THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 13, pp. 8511–8519, March 31, 2006

Dual Role for Transactivator Protein C in Activation of *mom* Promoter of Bacteriophage Mu*

Received for publication, December 2, 2005, and in revised form, January 18, 2006 Published, JBC Papers in Press, January 30, 2006, DOI 10.1074/jbc.M512906200

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Transactivator C protein of bacteriophage Mu activates the *mom* gene of the phage by an unusual mechanism. DNA binding by C to its site results in unwinding of the neighboring sequences, realigning the out-of-phase promoter elements to facilitate RNA polymerase (RNAP) binding. High level stimulation of a C-independent constitutive promoter mutant (where RNAP is already bound) by the transactivator suggested an additional mechanism of transcription activation at a step after RNAP recruitment. In this study, we have investigated the various steps of promoter-polymerase interactions during transcription initiation by using both the promoter mutant and a positive control (pc) mutant of C protein. The transactivator does not influence formation of the open complex or its stability after facilitating the RNAP binding. However, at a subsequent step, the protein exerts an important role, enhancing the promoter clearance by increasing the productive RNAP·promoter complex. The pc mutant of the transactivator C is compromised at this step, supporting the additional downstream role for C in *mom* transcription activation. We suggest that this unusual multistep activation of P_{mom} has evolved to ensure irreversibility of the switch during the late lytic cycle of the phage.

Regulation of transcription initiation is the major determining event employed by the cell to control gene expression and subsequent cellular processes. A large number of genes are subjected to transcription activation at the initiation step. The weak promoters with low basal transcription activities are activated by activators that influence the time and magnitude of transcription. Most activators stimulate transcription either by enhancing the binding of RNA polymerase (RNAP)[3] to the promoter or open complex formation (1). A few activators are also known to activate transcription at the post-isomerization steps of transcription initiation (2, 3). Bacteriophage Mu *mom* gene, which encodes a unique DNA modification function (4), is cytotoxic when expressed early or in large quantities (5, 6). Mu has evolved a complex, well controlled, and elaborate regulatory network for *mom* expression to ensure its synthesis only in the late lytic cycle. The intricate regulatory mechanisms involve, in addition to other features, repression of the promoter by OxyR, countering the repression by Dam methylation, transcription, as well as translational activation (4, 7–9). In the promoter region, there are two divergent promoters *P1* (also named P_{mom}), responsible for *mom* transcription, and *P2*, which directs transcription in the opposite orientation (10). The −10 and −35 elements of P_{mom} are away from the promoter consensus sequence, and the spacing between them is suboptimal 19 bp (Fig. 1*A*). The length and the structure of the spacer DNA, between −10 and −35 elements, is also shown to be important in determining the promoter strength (11). In P_{mom}, a stretch of six T residues just upstream to the −10 element confers an intrinsic curvature to the promoter, preventing RNAP binding (12). RNAP instead binds to *P2*, whose transcript does not encode any protein. The *P2* promoter thus appears to function solely as a regulatory element for P_{mom} (10, 13). The transcription activator C protein, a product of the middle gene in the phage gene expression cascade, is essential for all of the late gene transcription, including *mom*. The C protein binds to its palindromic sequence located adjacent to the −35 element of P_{mom} and overlapping the −10 element of promoter *P2* of the complementary strand (Fig. 1*A*). Binding of C with high affinity to its site results in the untwisting of the downstream region, leading to realignment of the otherwise "out-of-phase" promoter elements to facilitate RNAP binding (14, 15). This unusual activation mechanism has been confirmed subsequently by experiments involving spacer deletion and synthetic promoter constructs (16).

These findings reveal a new mechanism to recruit RNAP to a very weak promoter with an unfavorable configuration for polymerase binding and to initiate transcription. However, details of various steps of transcription initiation at P_{mom} subsequent to RNAP binding are not known. The role of the activator, if any, in later steps of transcription initiation have not been addressed, as binding of RNAP, itself, is dependent on the DNA untwisting activity of the activator. Enhancement of transcription by the transactivator in a C-independent promoter mutant suggested (see "Results") an additional downstream function for the activator. Here we have employed the promoter mutant (in which RNAP is able to bind without C) and a pc mutant of the transactivator to address the overall mechanism of *mom* transcription activation. The results demonstrate that the activator C has a role at more than one step during transcription initiation at P_{mom}, necessary to ensure complete transcription activation.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—The NTPs were from Promega. All of the column materials used for protein purification were from Amersham Biosciences. α-^{32}PATP (3000 Ci/mmol) was purchased from PerkinElmer Life Sciences. The oligos were synthesized by Sigma. DNase I was from Worthington, *Escherichia coli* DNA polymerase (Klenow polymerase) and other enzymes were from New England Biolabs.

Strains and Plasmids—Table 1 lists the bacterial strains and plasmids used in this study. Transcription templates were prepared by releasing a 220-bp EcoRI and BamHI promoter fragment from the respective promoter-*lacZ* fusion plasmids. The R105D mutant was previously gener-

* This work was supported by a grant from the Department of Science and Technology, Government of India (to V. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[3] The abbreviations used are: RNAP, RNA polymerase; pc, positive control; EMSA, electrophoretic mobility shift assay; DP, DNA·RNAP complex; CAP, catabolite activator protein.

TABLE 1
Strains and plasmids

Strains/Plasmids	Characteristics[a]	References
E. coli BL26 (DE3)	F^- *ompT gal [dcm] [lon]* $hsdS_B$ (r_B^-, m_B^-) Δ*lac* (DE3) *nin5 lac*UV5- T7 gene *1*	Laboratory stock
E. coli MG1655	F^- λ^- *ilvG*$^-$ *rfb*$^-$50 *rph*$^-$1	Laboratory stock
E. coli DH10B	Δ*(mrr-hsd RMS-mcrBC) mcrA recA1*	Laboratory stock
pVR7	Ap^r *C* under T7 promoter in pET11d	17
pVR7 R105D	Ap^r *C* containing R105D substitution under T7 promoter in pET11d	18
pVN184	Cm^r *C* under *tet* promoter in pACYC184	10
pVN184R105D	Cm^r *C* containing R105D substitution under *tet* promoter in pACYC184	This work
pLW4	Ap^r *mom-lacZ* fusion in pNM480	10
pLW4*tin7*	Ap^r *tin7-lacZ* fusion in pNM480	10

[a] Antibiotic resistance to ampicillin and chloramphenicol is indicated by Ap^r and Cm^r, respectively.

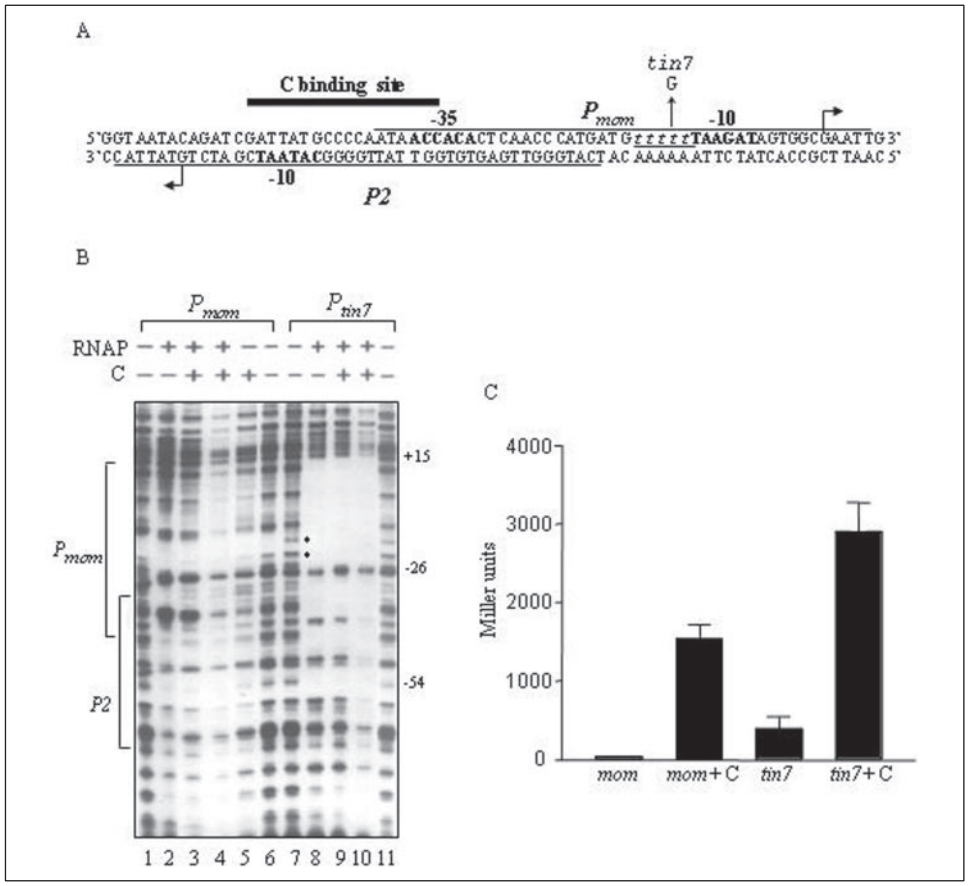

FIGURE 1. **Transcriptional regulation of P_{mom}.** *A*, schematic representation of the *mom* regulatory region. The C binding site is indicated with a *horizontal bar*, and −10 and −35 elements are in *bold*. The negative element, the T stretch, neighboring the −10 of P_{mom}, is shown in *lowercase letters* and *underlined*. P_{tin7} contains a T→G substitution (10), indicated by the *vertical arrow*. Transcription start sites of both of the promoters are indicated. *B*, RNAP occupancy at P_{mom} and P_{tin7}. DNase I footprinting of RNAP and C protein on P_{mom} and P_{tin7} was carried out as described under "Experimental Procedures." Plasmids pLW4 and pLW4*tin7* were incubated with RNAP, and increasing concentrations of C were added and then treated with DNase I. The *bottom strand* was mapped by primer extension. *Lanes 1, 6, 7,* and *11* show the DNase sensitivity of the *mom* and *tin7* promoter DNAs. The sensitivity toward nuclease is different in P_{mom} and P_{tin7} (indicted by *asterisks next to lane 7*) because of the T→G substitution and removal of intrinsic distortion (12). *C*, transcription activity of P_{mom} and P_{tin7} and effect of C protein, assessed by β-galactosidase assays.

ated in pVR7 (18). The BstXI-NruI fragment of the C protein open reading frame in pVN184 was replaced with the BstXI-EcoRV fragment of pVR7R105D to generate pVNR105D.

β-Galactosidase Assay—β-galactosidase assays were carried out in *E. coli* DH10B, as described by Miller (19). For P_{mom} transcription activity assay, plasmid pLW4 containing *mom-lacZ* fusion was used, and for P_{tin7}, pLW4*tin7* containing *tin7-lacZ* fusion (Table 1) was used as the reporter construct. To assess the effect of C or R105D, protein-expressing plasmid pVN184 or pVNR105D (Table 1) was used with the reporter plasmids in different combinations. The data presented is based on three independent measurements of the activity.

Protein Purification—Wild-type and mutant C proteins were purified from *E. coli* BL 26 (DE3) carrying plasmid pVR7 or pVR7R105D as described by Ramesh *et al.* (20). The protein preparations were >95% pure, as judged by silver-stained SDS-polyacrylamide gels. RNAP was purified from *E. coli* MG1655 according to the method of Kumar and Chatterji (21) and found to be >90% pure and devoid of any contaminating nucleases.

DNase I Footprinting—Experiments were carried out as described by Basak *et al.* (12). Two μg (0.36 pmol) of plasmid pLW4 or pLW4*tin7* was incubated with 20 nM RNAP, with or without C protein (20 and 40 nM) in footprinting buffer (20 mM Tris-HCl, pH 7.2, 1 mM EDTA, 5 mM $MgCl_2$, and 50 nM NaCl). DNase I (final concentration 0.1 ng/μl) was added and incubated for 30 s at 22 °C. Reactions were stopped by adding stop buffer (0.1 M Tris-HCl, pH 7.5, 25 mM EDTA, and 0.5% SDS). Samples were extracted successively with phenol/chloroform and chloroform and then precipitated with ethanol in the presence of glycogen as a carrier. Primer extension was by the method of Gralla (22) and carried out using *mom* forward primer as described earlier (15). Briefly, the footprinted DNA samples were used as templates for extension with Klenow polymerase after annealing with the *mom* forward primer (5′-GAAACGAGCGCATATA-3′). The reactions were analyzed in 6% urea polyacrylamide gel as described previously (10).

Electrophoretic Mobility Shift Assay (EMSA)—For EMSA, a 72-bp 5′ ^{32}P-labeled *mom* or *tin7* promoter DNA fragment was used. To study

the RNAP·DNA closed complex, an increasing amount of RNAP was incubated with DNA for 15 min on ice with or without C protein in transcription buffer (40 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 100 mM KCl, 100 μg/ml bovine serum albumin). Samples were analyzed on 3.5% non-denaturing polyacrylamide gel in 0.5× Tris borate-EDTA buffer. The gel was fixed in methanol:acetic acid:water (1:2:7) and subjected to phosphorimaging and densitometry. DNA·RNAP complex (DP) and free DNA (D) were quantified using Image Gauge (version 2.54) software. The intensity of the band was normalized with the band area to get the intensity/area value. The gel background value was subtracted from the quantified values of each band. According to the equation D + P ↔ DP, K_B = [DP]/[D][P], or [DP]/[D] = K_B·[P]. [DP]/[D] values were plotted as a function of RNAP concentrations ([P]), where the slope of the plot is the measure of K_B of RNAP binding.

For RNAP·DNA heparin-resistant complex, the closed complex was first formed as described above. To one set, C was added just before transferring the reaction to 37 °C. Aliquots were taken out at different time intervals; free RNAP was competed out with heparin (0.1 mg/ml) and analyzed on a 3.5% running non-denaturing polyacrylamide gel.

Assay for open complex formation at P_{mom} was carried out using the *mom* promoter fragment in which the −10 element of the *P2* is disrupted (*WT-P2*) (16). Increasing concentrations of RNAP and C or R105D were incubated with the DNA. Heparin was added to the open complexes and then subjected to EMSA.

Open Complex Stability Assay—The stability of the RNAP·promoter open complex was assayed as described by Anthony and Burgess (23). Open complexes were formed using the 40 nM *tin7* promoter DNA and 100 nM RNAP in 60 μl of reaction volume for 15 min at 37 °C. Heparin was added with or without 300 nM C protein. Aliquots were taken out at different time intervals, and transcription was initiated by the addition of 0.1 mM NTPs and (300 counts/min/pmol of ATP) [α-^{32}P]ATP. After 30 min, the reactions were stopped by adding stop buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol) and analyzed on an 8% polyacrylamide gel containing 8 M urea.

Promoter Clearance Assay—The assay was carried out as described by Glinkowska *et al.* (24). Open complexes were formed as described above and incubated with heparin for 1 min. To one set, 300 nM C protein was added. Transcription was initiated by the addition of NTPs and [α-^{32}P]ATP. Aliquots were withdrawn at different time intervals, and reactions were stopped by stop buffer. The products were analyzed on an 8% polyacrylamide gel containing 8 M urea. To assess the effect of C on abortive transcription, a promoter clearance assay was carried out. Half of the reactions were analyzed on an 8% urea polyacrylamide gel to resolve the runoff product and the other half on a 25% urea polyacrylamide gel to resolve abortive transcripts. In case of the *mom* (*WT-P2*) promoter, RNAP·promoter open complexes were formed in the presence of C or R105D. Single round transcription was carried out by adding heparin and NTPs with [α-^{32}P]ATP for 30 min. All of the assays were repeated at least three times, and standard deviations were included where required.

RESULTS

Transcription from C-independent Promoter Is Activated by C—The phage Mu *mom* gene is located at the right-most end of the genome. Earlier studies have established that P_{mom} is not expressed until the late lytic cycle of the phage Mu (5), and its activation is dependent on transactivator C (25). In a spontaneous mutant of P_{mom} (designated P_{tin7}), however, efficient transcription is observed (10). In this case, single base change T→G at the −14 position converted a very weak promoter into an extended −10 promoter (26). RNAP binding to P_{mom} is not observed in the absence of the transactivator, and instead, a weak protection is observed at the *P2* region (Fig. 1*B*, *lane 2*) (10). In contrast, with P_{tin7}, a DNase I footprint protecting the −10 and −35 regions of the *mom* promoter is seen (Fig. 1*B*, *lane 8*). With the increasing amount of C, however, RNAP binds to the P_{mom} (Fig. 1*B*, *lanes 3* and *4*). These results are in accordance with the earlier experiments using $KMnO_4$ footprinting (10, 12). As a result of the binding of the polymerase to P_{tin7}, even in absence of C, a high level of transcription was observed in reporter β-galactosidase assays (Fig. 1*C*). Significantly, the transcription is further enhanced in the presence of C (Fig. 1*C*). This behavior of C is in contrast to that of the CAP response at a transactivator-independent mutant *lac* promoter. RNAP alone efficiently binds to the mutant promoter, and the addition of CAP did not significantly enhance the transcription, as the transactivator is required only for the initial binding of RNAP (27). The activation of the *tin7* promoter by C indicated that the protein has a role at a step beyond RNAP recruitment during transcription initiation.

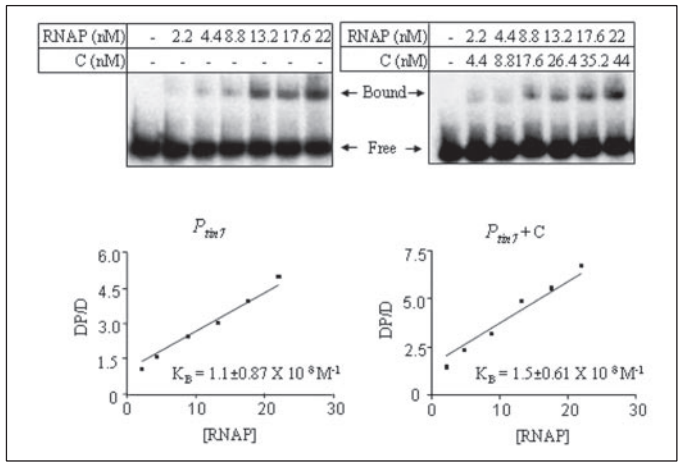

FIGURE 2. **Determination of K_B of RNAP binding to the *tin7* promoter in the absence and presence of C protein.** Increasing concentrations of RNAP (indicated in nM) was incubated with the *tin7* promoter fragment in the absence and presence of C. The amount of free and RNAP-bound DNA was quantified and analyzed to determine K_B of RNAP binding. DP:D values, the ratio between bound and free DNA, were plotted as a function of the concentration of RNAP, and the slopes of the plots are the K_B values (see "Experimental Procedures") indicated *within* the *graphs*.

Effect of C on RNAP·Promoter Closed Complex—The function of C at different steps of promoter-RNAP interactions was investigated further. Although the RNAP footprinting experiments on P_{tin7} DNA showed efficient binding of RNAP in the absence of C (Fig. 1*B*, *lane 8*) (10, 12), the protein could enhance or stabilize the binding. To assess whether C further influences the binding of RNAP at P_{tin7} and also to obtain more quantitative information, EMSAs were carried out. The end-labeled *tin7* promoter fragment of 220 bp was incubated with increasing concentrations of RNAP in the presence and absence of C protein and analyzed by EMSA to estimate the kinetics of the closed complex formation. Based on several experiments and the pattern obtained, the equilibrium binding constant or K_B of RNAP binding was determined (Fig. 2). The K_B in the absence of C protein is 1.1 ± 0.87 × 10^8 M^{-1} and in presence of C is 1.5 ± 0.61 × 10^8 M^{-1}, indicating that the closed complex formation at the C-independent promoter *tin7* is not further increased by the transactivator. It should be noted that P_{mom} is not amenable for K_B measurement, as RNAP on its own is unable to form a closed complex (see Fig. 1*B*, *lane 2*).

Effect of C on Open Complex Formation and Stability—Conversion of a closed to open complex has the potential to be a rate-limiting step in transcription initiation. Many activators influence this step by increas-

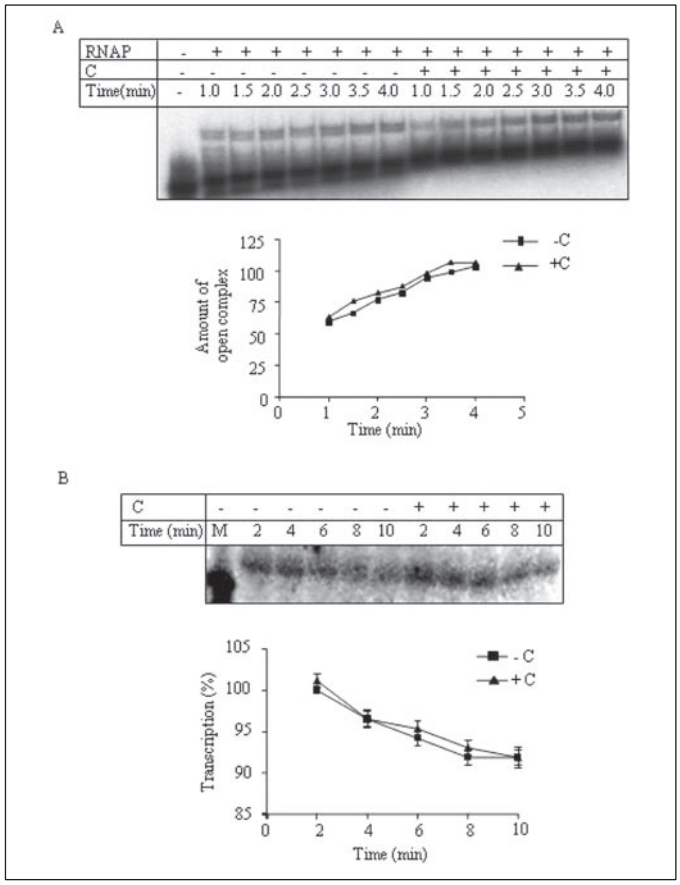

FIGURE 3. **Effect of C protein on open complex formation and stability.** *A*, open complex formation was analyzed by incubating RNAP (15 nM) with the radiolabeled *tin7* promoter fragment (5 nM) to form an open complex. To one set, 30 nM C was added. At different time intervals (indicated in min), samples were taken out and subjected to EMSA (see "Experimental Procedures"). The lower bands represent free DNA, and the upper bands represent heparin-resistant RNAP·promoter complex. The protein-bound DNA was quantified and plotted as a function of time. A representative set of data is presented based on several sets of experiments. *B*, stability of the open complex was analyzed by *in vitro* transcription. The linear *tin7* promoter fragment (40 nM) was incubated with 100 nM RNAP to form an open complex and then with heparin in the absence or presence of 300 nM C protein. At different time intervals, as indicated in the figure, aliquots were taken out for transcription. Percent transcriptions were plotted as a function of time, taking transcription in the absence of C at 2 min as 100%. S.D. values are based on three independent experiments.

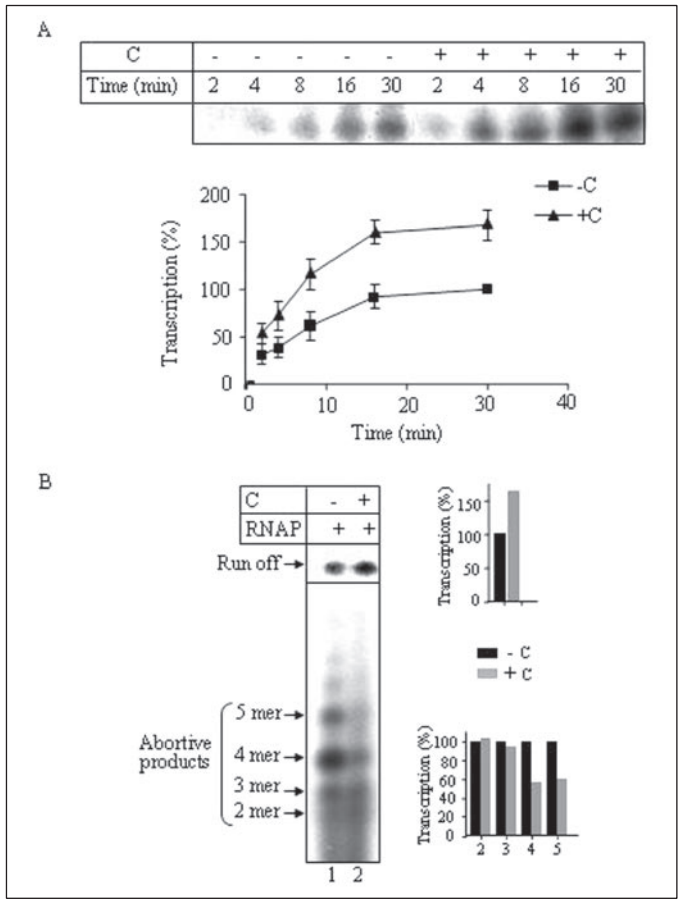

FIGURE 4. **Effect of C protein on the promoter clearance and abortive transcription at P_{tin7}.** *A*, promoter clearance assays were carried out as described under "Experimental Procedures." Open complexes were challenged with heparin, and then transcriptions were initiated by the addition of NTP, with or without 300 nM C. The products were analyzed on urea polyacrylamide gel. The plot shows quantitative representation of the promoter clearance. Transcriptions carried out in the absence of C for 30 min were taken as 100% to plot percent transcription. *B*, analysis of abortive transcription products. Transcription was carried out as described above for 30 min. Abortive and productive transcripts were analyzed on 25 and 8% urea polyacrylamide gels, respectively. The *bar diagrams* next to the data are quantitative representations of runoff (*top*) and abortive transcripts (*bottom*). The *numbers* on the *x*-axis of the *bottom diagram* represent the length of the different abortive transcripts. Transcription without C protein was taken as 100%.

ing the rate of promoter melting. For example, in the case of the λ P_{RM} promoter, λ cI protein increases the rate of isomerization (28). We investigated the effect of C protein on open complex formation. RNAP was incubated with the ^{32}P-labeled *tin7* promoter fragment to form open complexes for different time spans in the absence and presence of C protein and then subjected to EMSA. There was no significant difference in the rate or amount of open complexes formed in the absence and presence of C protein, indicating that C does not have any influence on the isomerization step (Fig. 3*A*).

The dissociation of RNAP from the promoter leads to the decay of the open complex. An unstable or more stable complex would be a rate-limiting condition for the next step of the reaction. In the case of the *rrnB P1* promoter, Fis stabilizes the unstable open complex and thereby activates transcription (29). To assess the stability of the open complex at P_{tin7}, RNAP·DNA open complexes were incubated (in the absence and presence of C) with heparin for different time intervals. At different time points, aliquots were taken and transcription was initiated by adding NTPs. Longer incubation in the presence of heparin would result in more decay of the isomerized complexes and less transcription. The amounts of transcripts, representative of the existing complexes at each time point, were the same in the presence and absence of C protein (Fig. 3*B*). From these experiments, it is apparent that the transactivator has no appreciable effect on the isomerization or stability of the open complex.

Effect of C on Promoter Clearance at P_{tin7}—To investigate the influence of C protein after open complex formation, *i.e.* at the step of promoter clearance at P_{tin7}, typical promoter clearance assay was performed (24, 30). RNAP·DNA open complexes were incubated with heparin, and transcription was initiated by the addition of either NTPs alone or NTPs and C protein. The addition of C after heparin allowed us to assess the effect of the transactivator at subsequent steps following promoter melting. Comparison of the amount of transcripts in two reactions at different time intervals represents the effect of C protein on promoter clearance. The data presented in Fig. 4*A* shows increase in the amount of transcripts in the presence of C protein, indicating that C protein enhances promoter clearance from P_{tin7}.

C Enhances Promoter Clearance by Reducing Abortive Transcription from P_{tin7}—In the experiment described above, to evaluate promoter clearance, although equal amounts of RNAP were used and the extent of open complex formation was the same, transcription in the absence of C

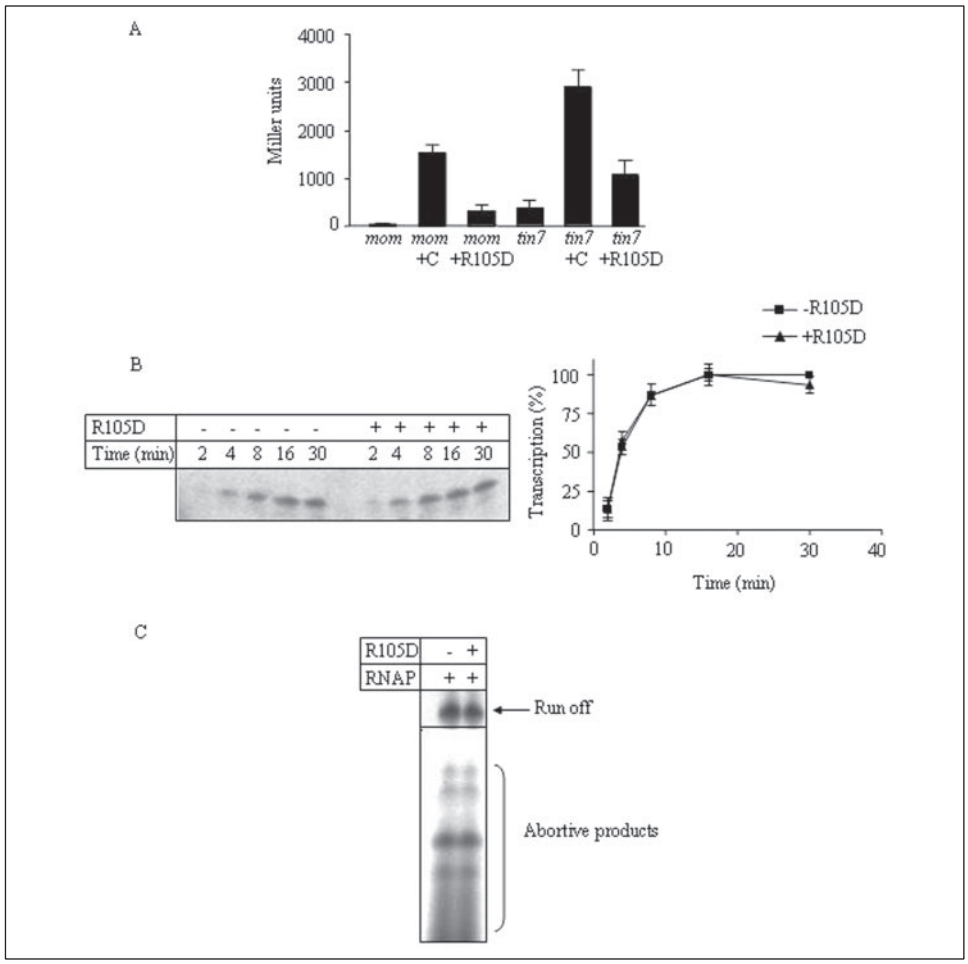

FIGURE 5. **Effect of R105D mutant on transcription.** *A*, transactivation efficiencies of C and R105D at the *mom* and *tin7* promoters were estimated using β-galactosidase assays as described under "Experimental Procedures." *B*, effect of R105D on promoter clearance at P_{tin7}. Assays were carried out as described in the legend to Fig. 4*A* and "Experimental Procedures," in the absence and presence of 300 nM R105D mutant protein. The *graph* shows quantitative representation. *C*, effect of R105D on abortive transcription at P_{tin7}. Experiment was carried out as described in the legend to Fig. 4*B*, with or without 300 nM R105D protein.

did not reach the level observed in the presence of C. Thus, it appeared that some of the RNAP molecules in the absence of C do not complete transcription. The non-productive RNAP·promoter complex would synthesize more abortive RNA products and hence fail to enter the elongation phase. To assess the effect of C on abortive transcription at P_{tin7}, both the productive and abortive transcripts of the promoter clearance assay were analyzed. In the absence of C, more abortive transcripts were synthesized at P_{tin7} (Fig. 4*B*, *lane 1*), and C protein reduced the abortive phase of initiation with a concomitant increase in the runoff transcripts (Fig. 4*B*, *lane 2*).

Enhanced Promoter Clearance Is Compromised in a Positive Control Mutant—To address further the role of C at the promoter clearance step, we carried out experiments with a pc mutant of the transactivator. The HTH motif of the C protein, present toward the carboxyl-terminal region, is responsible for DNA binding (18), and Arg-105 lies in this motif. The R105D mutant of C binds DNA with an affinity comparable with wild-type C protein (18). However, it showed reduced transactivation at both the *mom* and *tin7* promoters measured by reporter β-galactosidase assays (Fig. 5*A*) (18). To dissect out the effect of the mutation on C-mediated transcription activation, the promoter clearance assay was carried out with the pc mutant. The mutant transactivator did not enhance RNAP clearance from P_{tin7}, unlike the wild-type C protein (Fig. 5*B*). The high level of abortive transcription observed in the absence of the transactivator was unaltered in the presence of R105D (Fig. 5*C*).

C Protein Reduces Abortive Transcription at P_{mom}—To assess the role of C protein in abortive initiation and promoter clearance steps at P_{mom}, we compared the reactions between wild-type C and R105D. To avoid the complications arising because of transcription from the divergent *P2*, we used the *mom* promoter fragment, where the *P2* −10 element has been disrupted (16). RNAP·promoter open complexes were formed in the presence of C or R105D, and single round transcription was initiated by adding heparin and NTPs. The amount of productive transcripts was lower in the presence of R105D with the concomitant increase in abortive RNA products (Fig. 6*A*, *lane 3*). Conversely, transcription in the presence of C protein had less abortive transcripts and enhanced full-length transcripts (Fig. 6*A*, *lane 2*). To verify that the effect of R105D, seen by the *in vitro* transcription experiment, is not due to compromised RNAP recruitment at P_{mom}, we examined the formation of the heparin-resistant RNAP·promoter complex in the presence of C or R105D. The EMSA experiment, described in Fig. 6*B*, reveals that mutant R105D leads to the formation of the RNAP·promoter open complex to the same extent as that of C protein itself, indicating that R105D is not compromised in the binding of RNAP or isomerization but only fails to enhance promoter clearance. These experiments together establish a new role for C, besides RNAP recruitment, at a step beyond promoter melting during transcription activation of the *mom* promoter.

DISCUSSION

The initiation of transcription involves a series of steps of promoter-RNAP interactions before RNAP engages itself in transcribing the template. Each one of these steps could be regulated (31), and most of the transcription activators influence one of the steps in the transcription initiation. We have demonstrated here that C protein of bacteriophage Mu activates P_{mom} in a unique fashion by acting at two steps of the

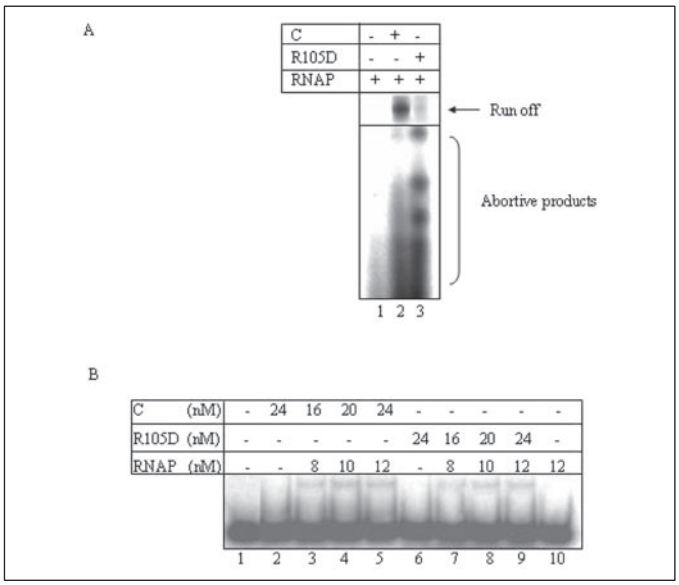

FIGURE 6. **Effect of C and R105D on transcription at the *mom* promoter.** The reactions were carried out as described under "Experimental Procedures." *A*, abortive and productive transcription in the presence of C and the R105D mutant. Open complexes were formed on the 40 nM *mom* promoter DNA fragment using 100 nM RNAP with 300 nM C or R105D mutant protein. Transcription was initiated by the addition of heparin and NTPs. *B*, RNAP recruitment by C and R105D at the *mom* promoter. End-labeled DNA (5 nM) was incubated with increasing amounts of RNAP and C or R105D (indicated in nM) to form the open complex and then challenged with heparin. Heparin-resistant complexes were subjected to EMSA. RNAP alone does not bind to P_{mom} (*lane 10*) and binds only in the presence of wild-type C (*lanes 3–5*) or R105D mutant (*lanes 7–9*).

transcription initiation. RNAP, on its own, is unable to bind to P_{mom}. Site-specific binding of C and the consequent realignment of promoter elements lead to RNAP binding (14, 15). After the closed complex formation, C does not influence the isomerization step or the stability of the open complex but enhances promoter clearance. C reduces abortive initiation and leads to the efficient conversion of initiation to the elongation complex, thereby strongly and irreversibly activating transcription.

A majority of the activators influence the recruitment of RNAP in the transcription initiation. The activators either increase RNAP binding to the promoter or, when it is already bound, influence isomerization from the closed to open promoter complex (1). For example, CAP increases the initial binding of RNAP at the *lac* promoter (32), and λ cI protein positively influences the rate of isomerization at the λ P_{RM} promoter (28). A few activators have also been shown to act at a post-recruitment step of transcription initiation, as observed in the case of Arc, which enhances promoter clearance from the P_{ant} promoter of bacteriophage P22 during late lytic growth (2). It is also well established that a given activator could influence different steps of transcription initiation in different promoters. Most of the CAP-dependent promoters are regulated at the RNAP recruitment step; however, at the *malT* promoter, CAP enhances promoter clearance (33, 3). Using the CAP and mutant *lac* promoter, it has been demonstrated that regulatory proteins could act as activators or repressors in different steps of the transcription initiation pathway, depending on the energetic differences of the intermediate complexes (34). Another well studied example is Fis, which stimulates RNAP binding by interacting with the α-carboxyl- terminal domain at *proP* P_2 (35). Two other promoters, *leuV* and *rrnB* P_1, are activated by Fis at the steps of isomerization (36) and open complex stability (29), respectively. In the vast repertoire of transcription activators, there are few that act at multiple steps of transcription initiation at a single promoter. The most well known example is CAP-mediated activation of the initial binding of RNAP and subsequent isomerization at the *gal P1* promoter (37). Recruitment of RNAP and subsequent promoter clearance at the λ P_R promoter are enhanced by DnaA (24). Fis has been reported to sequentially stimulate transcription initiation steps at the *tyrT* promoter by facilitating initial binding of RNAP, unwinding of DNA at the transcription start point, and subsequent promoter clearance (38). At the P_{RE} promoter of the λ phage, cII protein enhances both the formation and stability of the RNAP·promoter open complex (39). Transactivator C thus belongs to a small group of activators acting at multiple steps of promoter-polymerase interactions in a single promoter.

A majority of activators interact with one or more subunits of RNAP to influence K_B, k_f, subsequent ternary complex formation, or switch over to elongation. Employing a variety of assays, including gel filtration, chemical cross-linking, far western, surface plasmon resonance refractometry, and yeast two-hybrid assays, we have been unable to detect interaction of C with the RNAP core or holoenzyme. Thus, it appears that the C interaction with RNAP, if it occurs, is very transient and not easily amenable for experimental analysis.

The complex regulatory network of *mom* prevents its premature or unnecessary expression and ensures expression only in the late lytic cycle. It is not difficult to visualize the need for elaborate negative regulatory mechanisms to keep *mom* expression under tight control, considering the cytotoxic effects of the gene product. In addition to OxyR-mediated repression, promoter occlusion by divergent *P2* and intrinsic distortion near the −10 element effectively prevent basal expression of *mom*. These measures, in conjunction with weak promoter elements and their suboptimal spacing, establish very tight negative regulation. However, after promoter derepression during the late lytic cycle, the importance of the multistep transactivation process in turning on *mom* is rather intriguing. In hindsight, one could envision the importance of such a mode of transcription activation for a gene, which needs to be expressed for a very brief duration during the life cycle of a phage. Unlike P_{mom}, most promoters, weak or strong, exhibit a basal level of activity. In all of these cases, where operons are involved in biosynthesis or degradation, activation or repression steps are readily reversible under various environmental signals. Indeed most genetic switches operate based on "on" or "off" mechanisms, wherein existing basal levels are altered. These metabolic switches are designed with built-in reversibility. In contrast, the turning on of P_{mom} has to be irreversible, occurring during the late lytic cycle after the phage replication and transcription of all of the other genes. At the first step of activation, C binding would occlude RNAP binding to *P2* and recruit it to P_{mom}, overcoming intrinsic distortion. By enhancing the promoter clearance of RNAP, C protein ensures the switch is irreversible during the last phase of the lytic developmental cascade.

Acknowledgments—We are thankful to B. D. Paul for the pc mutant of C (R105D) and S. Hattman for the plasmids pLW4 and pLW4tin7.

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