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In Vitro Transcriptional Activation of the Phage Mu *mom* Promoter by C Protein

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The phage Mu gene C encodes a 16.5-kDa site-specific DNA-binding protein that functions as a *trans*-activator of the four phage "late" operons, including *mom*. We have overexpressed and purified C and used it for DNase I footprinting and transcription analyses in vitro. The footprinting results are summarized as follows. (i) As shown previously (V. Balke, V. Nagaraja, T. Gindlesperger, and S. Hattman, Nucleic Acids Res. 12:2777–2784, 1992) in vivo, *Escherichia coli* RNA polymerase (RNAP) bound the wild-type (wt) *mom* promoter at a site slightly upstream from the functionally active site bound on the C-independent *tin7* mutant promoter. (ii) In the presence of C, however, RNAP bound the wt promoter at the same site as *tin7*. (iii) C and RNAP were both bound by the *mom* promoter at overlapping sites, indicating that they were probably on different faces of the DNA helix. The minicircle system of Choy and Adhya (H. E. Choy and S. Adhya, Proc. Natl. Acad. Sci. USA 90:472–476, 1993) was used to compare transcription in vitro from the wt and *tin7* promoters. This analysis showed the following. (i) Few full-length transcripts were observed from the wt promoter in the absence of C, but addition of increasing amounts of C greatly stimulated transcription. (ii) RNA was transcribed from the *tin7* promoter in the absence of C, but addition of C had a small stimulatory effect. (iii) Transcription from linearized minicircles or restriction fragment templates was greatly reduced (although still stimulated by C) with both the wt and *tin7* promoters. These results show that C alone is capable of activating rightward transcription in vitro by promoting RNAP binding at a functionally active site. Additionally, DNA topology plays an important role in transcriptional activation in vitro.

The bacteriophage Mu *mom* gene encodes an unusual DNA modification function (6, 7, 26–28), which is regulated in a complex fashion (2, 9, 10, 15–17). The host *Escherichia coli* DNA-(N^6-adenine)-methyltransferase, which methylates the sequence GATC (8), is required for transcriptional activation of the *mom* operon (7, 9, 15, 22, 25). Two phage Mu gene products, C and Com, are involved in positively regulating *mom* expression. C is required for transcriptional activation (11, 14, 18), while Com is a site-specific mRNA-binding protein (13, 29, 31) required for translation of the *mom* open reading frame (12, 30). The *C* gene has been cloned and sequenced (14, 18); it encodes a 16.5-kDa polypeptide (140 amino acids) that is a site-specific DNA-binding protein (1, 3, 21). Comparison of the four C-activated Mu late promoters revealed that each of the late transcripts initiates near a conserved sequence (20). We suggest that the critical recognition element in this sequence is an inverted tetranucleotide repeat, TTAT. . .ATAA, which is separated by a GC-rich spacer of 5 to 6 nucleotides (nt); consistent with this is the observation that C appears to be a dimer in solution (13a).

Bölker et al. (3) used MPE · Fe(II) footprinting in vitro to identify a single C-binding site located between nt 1006 and 1024 from the right end of Mu, corresponding to −35 to −53 with respect to the *mom* transcription initiation site (the inverted tetranucleotide repeat, TTAT. . .ATAA, is included within these coordinates). In the absence of the C protein in vivo, RNA polymerase (RNAP) binds in the *mom* promoter region at a site which is functionally inactive for *mom* (rightward) transcription (1). In this report, we show that RNAP requires the C protein for functional binding and transcription of the *mom* promoter in vitro. C-activated transcription of the Mu *lys* promoter was demonstrated earlier by Margolin and Howe (19), who used partially purified protein fractions.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* SA1751 [λint^+ *xis439 cI857*(*cro-chlA*)$_{\Delta H1}$] and a minicircle vector, pSA508, were generously provided by Sankar Adhya prior to publication (5). In the presence of phage λ integrase and *E. coli* integration host factor, pSA508 undergoes intramolecular site-specific recombination and produces two smaller circular DNAs. *E. coli* LL306 Δ(*pro-lac*) *nalA recA supE44 thi* was from Lasse Lindahl (32). *E. coli* MH9028 was kindly provided by Martha Howe; this strain harbors two compatible plasmids. One is the C-overproducing plasmid, pWM18 (19), which contains the *C* gene coding region located 3′ to the phage T7 gene 10 promoter and ribosome binding site. The second plasmid, pGP1-2, expresses phage T7 RNAP from the λ P_L promoter (regulated by the thermolabile phage λ*cI857* repressor, also encoded on the plasmid). Plasmids pLW4 and pLW4*tin7* were described previously (1). These plasmids contain a small portion of the 3′ end of the Mu *gin* gene, the *mom* promoter region, and a portion of the 5′ end of the *com* gene fused inframe to the *E. coli lacZ* gene. Plasmid pLW4 produces enzymatically active β-galactosidase (as a Com-LacZ protein fusion [4]), but only in the presence of C; plasmid pLW4*tin7*, which has a single base substitution (T to G) at −14, constitutively produces enzyme activity, but at a low level.

Materials and general methods. Enzymes used for DNA cleavage and cloning were purchased from New England Biolabs or Bethesda Research Laboratories. DNase I was from Bethesda Research Laboratories, and *E. coli* RNAP holoenzyme was purchased from Pharmacia.

Standard protocols were used for plasmid isolation, restriction digestions, ligation, plasmid transformation, gel electro-

phoresis, and electroelution of fragments from agarose gels (23). For DNA ligation, fragments were isolated from gels with Geneclean (Bio 101). Dideoxy sequencing of DNA was with a Sequenase 2.0 kit from United States Biochemical and ^{32}P-end-labeled primers that were made with [γ-^{32}P]ATP (10 mCi/ml; >6,000 Ci/mmol [NEN]) and T4 polynucleotide kinase (United States Biochemical) according to the supplier's instructions.

Tris, dithiothreitol (DTT), polyethylene glycol 8000, yeast tRNA, ampicillin, and other chemicals were from Sigma Chemical Corp. Acrylamide solution for sequencing gels was from AMRESCO. The *lac* primer (New England Biolabs) is complementary to the nontranscribed (top) strand; prior to use, the primer was passed through a PD-10 Sephadex G-25M column (Pharmacia).

Cloning the *mom* promoter region into the minicircle vector, pSA508. In order to clone the wild-type (wt) and C-independent *tin7* versions of the *mom* promoter into the minicircle vector, plasmids pLW4 and pLW4*tin7* were digested with *Eco*RI and *Pst*I. The 200-bp promoter-containing fragment from each was isolated from a 1% agarose gel and ligated into the corresponding restriction sites in the minicircle vector, pSA508. The resultant plasmids (see Fig. 1) were transformed into competent LL306 cells and grown at 37°C on Luria-Bertani–ampicillin agar (100 μg/ml). Plasmid DNA was prepared and tested for the presence of the correct insert by digestions with *Eco*RI and *Cla*I and with *Pst*I and *Xba*I. Appropriate clones of the two plasmids were then transformed at 30°C into *E. coli* SA1751 and grown on Luria-Bertani–ampicillin agar.

Cultures of SA1751 containing pSA508 with a wt or a *tin7* promoter insert were grown in Super Broth (23) with ampicillin (100 μg/ml) at 32°C until late log phase (optical density at 600 nm = 8). Induction of the phage λ integrase was accomplished by rapidly heating the cultures to 42°C; then, after 30 to 45 min, the cultures were returned to 32°C for a further 30 min. Under these conditions, site-specific recombination produces a minicircular plasmid containing the *mom* promoter (Fig. 1). Plasmid DNA was obtained by alkaline lysis. The minicircle monomer was separated from other recombination products by electrophoresis on a 1% agarose gel and by electroeluting the minicircle band into dialysis tubing. Ethidium bromide was removed by extraction with *n*-butanol, and the DNA was isolated after successive phenol and phenol-chloroform-isoamyl alcohol extractions, followed by ethanol precipitation. DNA concentration was estimated by coelectrophoresis with known amounts of standards.

The minicircle DNA was digested with *Pst*I. Following electrophoresis on a 1% agarose gel, the linear monomer band was excised and extracted from the gel.

In vitro transcription. Each 10-μl reaction mixture contained 2 nM DNA in 40 mM Tris-acetate (pH 7.9); 10 mM magnesium acetate; 0.1 mM EDTA; 100 mM potassium glutamate; 0.2 mM DTT; 0.5 mM (each) ATP, GTP, and CTP; 0.2 mM UTP; and 10 μCi of [α-^{32}P]UTP (40 mCi/ml; 800 Ci/mmol [Amersham]). *E. coli* RNAP was used at a concentration of 65 nM (unless otherwise indicated); the concentration of C was varied as indicated. Each reaction mixture was prepared on ice prior to the addition of C or RNAP. After the addition of C, the tubes were shifted to 37°C for 5 min; then, RNAP was added, and the incubation at 37°C was continued for an additional 10 min. The reactions were terminated by the addition of 10 μl of tRNA (250 μg ml)–50 mM EDTA and placement on ice. Eighty microliters of distilled, deionized water was added, and the samples were deproteinized by phenol and phenol-chloroform-isoamyl alcohol extractions.

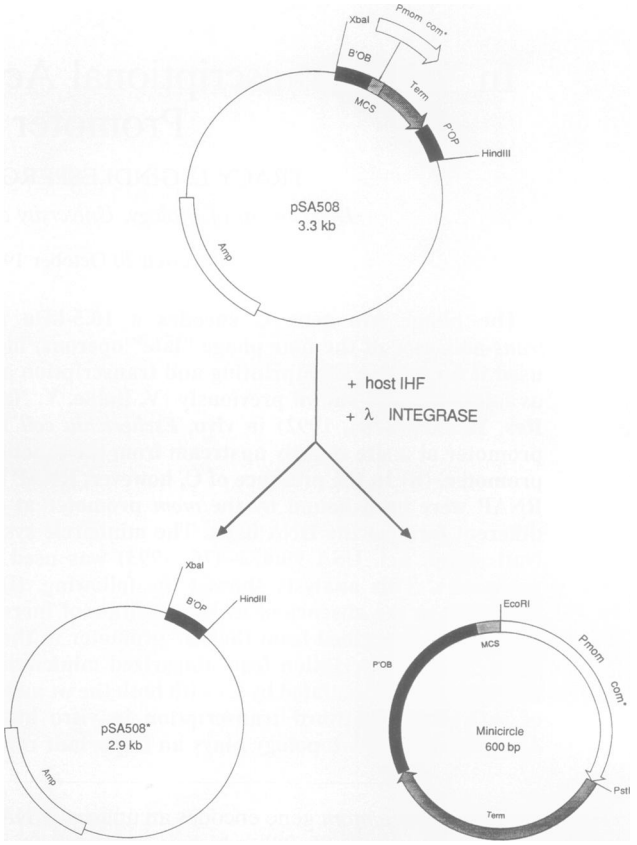

FIG. 1. Cloning strategy to create minicircular DNA containing the *mom* promoter. The 200-bp *Eco*RI-*Pst*I fragment of pLW4 or pLW4*tin7* containing the *mom* promoter was cloned into the same sites in the vector pSA508. Term indicates the Rho-independent terminator of the *E. coli rpoC* gene (5), and MCS marks the multiple cloning site. After recombination, the promoter-containing minicircle was purified as described in Materials and Methods.

The aqueous phases were ethanol precipitated overnight at −20°C. After harvesting by centrifugation, the RNA pellet was resuspended in 5 μl of sequencing gel loading dye and electrophoresed on a 4% acrylamide–8 M urea sequencing gel at 65 W for 2 h. The gel was dried and subjected to autoradiography.

DNase I footprinting-primer extension. Footprinting and end-labeled primer extension reactions were performed on supercoiled plasmid as previously described (24) with the following modifications. A total of 2 μg (approximately 34 pmol) of DNA of plasmid pLW4 or pLW4*tin7* was incubated with RNAP or C (or both) in 20 μl of footprinting buffer (20 mM Tris-HCl [pH 7.5], 0.5 mM EDTA, 5 mM $MgCl_2$, 70 mM KCl, 20 mM NaCl, 1 mM $CaCl_2$, 1 mM DTT, 3% glycerol, and 2% polyethylene glycol 8000). After 5 min at 22°C, 1 μl of DNase I (final concentration of approximately 0.05 to 0.1 U/μl in footprinting buffer) was added to the reaction mix for 45 s at 22°C. Reactions were stopped by the addition of 20 μl of Stop Buffer (150 mM NaCl, 25 mM EDTA, 100 mM Tris-HCl [pH 7.5], 0.5% sodium dodecyl sulfate [SDS]). The samples were extracted successively with phenol and phenol-chloroform-isoamyl alcohol and then precipitated with ethanol. The DNA was resuspended in 70 μl of water and divided into two aliquots. An aliquot was incubated with an oligonucleotide primer complementary to the top (amino acid-coding) DNA

strand; the primer was previously end labeled with [γ-^{32}P]ATP (NEN; 6,000 Ci/mmol), and T4 polynucleotide kinase (United States Biochemical). Following alkali denaturation at 80°C, the primer was annealed at 50°C in 50 mM Tris-HCl (pH 7.2)–10 mM $MgSO_4$–0.2 mM DTT. After addition of 5 mM deoxynucleotide triphosphates, 1 μl (1 U) of Klenow fragment was added, and the extension was carried out at 45°C for 10 min. The reactions were terminated by addition of 17 μl of 20 mM EDTA–4 M ammonium acetate. Samples were precipitated with 2 volumes of 95% ethanol at −20°C overnight. After pelleting, the samples were resuspended in 5 μl of gel loading buffer and applied to a 6% denaturing acrylamide gel. Control untreated template DNA was sequenced by the dideoxy method with phage T7 Sequenase with the same end-labeled primer as employed for the footprinting samples. After 4 h at 65 W, the gel was dried and autoradiographed.

Overproduction and purification of C. *E. coli* MH9028 was grown at 30°C in dYT medium (18) supplemented with ampicillin (100 μg/ml) and kanamycin (30 μg/ml). At an optical density at 600 nm of 0.8, the culture was shifted to 42°C and incubated for 4 h. After harvesting at low speed, the cells were resuspended in buffer I (20 mM Tris-HCl [pH 7.5], 1 mM Na_2-EDTA, 1 mM DTT, 10% [vol/vol] glycerol, 50 mM NaCl) and stored at −80°C until ready for use. Thawed pellets were harvested, and the cells were suspended in buffer I plus 1 mM phenylmethylsulfonyl fluoride (unless specified otherwise, all steps were at 4 to 5°C). The cells were disrupted by sonication, and debris was removed by centrifugation at 12,000 × *g*. An S-100 fraction was prepared by centrifugation for 2 h at 45,000 rpm in a Beckman type 50 rotor. The supernatant was collected and adjusted to a final concentration of 0.2 M in NaCl. Polymin P was added with stirring to a final concentration of 0.25% (vol/vol). After 30 min, the solution was centrifuged for 20 min at 15,000 rpm in a Sorvall SS-34 rotor. The supernatant was collected, and solid ammonium sulfate was slowly added to 40% saturation. After low-speed centrifugation, the supernatant was discarded, and the pellet was suspended in buffer 2 (20 mM Tris-HCl [pH 7.3], 0.1 mM EDTA, 7 mM 2-mercaptoethanol, 5% [vol/vol] glycerol, 150 mM NaCl). Molecular sieve chromatography was carried out on Sephadex G-100 equilibrated in the same buffer; 20-μl aliquots were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (16% acrylamide, 90 V, 2 h) and Coomassie blue staining. C-containing fractions were pooled and applied to a phosphocellulose P-11 column equilibrated in buffer 2. After washing, C was eluted in a 150 to 500 mM NaCl gradient; SDS-PAGE and Coomassie blue staining were used to identify the C-containing fractions. These were pooled and diluted 1:1 with buffer P (20 mM potassium phosphate buffer [pH 7.3], 7 mM 2-mercaptoethanol, 0.1 mM EDTA, 5% [vol/vol] glycerol). The solution was passed through a hydroxylapatite column (equilibrated in buffer P), and the flowthrough and wash fractions were collected. These were combined and loaded onto a phosphocellulose P-11 column and fractionated as described above. Selected C-containing fractions were combined and concentrated against solid Sephadex G-100, dialyzed against buffer 2 in 50% glycerol, and then stored at −20°C. On the basis of SDS-PAGE and silver staining, C was at least 90% pure.

RESULTS

DNase I footprinting of C. The Mu C protein was overproduced and purified as described in Materials and Methods. As shown in Fig. 2, the final preparation was at least 90% pure.

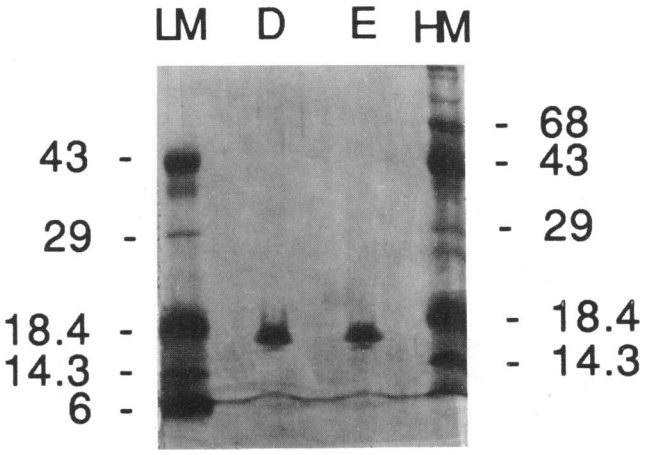

FIG. 2. SDS-PAGE analysis of purified C. Twenty-microliter aliquots of two fractions (D and E) of purified, concentrated C (1 mg/ml) were separated by electrophoresis through an SDS–16% acrylamide gel and visualized by silver staining. The positions of protein molecular weight markers (in kilodaltons) are indicated.

The minor band migrating slightly faster than C appears to be a degradation product of C, since it accumulated after long periods of storage at −20°C.

Site-specific in vitro DNA binding activity of our purified C fractions was verified by DNase I footprinting with plasmid pLW4 and pLW4*tin7* DNAs as substrates. As shown in Fig. 3, with increasing levels of C, DNA in the wt *mom* promoter region was protected against DNase I attack from positions −28 to −55 on the top strand (relative to the *mom* transcription start site). The sequence of the protected region is shown in Fig. 4; the same protection was observed with the *tin7* promoter (see below). These results are consistent with those of Bölker et al. (3), who reported an in vitro footprint from −35 to −53 with MPE · Fe(II), because DNase I footprints are typically larger than those obtained with chemical agents.

We have shown previously that *E. coli* RNAP binds to the wt *mom* promoter region at a site (P2) that is not functionally active in rightward transcription (1). In the presence of C in vivo, however, RNAP binds at a slightly downstream site (P1) that is the functional promoter. In contrast, the partially C-independent *tin7* promoter binds RNAP at P1 even in the absence of C. In order to study these interactions in vitro, we carried out a DNase I footprinting analysis. DNAs from pLW4 and pLW4*tin7* were treated in the presence of C alone, RNAP alone, or C and RNAP together (Fig. 5). In the presence of RNAP alone, the wt promoter was protected from −11 to −64 (the P2 site), whereas *tin7* showed RNAP protection from −41 to +16 (the P1 site). These coordinates are similar, but not identical, to those reported previously (1). In the presence of both RNAP and C, wt and *tin7* DNAs exhibited identical DNase I protection patterns; that is, the region from −55 to +16 was resistant to attack, while residues at −22 and −24 were hypersensitive. Thus, the protection pattern is a composite of both RNAP and C bound to the same DNA molecule, most likely on different faces of the helix; therefore, the presence of C alters the site at which RNAP binds the wt *mom* promoter.

It should also be noted that the DNase I sensitivities of free wt and *tin7* promoter DNAs differ. As seen here (Fig. 5), *tin7* exhibited several hypersensitive sites between −10 and −17 that were not present with the wt; this has been observed

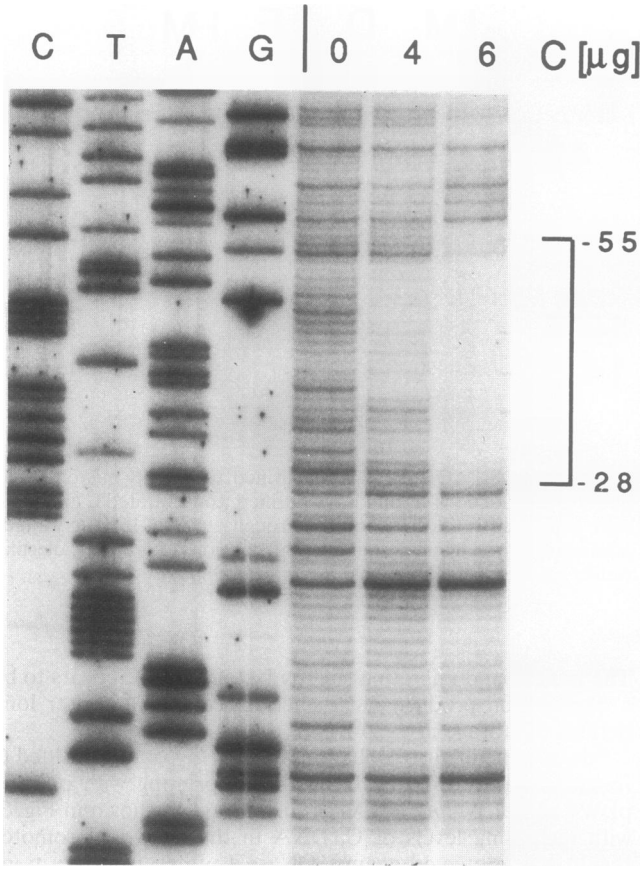

FIG. 3. In vitro DNase I footprinting (on the top strand) of C binding to the *mom* promoter. Plasmid pLW4 (2 μg; 17.2 nM final concentration) was incubated in the absence or presence of increasing levels of C (0, 4, and 6 μg; 5.8 and 8.7 μM final concentrations, respectively, calculated on the basis of C being a dimer) and treated with DNase I (see Materials and Methods). Cleavage sites were mapped by extension with end-labeled primer and sequencing gel electrophoresis. The vertical brackets denote the region of C protection.

previously (1). These sites are in the vicinity of the *tin7* mutation, a T-to-G substitution at −14, which changes a T_6 run to T_3GT_2.

In vitro transcription. The above results indicated that C alone was sufficient to guide RNAP to the correct binding site in the wt *mom* promoter. To determine whether this complex is functionally competent for transcription, we carried out an in vitro analysis with minicircle DNA as the substrate. This system was chosen so that transcription would occur only from the *mom* promoter (in order to avoid any complications resulting from competition for RNAP at other more transcriptionally active sites). It should be recalled that the *mom* promoter contains a poor −35 sequence and a suboptimal spacer length of 19 nt between the −10 and −35 hexamers (Fig. 4).

Various minicircle DNA templates were preincubated with increasing levels of C. RNAP was then added, and transcription was allowed for 10 min. As can be seen in Fig. 6, few full-length transcripts (133 nt, based on the known position of the Rho-independent *rpoC* gene transcription terminator [Fig. 1]) were observed from the wt promoter in the absence of C; however, addition of increasing amounts of C greatly stimulated transcription. (The occurrence of a doublet is ascribed to multiple terminations within the U run of the terminator, rather than to multiple initiations; this is supported by the observation that in vitro transcription from the *mom* promoter on another DNA template showed only a single start site [1].) In contrast, RNA was transcribed from the *tin7* promoter in the absence of C, and addition of C had a small stimulatory effect. Also, we found no evidence for leftward transcription from either promoter (data not shown). These results show that C alone is capable of activating in vitro rightward transcription by RNAP.

To test the possible effect of DNA topology on *mom* transcription, covalently closed and unit-length linearized (at the *Pst*I site) minicircles, as well as *Eco*RI-*Pst*I fragments of pLW4 and pLW4*tin7* were compared as templates. If transcribed, both types of linear molecules would produce a 97-nt runoff transcript. As expected, C stimulated transcription on both wt and *tin7* supercoiled minicircles (Fig. 7); a small amount of 133-nt transcript was observed with the linearized DNA, which we attribute to the presence of a low level of uncut minicircle. However, the transcription levels from both wt and *tin7* linearized minicircles, or from restriction fragments, were greatly reduced (although still stimulated by C). These results suggest that DNA topology plays an important role in the transcriptional activation of the *mom* promoter in vitro, possibly by imposing restraints on the contacts that C and RNAP can make with the DNA and/or with each other.

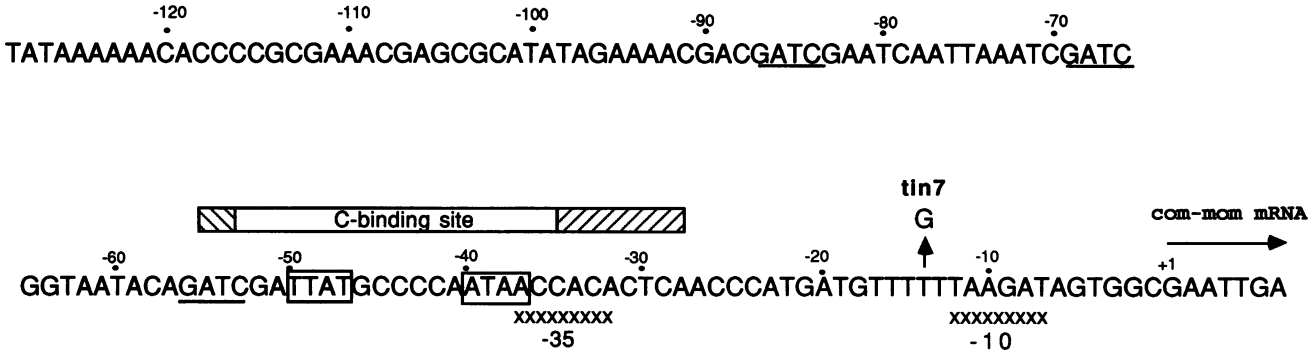

FIG. 4. Sequence of the *mom* promoter region. The C binding site is indicated; the open box denotes the region protected by C against cleavage with MPE · Fe(II) (3), and the crosshatched boxes denote the boundaries of protection against DNase I. The positions of the *tin7* mutation and the transcriptional start site are also shown. The three GATC sequences important in regulation of *mom* transcription are underlined.

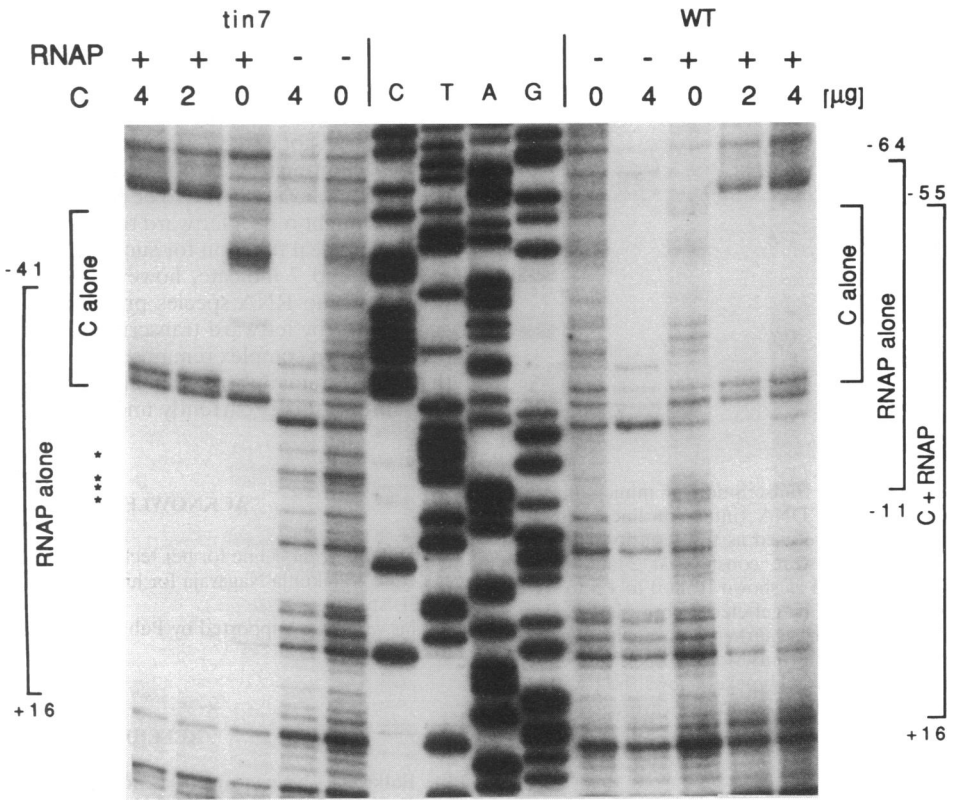

FIG. 5. In vitro DNase I footprinting of RNAP and C (see legend to Fig. 3). RNAP, when added, was constant (600 nM), and the amount of added C varied (2 and 4 μg; 2.9 and 5.8 μM final concentrations, respectively). Hypersensitive sites present only on *tin7* in the absence of C and RNAP are denoted with asterisks; the vertical brackets denote the protected regions. Sequencing lanes from *tin7* DNA are shown as controls.

DISCUSSION

In this paper, we describe the purification of the Mu late gene transcriptional activator protein, C; its binding to the regulatory region of the *mom* operon; and its in vitro activation of transcription by *E. coli* RNAP. Our DNase I footprinting data are in agreement with Bölker et al. (3), who used MPE · Fe(II) cleavage. They defined the C protection boundaries from −35 to −53 on linear DNA, whereas we observed DNase I protection from −28 to −55 on covalently closed circular DNA (Fig. 3). We attribute these differences to the greater accessibility of the chemical cleavage agent, not to the topological differences of the two DNA substrates.

The results of the in vitro DNase I footprinting of RNAP are also consistent with the existence of two functionally distinct binding sites for RNAP. In vivo (and in vitro) probing of $KMnO_4$-sensitive sites (1) revealed that in the absence of C, RNAP bound wt DNA in a complex (site P2) that is not functional in transcribing the *mom* operon. However, in the presence of C, RNAP bound another site, P1 (downstream and overlapping P2), that is the functional promoter binding site. With *tin* mutants, partially independent of C, RNAP bound to P1 even in the absence of C (1). We have defined the boundaries of these two RNAP-binding regions by in vitro DNase I footprinting. With the wt plasmid, a DNase I footprint corresponding to P2 was observed from −64 to −11 on the top strand. In contrast, the footprint corresponding to P1 spanned −41 to +16 on *tin7*. These results confirm and extend those made with $KMnO_4$ (1). Because P2 and P1 overlap, it is likely that RNAP binding to the wt in P2 precludes its binding to P1.

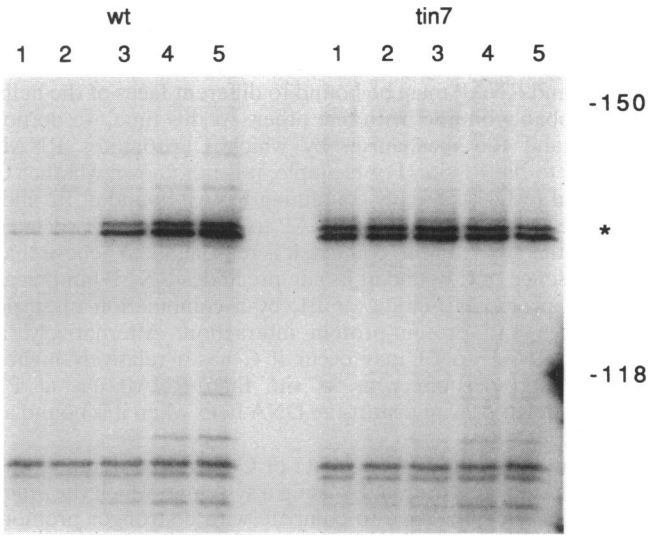

FIG. 6. In vitro transcription of minicircular DNAs. Minicircle DNA with the wt or *tin7* promoter (2 nM) was incubated with RNAP (65 nM) and increasing levels of C (0, 29, 144, 288, or 575 nM [lanes 1 to 5, respectively]) as described in Materials and Methods. The RNA products were then separated by gel electrophoresis and visualized by autoradiography. The positions of RNA size markers (in nucleotides) are indicated. Full-length minicircle transcripts (133 nt) are marked with an asterisk; this size is predicted from the known position of the *rpoC* gene terminator (5).

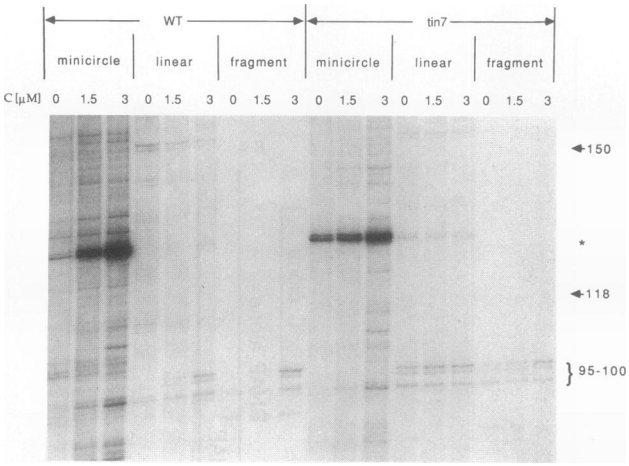

FIG. 7. Comparison of in vitro transcription of minicircular and linear DNA templates. Minicircle DNA (intact or linearized) and promoter-containing fragments were used as transcription templates. DNA and RNAP concentrations were constant at 2 and 65 nM, respectively. C concentration varied as shown from 0 to 1.5 to 3 μM. The RNA products were separated by gel electrophoresis and visualized by autoradiography. RNA size markers (in nucleotides) are indicated. The position of full-length transcript (133 nt) from circular templates is indicated by an asterisk; a bracket shows the position of RNA molecules transcribed from linear templates (average length = 97 nt).

The DNase I footprinting analysis was extended to reactions containing both RNAP and the transcriptional activator protein, C. With the wt plasmid, pLW4, the top strand footprint with RNAP and C together extended from −55 to +16 (Fig. 5). With *tin7* as the substrate, the same protection was observed. This protection pattern differs from either RNAP alone or C alone, and it appears to be a composite of protection by both RNAP and C bound to the same DNA molecule. Because the two individual regions of protection overlap, we believe that C and RNAP must be bound to different faces of the helix and probably interact with one other. At this time, we do not understand the mechanism by which C promotes RNAP binding to the P1 site. For example, it is not known whether C can bind to DNA already containing RNAP bound at P2 and, if it does, whether it moves RNAP to P1 or simply dissociates it from the DNA. In this context, it is reasonable to believe that the presence of C bound at its site precludes RNAP binding at P2 and promotes binding at P1, by a combination of steric hindrance and protein-protein interaction. Alternatively, a shift of RNAP to P1 may occur if C has a relatively higher on-rate and occupancy at its site than RNAP has at P2 (although RNAP can distort the DNA helix when it is bound at P2 [1]).

The in vitro study of the effect of C on RNAP binding was extended to transcription. By using a system in which the *mom* promoter does not have to compete with a stronger promoter(s) for RNAP, we were able to demonstrate that C is necessary and sufficient to activate transcription in vitro (Fig. 6). As observed previously in vivo (1), the *tin7* promoter was transcribed in the absence of C, while little (if any) RNA was made from the wt *mom* promoter. In addition, we have shown that DNA topology plays an important role in the in vitro activation of the *mom* promoter (Fig. 7). Although some C-stimulated transcription did occur from linearized DNA templates, the levels were much lower than those from supercoiled circular templates. It is possible that when the DNA topology is altered, C-RNAP contacts are disturbed, thus limiting transcriptional activation. Further studies on the effect of topology are in progress.

Using $KMnO_4$ as a probe, we had noted that in the absence of C, RNAP appeared to form an open complex at a site (P2) that was nonfunctional in rightward transcription in vivo (1); a similar complex appeared to be formed in vitro. We speculated that this might reflect leftward transcription, although we know of no biological function for such activity. The results obtained in Fig. 6 and 7 indicate, however, that in the absence of C, there were no RNA species produced in vitro that we could account for by leftward transcription. Thus, the nature of the putative open complex remains to be determined. It is possible that RNAP remains locked in an abortive initiation cycle when C is absent; this is currently under investigation.

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