. DNase I reaction also revealed substantial differences in cleavage sensitivity patterns (indicated by the *asterisks* in Fig. 2*c*, *lanes 1* and *2*). DNase I cleavage gave rise to two hypersensitive sites, at −9G and −17T (top strand) in the WT promoter, compared with hypersensitive sites at −12T and −13T (in the T$_6$ run) of the *tin7* promoter. These results show that the two promoter regions differ in their susceptibility to nuclease cleavage, indicating that the DNA conformations are different.

Because the T4G (*tin7*) mutant showed a difference in DNA conformation with respect to wild type, the effect of base substitutions at other position in the T$_6$ run were examined. To this end, negatively supercoiled DNAs of various mutants were subjected to *in vitro* cleavage with DNase I. Mutants T2G, T3G, T2GT3G, and T4G (*tin7*) were selected as representatives for this analysis (Fig. 2*c*). The mutants showed hypersensitivity patterns different from one another, as well as from the WT. For example, the top strand residue −14T was cleaved more

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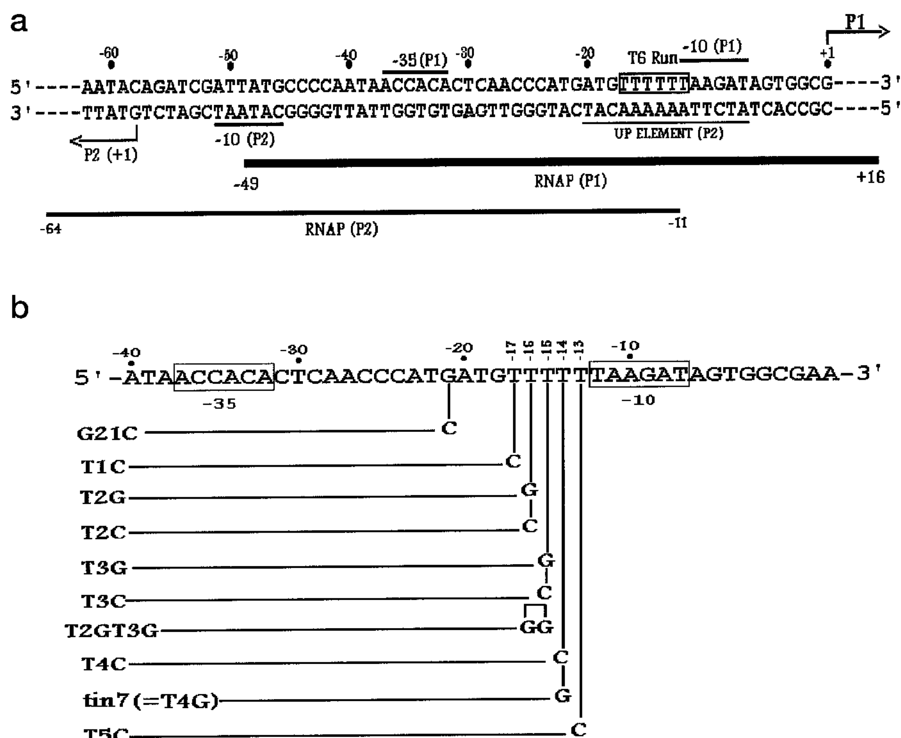

FIG. 1. *a*, regulatory region of bacteriophage Mu *mom* gene. The −10 and −35 elements of *mom*P1 are *overlined* (top strand). The −10 hexamer and the proposed UP element for *mom*P2 are *underlined* (bottom strand). The transcription start sites for both *mom*P1 and *mom*P2 are indicated with *arrows*; the T_6 run (top strand) is enclosed in an *open rectangle*. Regions protected by RNAP in *mom*P1 and *mom*P2 are indicated. *b*, sequence of the *mom*P1 promoter. Substitution mutations in the T_6 run of the spacer region of the *mom*P1 promoter are indicated. The T residues at positions −17 through −12 are designated *T1* through *T6*, respectively.

frequently in T2G, whereas −12T, −14T, and −16T were hypersensitive in T3G, and −12T and −14T were hypersensitive in T2GT3G. The narrower minor groove of the A/T tract is altered by the G substitutions, leading to its widening at these positions. As a consequence, different DNase I-hypersensitive sites are observed (marked by *asterisks* in Fig. 2*c*) in each of the mutants; $(OP)_2Cu$ footprinting analysis gave analogous results (not shown). Because both DNase I and $(OP)_2Cu$-mediated DNA cleavages are in the minor groove (39, 40), the T_6 run mutations appear to change the DNA conformation in the minor groove. This is further supported by an altered migration pattern of DNA fragments in polyacrylamide gels (data not shown).

Mutations in the T_6 Run Result in Increased Promoter Activity—The differences in chemical nuclease and enzymatic cleavage patterns of WT and mutant T_6 run promoters reflect structural differences among these promoters. To determine whether the changes also influence promoter activity, promoter-*lacZ* fusion constructs were generated for all of the mutants, and promoter strength was assessed indirectly by measuring β-galactosidase activity in cells harboring these plasmids. Moreover, the C-activated level was analyzed in cells also harboring a compatible C-producing plasmid, pVN184.

T_6 run mutants T2G, T2C, T3G, T3C, T2GT3G, and T5C produced 5–26-fold higher levels of enzyme compared with the WT promoter (Table I); in contrast, T1C and T4C showed little or no increased expression. However, all of the mutants remained responsive to transactivation by C protein (Table I), producing enzyme levels comparable with that of the activated WT *mom*P1 promoter. Thus, all of the mutant promoters are C protein-dependent for their full activity, indicating that the C transactivation mechanism was unaltered. As a control, the mutation G21C was created (shown in Fig. 1*b*) upstream from the T_6 run yet within the spacer region. As expected, this mutant showed levels of β-galactosidase activity comparable with the WT *mom*P1 promoter in the absence and in the presence of C protein (Table I). The variable increase in *mom*P1 promoter activity among these mutants could be due to differences in their perturbations of DNA structure as shown in Fig. 2. None of the mutant promoters showed activity as high as that of T4G (*tin7*), which showed an increase that was between 46- and 80-fold depending on the type of fusion examined (Tables I and II). These results suggest that in addition to DNA distortion, an alternative mechanism might be operating in *tin7*, most likely having an extended −10 promoter because of the specific base substitution at −14 position (discussed further below).

The above experiments were carried out using a *mom*P1 promoter directing production of a Com-LacZ translational fusion. We carried out similar experiments with a *mom*P1 promoter-*lacZ* transcriptional fusion vector. This was constructed by subcloning *mom*P1 mutations (T4G, T4A, T4C, T3G, T3A, and T3C, produced in a *mom*P1-containing synthetic oligonucleotide duplex) into a site 5′ to a promoterless, reporter *lacZ* gene (see "Experimental Procedures"), and *mom*P1 promoter activity was assayed by measuring β-galactosidase activity. As seen in Table II, the substitutions generated variable increases in enzyme level, in good agreement with the results observed with the pLW4 plasmid system. Most interesting are the three T4X substitutions. First, the T4G mutant had the highest level of C-independent expression, 80-fold above the WT. In contrast, T4A had a 6-fold increase, whereas T4C showed no increase. Thus, the three different T4X substitutions produced three different phenotypes. We suggest that the high level of constitutive expression by T4G (*tin7*) is due to its having an extended −10 promoter, in addition to the alteration in DNA conformation. In contrast, the T4A (as well as the T3A) substitution appears to only affect *mom*P1 DNA conformation, indicating that T-A to A-T base pair alterations can also affect conformation. At first glance, it was surprising that the T4C mutant did not show increased *mom*P1 expression; however, as will be shown below, the T4C mutant does not exhibit any structural difference from the WT based on *in vitro* cleavage. Finally, it should be noted that the T2G and T3G mutations create a TG at positions −17 and −16 and at positions −16 and −15, respectively. Although these mutations exhibited enzyme levels severalfold higher than the WT, they do not appear to provide extended −10 functional capability.

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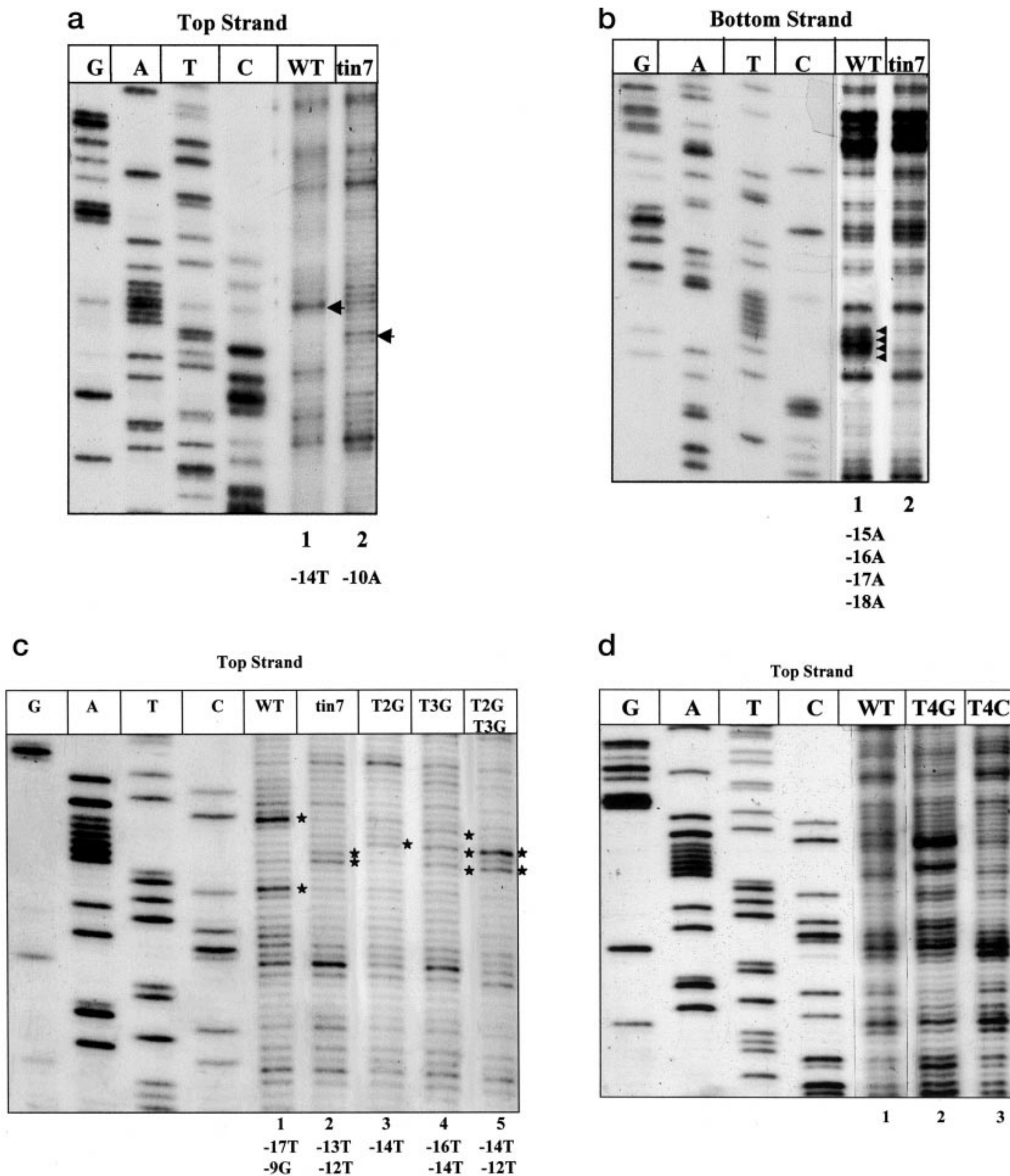

FIG. 2. **Nuclease sensitivity pattern of WT and mutant *mom*P1 promoters.** The $(OP)_2Cu$ cleavage reactions of the WT (pLW4, *lanes 1*) and *tin7* mutant (p*tin7*, *lanes 2*) promoters and the sensitivity pattern of the top (*a*) and the bottom (*b*) strands are shown. *c*, DNase I cleavage reactions of WT (*lane 1*), *tin7* (*lane 2*), T2G (*lane 3*), T3G (*lane 4*), and T2G T3G (*lane 5*) promoters in the top strand is shown. *d*, DNase I cleavage reactions in the top strand of WT (*lane 1*), *tin7* (*lane 2*), and T4C (*lane 3*) mutant promoters. Hyper-reactive residues are indicated with *arrowheads* and *asterisks*. *G*, *A*, *T*, and *C* refer to Sanger's dideoxy sequencing ladder of the region of pLW4 using end-labeled *mom* forward and reverse primers.

In view of the different levels of *mom*P1 expression observed with the three T4X mutations, we compared their *in vitro* sensitivity to cleavage by DNase I. As shown in Fig. 2*d*, the T4C promoter region showed a cleavage pattern similar to that of the WT, which is in sharp contrast to all of the other T_6 run mutants. Because the T4C substitution does not appear to alter the WT *mom*P1 DNA conformation, we suggest that it possesses the same unfavorable distortion as the WT and, hence, requires protein C activation for any transcription.

Formation of Open Complexes by T_6 Run Mutants—Increased β-galactosidase levels with certain T_6 mutant *mom*P1 promoters indicate increased transcription initiation capability. Interaction of RNAP at a promoter can be ascertained by assessing open complex formation using an *in vivo* $KMnO_4$ footprinting technique (36). *E. coli* DH10B cells harboring a T_6 mutant pLW4 plasmid in the absence or the presence of the C protein-producing plasmid, pVN184, were probed (see "Experimental Procedures"). In accordance with its high level of constitutive promoter activity in the absence of C protein, *tin7* showed hypersensitive bands (Fig. 3, *lane 4*) characteristic of open complex formation; the observed pattern was in good agreement with the results of Balke *et al.* (17). Open complex formation in the absence of C protein was also observed with mutants T2G and T2GT3G (Figs. 3, *lane 12*, and 4*a*, *lane 10*). However, open complexes were not detected with T3G, T3C, and T2C (Fig. 3, *lanes 8*, *10*, and *14*, respectively), which correlates with their relatively lower levels of β-galactosidase expression (in the absence of C protein). As could be predicted from the promoter strength analysis (Table I), the WT *mom*P1 promoter and the T4C mutant were unable to produce detectable open complexes (Fig. 3, *lanes 2* and *6*, respectively). In the presence of C protein, however, all promoters showed open complex formation (Fig. 4, *a* and *b*). This result rules out an artifactual inability to detect open complexes with the mutant

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TABLE I
Production of β-galactosidase activity in E. coli DH10B cells containing a pLW4-momP1 promoter-lacZ fusion derivative ± compatible plasmid pVN184

See "Experimental Procedures" for growth of cells and enzyme assays. Plasmid pVN184 produces C protein constitutively at a low level. *E. coli* DH10B cells alone and harboring pVN184 do not show any enzyme activity.

*mom*P1 mutant	LacZ (−C protein)	Relative activity[a]	LacZ (+C protein)	Fold activation[b]
	Miller units		*Miller units*	
WT	23	(1.0)	2,264	99
T1C	8.4	0.4	1,605	191
T2C	135	5.8	2,027	15
T3C	118	5.1	1,509	13
T4C	42	1.8	2,952	71
T5C	125	5.4	3,226	26
T2G	396	17.2	2,855	7.2
T3G	183	8	3,164	17
T2GT3G	595	26	2,989	4.1
T4G(*tin7*)	1,053	46	4,913	4.7
G21C	18	0.7	2,039	112

[a] The relative β-galactosidase activity with the WT promoter in the absence of C protein is defined as 1.0. It corresponds to 23 Miller activity units.
[b] Fold activation is defined as the ratio of β-galactosidase activity produced by a *mom*P1 mutant plasmid in the presence *versus* the absence of the compatible C protein-producing plasmid, pVN184.

TABLE II
Production of β-galactosidase activity in E. coli LL306 cells containing a pLC1-momP1 promoter mutant plasmid

See "Experimental Procedures" for growth of cells and enzyme assays.

*mom*P1 mutant	LacZ	Relative activity[a]
	Miller units	
WT	20	(1.0)
T4C	22	1.1
T4A	125	7
T4G(*tin7*)	1,600	80
T3C	70	3.5
T3A	72	3.6
T3G	220	11

[a] The relative β-galactosidase activity with the WT promoter is defined as 1.0. It corresponds to 20 Miller activity units.

constructs used in these experiments.

T_6 Run Mutants Show Increased P1 Transcript Levels—Because only mutants with higher (>8-fold the WT basal level) expression of β-galactosidase activity showed open complex formation, we employed a more direct method of assessing promoter strength. For this purpose, we assayed for *mom*P1-specific mRNA transcripts in total RNA isolated from WT, T4G (*tin7*), and T_6 mutant (T2G, T3G, T3C, and T2GT3G) plasmid-containing cells (see "Experimental Procedures"). The results of such an experiment are shown in Fig. 5*a*. In all of the mutants examined the transcription start site was identical to that of the wild type *mom*P1 promoter, indicating that the mutations did not lead to the formation of new promoters. Those mutants (*e.g.* T3G and T3C) that failed to show open complexes in the $KMnO_4$ probing experiments did produce increased amounts of *mom*P1-specific transcripts compared with the WT promoter (Fig. 5). There was a good correlation in the fold increase in *mom*P1-specific transcript levels and the relative promoter strengths of these mutants with respect to the WT levels (compare Tables I and II and Fig. 5*b*).

Mutations Disrupting the momP2 −10 Hexamer Do Not Increase Activity of the WT (or T_6 Run Mutant) momP1 Promoter—The results presented above support the view that alteration in DNA conformation caused by disruption of the T_6 run results in increased basal activity of the *mom*P1 promoter. However, the scenario is somewhat complicated by the fact that the *mom* regulatory region has two overlapping divergent promoters, *mom*P1 and *mom*P2. The T_6 run substitution mutations generated in *mom*P1 also disrupt the A_6 tract in the complementary strand (Fig. 1), which is proposed to function as part of an UP element directing leftward transcription from the *mom*P2 promoter (18). Hence, an alternate possibility for the increased activity of *mom*P1 in *tin7* and other T_6 run mutants could be due to weakening of the UP element of *mom*P2. Therefore, *mom*P2 transcript levels were measured by isolating total RNA and extending it with end-labeled *mom* forward primer using reverse transcriptase. The results are shown in Fig. 6, where *mom*P2 transcripts were detected with *tin7*, as well as with some other T_6 (T2G, T3G, and T3C) run mutants. Thus, the substitution mutations in the T_6/A_6 run did not abolish *mom*P2 activity while having increased *mom*P1 activity. This conclusion was supported by results from independent experiments in which synthetic duplexes having mutations in the T_6/A_6 run (corresponding to T2G or T3G or T4G) were cloned into pLC1 in an orientation where *lacZ* gene transcription was under control of *mom*P2 (in these constructs the *mom*P1 −10 hexamer was also altered so as to reduce its potential expression). We observed that each of the single-base substitution mutations lowered the enzyme level less than 2-fold (data not shown). Thus, T_6 run mutations that increase *mom*P1 transcription do not do so by reducing *mom*P2 transcription.

To further examine the effect (if any) of *mom*P2 expression on *mom*P1 expression, the *mom*P2 −10 hexamer was mutated in the WT, *tin7*, and T2GT3G mutant constructs (Fig. 7*a*). In these mutants, loss of *mom*P2 function was confirmed by measuring leftward transcript levels produced *in vivo* by the parental and disrupted *mom*P2 promoters, using primer extension assays with total RNA extracted from cells harboring these plasmids (Fig. 7*b*). The results in Fig. 7*c* show that there was no increase in the WT, *tin7*, or T2GT3G *mom*P1 promoter activity in the *mom*P2 −10 disrupted background. These results indicate that the overlapping *mom*P2 promoter plays, at most, only a minor role in *mom*P1 activity, unlike other overlapping promoters. We conclude that mutations in the T_6 run that increase *mom*P1 expression function by alleviating DNA distortion.

DISCUSSION

We have addressed the importance of the run of six T nucleotides located in the *mom*P1 promoter (Fig. 1*a*) in the regulation of *mom* operon expression. An intrinsic DNA distortion caused by the presence of the T_6 tract overlapping the 5′ end of the −10 element could produce an unfavorable conformation for RNAP occupancy. Different T to G substitutions in this run showed different sensitivity patterns to nucleases as compared

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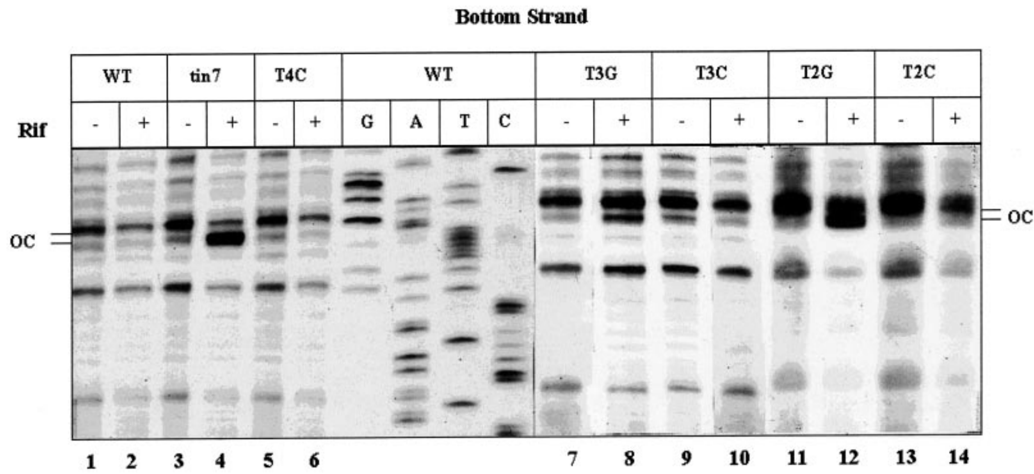

FIG. 3. ***In vivo* KMnO$_4$ footprinting analysis.** The presence (+) or absence (−) of rifampicin (*Rif*) to trap RNAP in the open complex is indicated. *OC* refers to the bottom strand hypersensitive sites upon open complex formation in *momP1*. Sequencing lanes are shown as *G*, *A*, *T*, and *C*.

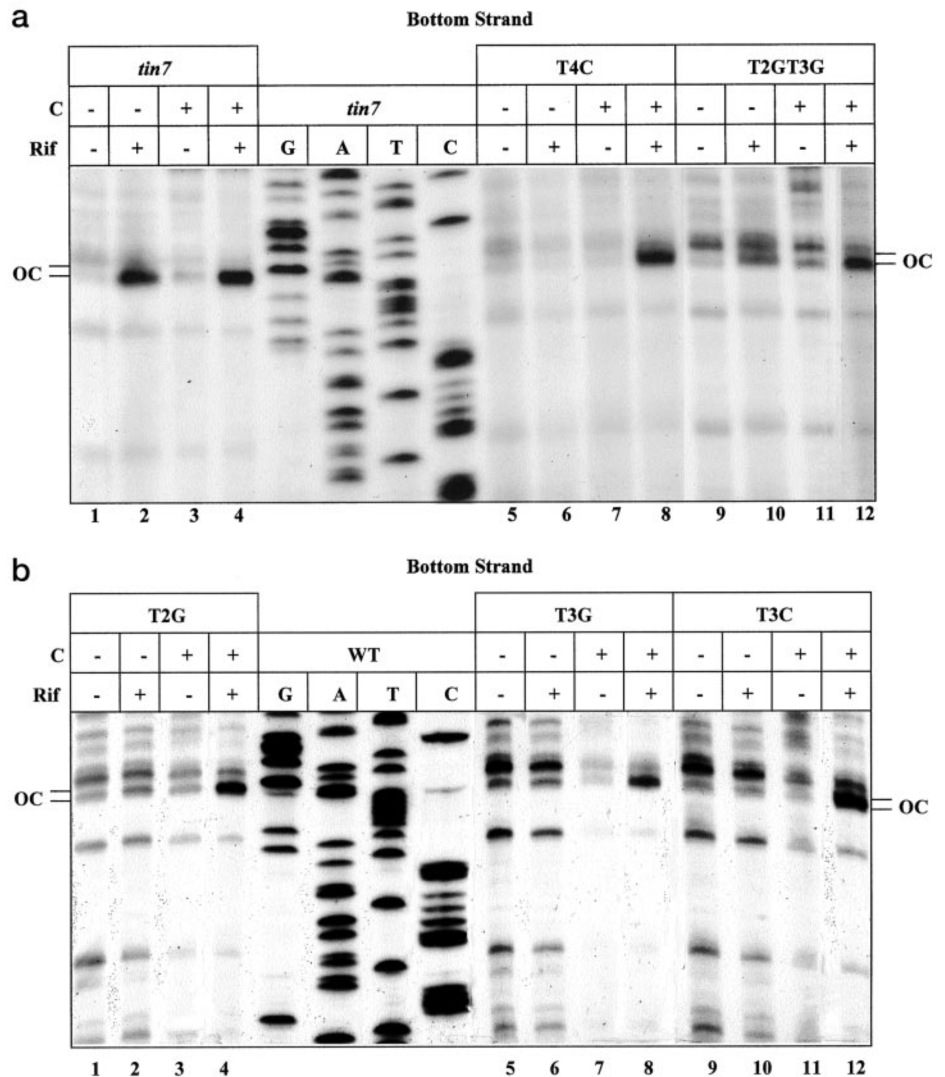

FIG. 4. **Open complex formation by T$_6$ run mutants.** The presence (+) or absence (−) of C protein (*C*) and rifampicin (*Rif*) are indicated. *OC* indicates the hypersensitive sites produced upon open complex formation in the bottom strand. *G*, *A*, *T*, and *C* refer to sequencing lanes. Analysis with *tin7*, T4C, T2GT3G (*a*) and T2G, T3G, T3C (*b*) were carried out.

with the WT *mom*P1 promoter (Fig. 2). This is attributed to a difference in the DNA structure caused by each substitution. These substitutions in the T$_6$ run also produce variable increases in the basal activity of mutant *mom*P1 promoters (Tables I and II). The increase in the promoter strength of some of the mutants was correlated with the formation of detectable open complexes and the levels of *mom*P1-specific transcripts in the absence of trans-activator protein C (Figs. 3–5).

One could argue that the increase in promoter activity observed in the T$_6$ run mutants could be a consequence of new base-specific contacts made by RNAP in the substituted positions instead of removal of intrinsic DNA distortion. However,

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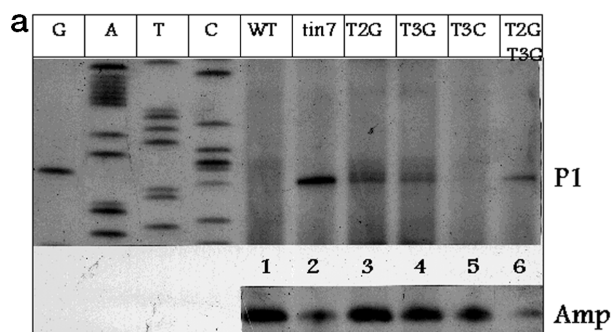

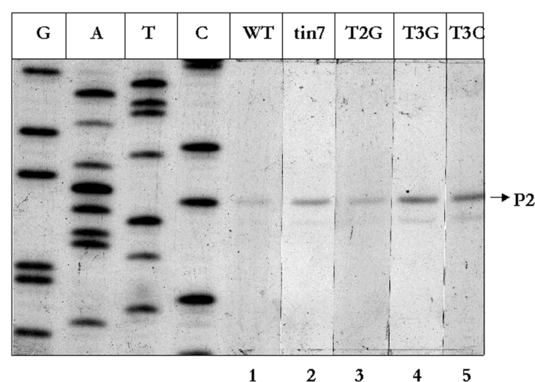

FIG. 6. **Detection of the *mom*P2 transcript by T_6 run mutants.** The experiment was carried out as described under "Experimental Procedures" using equal amounts of total RNA (20 μg) in all of the lanes and end-labeled *mom* forward primer for primer extension.

FIG. 5. ***mom*P1 transcript levels produced by T_6 run mutants.** Primer extension reactions were carried out using *mom* reverse primer and reverse transcriptase as described under "Experimental Procedures." *G*, *A*, *T*, and *C* refer to sequencing lanes. *a*, autoradiograph showing the *mom*P1 transcript and ampicillin (*Amp*) signals. *b*, levels of momP1 specific RNA (with respect to WT levels after normalizing with the ampicillin signal).

this seems unlikely because changing different residues (−13 to −16) led to the increase in promoter activity. Moreover, substitutions elsewhere in the spacer do not increase the activity, including the mutant T1C, whose substitution still retains the run of T_5 nucleotides. A point to be remembered and shown here is that *mom*P1 wild type promoter is unable to form an open complex on its own (Ref. 17 and Fig. 3). This is not due to the suboptimal spacer length (see the Introduction and Fig. 1) because single-base and two-base deletions in the spacer (18 and 17 bp, respectively) do not lead to any increase in promoter activity (data not shown). Thus, it is difficult to visualize RNAP contacting each and every residue between −13 to −16 when it is unable to make favorable contacts in an optimally spaced promoter. An altered DNA structure of the various T_6 run mutants leads to the formation of an open complex at these promoters. As an additional support for altered DNA conformation detected in nuclease probing experiments, the wild type promoter fragment (226 bp) migrated slower than the T2G mutant promoter fragment (226 bp) in a gel electrophoretic mobility assay. Taken together, we conclude that the transcription from promoters having substitutions in the T_6 run is due to the removal of an unfavorable distortion.

Of all of the mutants whose promoter strength we analyzed, T4G (*tin7*) showed the highest *mom*P1 transcriptional activity. This mutation, a T → G at position −14, produces a −15T, −14G, which is characteristic of extended −10 promoters (28, 41). Extended −10 promoters are usually constitutive, and they do not require a −35 element or an activator protein. In contrast to the T4G, the corresponding T4C substitution did not increase expression of *mom*P1 nor alter promoter DNA conformation compared with the WT. All three −15T (T3*X*) substitutions exhibited an increase in *mom*P1 basal activity compared with WT. However, although the T3G mutation created a TG at positions −16 and −15, it did not produce the same high level of expression exhibited by T4G (*tin7*); this is to be expected because the former TG is not positioned properly to create an extended −10 promoter. Thus, we suggest that a combination of both DNA conformational alteration and extended −10 promoter characteristics contribute to the T4G (*tin7*) phenotype, but the increase in basal activity of the other T_6 run mutants is due to the removal of an unfavorable distortion in *mom*P1 promoter DNA. Once this distortion is ameliorated, an otherwise very weak promoter can be transcribed in the absence of activator protein C. However, these promoters are still dependent on C for full activity, as shown by both the C-mediated increases in β-galactosidase activity (Table I) and the formation of open complexes (Fig. 4).

It has been shown earlier that it is primarily the length, not the sequence, of spacer DNA between the two promoter consensus sequences (−10 and −35 regions) that is important for activity of a promoter (42, 43). It has also been demonstrated that the sequences located either upstream or downstream of the −10 and −35 regions determine the kinetics of association of promoter with RNAP and efficiency of transcription initiation (44, 45). It is believed that the spacer DNA holds the −10 and −35 regions in the proper orientation for their recognition by the RNAP holoenzyme complex without having any specific contacts with RNAP. However, characterization of mutants of the P_{RM} promoter of phage λ bearing $dC_9 \cdot dG_9$ sequences in a stretch of the spacer DNA separating the contacted −10 and −35 regions showed reduced promoter activity both *in vitro* and *in vivo* (46, 47). These mutations were interpreted as altering the structure of the spacer DNA and, as a consequence, leading to a change in the orientation or local structure of the contacted −10 and −35 elements of the promoter. A library of synthetic promoters of *Lactococcus lactis* having randomized 17-bp optimal length spacer in between the consensus −10 and −35 elements was assayed for activity both in *L. lactis* and *E. coli* (48). In both host backgrounds, a large variation (~400 fold) in promoter activity was observed because of variations in the spacer sequence context. It seems that the overall three-dimensional topological structure of the promoter DNA that arises from a particular nucleotide sequence could be important for the activity of a promoter.

Complex regulatory mechanisms have evolved in bacteriophages to ensure the precise expression of phage genes. Expression of the bacteriophage Mu *mom* gene during the late

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FIG. 7. **Effect of *mom*P2 −10 hexamer mutations on *mom*P1 activity.** *a*, the sequence of the *mom* regulatory region is shown indicating the mutation in three different promoters (WT, *tin7*, and T2GT3G). *b*, detection of *mom*P2-specific mRNA in the wild type *mom* promoter (*lanes 1* and *3*) and in a disrupted *mom*P2 −10 hexamer background (*lanes 2* and *4*) using end-labeled *mom* forward primer. *Lane 1*, 15 μg of total RNA; *lane 2*, 15 μg of total RNA; *lane 3*, 30 μg of total RNA; *lane 4*, 30 μg of total RNA. *c*, *mom*P1 promoter strength (measured by β-galactosidase activity) in a disrupted *mom*P2 −10 hexamer background. The values plotted are the averages of four experiments.

transcription phase is a good example of one such regulatory scheme. Although *mom* gene expression seems to be dispensable for Mu growth or lysogeny, premature activation or constitutive expression of *mom* function is detrimental to the host cells (49, 50). Hence, it is not surprising that intricate mechanisms have been evolved for the regulation of *mom* expression at both the transcriptional and translational levels (16). Apart from these modes of regulation, recently, Sun and Hattman (18) have suggested another possible regulatory control over *mom* expression. It was suggested that the leftward transcription at the *mom*P2 promoter (Fig. 1 and Ref. 18) might prevent low level rightward transcription of *mom*P1 (and, hence, *mom* expression) in two possible ways. First, *mom*P2 might compete with *mom*P1 for RNAP binding in the absence of C protein. Second, leftward transcription produces an antisense transcript that might prevent *gin* mRNA elongation into *mom*. The present study rules out the first possibility because disruption of the *mom*P2 −10 hexamer did not lead to an increase in the basal level activity of *mom*P1 (Fig. 7*c*). On the other hand, disruption of the T_6 run in the spacer region of *mom*P1 promoter led to increased rightward transcription, indicating the importance of its role as a *cis*-acting negative element. Another possible role attributed to *mom*P2 is to act as a sink for capturing RNAP in the vicinity of *mom*P1, so that RNAP is ready for occupancy at *mom*P1 at the right time of *mom* expression (17). Our results do not exclude that possibility.

Existence of overlapping promoters in many systems add additional regulatory complexities (51–53). The *mom*P1 and *mom*P2 promoters (Fig. 1*a*) are overlapping and oriented in a divergent fashion. Normally such organization would lead to competition in the transcription machinery as exemplified in case of other overlapping/competing promoters (53). However, earlier DNase I footprinting analysis with the *mom* promoter revealed that RNAP is bound to *mom*P2 in the absence of C protein (17). Partitioning of RNAP between the two promoters was not observed, although neither promoter appears to be a strong one. Further, *mom*P2 disruption did not lead to increased *mom*P1 expression (Fig. 7*c*), underlining the importance of the T_6 run as a *cis*-acting negative element that prevents RNAP binding to *mom*P1. Thus, the primary role of the T_6 run is to prevent low level rightward transcription initiation at *mom*P1.

It is noteworthy that the P_{lys} promoter, another bacteriophage Mu late gene promoter, also has a T_6 stretch in the position corresponding to that in *mom*P1, but it is absent in the other two late promoters (P_i *and* P_p). Substitutions in two of the T bases in the P_{lys} spacer region show an UP phenotype depending on the substituted base (22). Because the premature expression of *mom* and *lys* is detrimental to the host cells, the DNA negative element seems to be a common fail-safe mecha-

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nism to keep these two late genes tightly regulated until the right time for their expression. Thus, the phage Mu seems to have evolved one common strategy to keep two potentially cytotoxic genes under control.

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