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Transcriptional Activator C Protein-mediated Unwinding of DNA as a Possible Mechanism for *mom* Gene Activation

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The bacteriophage Mu *mom* gene encodes the unique DNA-modification function of the phage. Regulation of the *mom* gene at the transcriptional level is brought about by the transactivator protein C of the phage. The *mom* promoter is an activator-dependent weak promoter having poor −10 and −35 elements separated by a 19 bp suboptimal spacer region. These features could constrain RNA polymerase occupancy at the promoter. Here, we have probed into the mechanism by which C protein acts as a transcriptional activator at P_{mom}. *In vivo* dimethyl sulfate footprinting studies demonstrate C protein-mediated asymmetric distortion of its specific site at the *mom* regulatory region. Using a coupled topoisomerase assay, we demonstrate that C protein induces the unwinding of DNA. This C-mediated unwinding seems to be localised to the 3′ flanking region of the C binding site located adjacent to and overlapping the −35 element of P_{mom}. These results suggest that C protein-mediated torsional changes could be reorienting the −10 and −35 elements to a favorable conformation for RNA polymerase occupancy at the *mom* promoter.

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Introduction

Accurate initiation of transcription often requires interactions of the activator protein(s) with specific sequences and RNA polymerase (RNAP) in addition to the promoter-polymerase interactions (Raibaud & Schwartz, 1984). This requirement for the mediation by activator proteins in the transcription activation process is a consequence of the promoter architecture. Activator proteins bind at various distances from the weak promoters to enhance binding or open-complex formation by RNAP (Busby & Ebright, 1994). A large number of activator proteins have been identified in *Escherichia coli* itself and their mode of interaction has been studied (Collado-Vides *et al.*, 1991; Ishihama, 1993). One would predict different transcription activation mechanisms to be operational considering the diversity of transcriptional activator proteins and their specific interactions. Apart from the protein-protein interactions between the activator and RNAP, protein-induced DNA distortion also contributes to the mechanism of transcription activation (Raibaud & Schwartz, 1984; Ishihama, 1993; Niu *et al.*, 1996).

Here, we have investigated the role of bacteriophage Mu C protein in the transcription activation of the *mom* gene promoter. The *mom* gene product encodes a novel DNA-modification function of the phage (Kahmann & Hattman, 1987). Expression of the *mom* gene is controlled in a complex manner at both the transcriptional and translational levels (Hattman, 1982; Bolker & Kahmann, 1989; Hattman *et al.*, 1991; Wulczyn & Kahmann, 1991). Positive control of *mom* gene expression is brought about by the C protein (Hattman *et al.*, 1985; Heisig & Kahmann, 1986), which is a site-specific DNA binding protein that functions as an activator of transcription from the four late promoters, P_{lys}, P_p, P_i and P_{mom}, of phage Mu (Bolker *et al.*, 1989; Chiang & Howe, 1993). The C binding site has been located from the −28 to the −57 position (Gindlesperger & Hattman, 1994; De *et al.*, 1998; Ramesh, 1997) in P_{mom} (Figure 1). C protein binds to its site as a dimer (De *et al.*, 1997) with a very high affinity (Ramesh *et al.*, 1994a), and Mg^{2+} acts as a cofactor for this site-specific binding (De *et al.*, 1998). In the absence of C protein, *E. coli* RNAP binds to an upstream apparently non-functional

Abbreviations used: RNAP, RNA polymerase; DMS dimethyl sulfate; $(OP)_2Cu$, 1,10-phenanthroline-copper.

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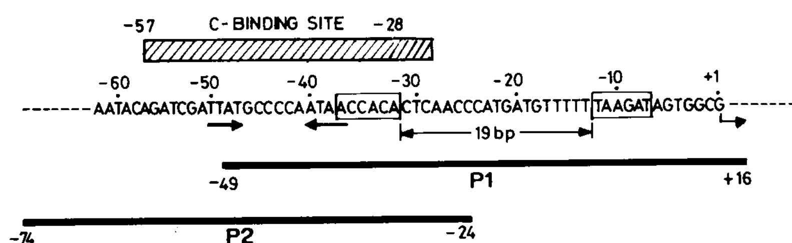

Figure 1. The Mu *mom* regulatory region. The C binding site located between −57 and −28 is shown as a hatched box. The tetranucleotide palindromic sequence within the C binding site is shown with two inverted arrows. The −10 and −35 promoter elements are boxed. The regions protected by RNAP in the presence (P1) and absence (P2) of C protein are indicated with thick lines. The 19 bp spacer region and the +1 start site of P1 are indicated.

promoter P2. In the presence of C, it binds to the downstream functional promoter P1 (Balke *et al.*, 1992), which shows the characteristics of an activator-dependent weak promoter (Raibaud & Schwartz, 1984; Nagaraja, 1993). P1 has a poor −35 region (ACCACA) and a suboptimal 19 bp spacer region between the −10 and −35 sequences (Figure 1).

The contact points for C protein at its specific binding site in P_{mom} have been determined using various footprinting agents (Ramesh & Nagaraja, 1996; Sun *et al.*, 1997). These studies indicate that C protein binds to one face of the helix, distorts DNA and makes major groove contacts. Now, we have carried out an in-depth analysis of C-mediated conformational changes in the *mom* promoter region. The C protein-mediated distortion is asymmetric in nature, and the binding leads to localized unwinding of DNA, which could be a prerequisite for the transactivation of P_{mom}.

Results

In vivo dimethyl sulfate footprinting analysis with C protein

Site-specific interaction of C protein at its target site in the *mom* promoter region was studied by *in vivo* footprinting analysis. This type of analysis would show contact points and distortions brought about by the protein upon binding to its cognate site. Such an analysis with supercoiled plasmid would serve two purposes. It provides a realistic assessment of (1), protein contact in the natural context and (2) the influence of overall topological constraints (if any) on protein binding. Dimethyl sulfate (DMS) serves as a useful probe for examining protein-DNA interactions *in vivo* by the primer-extension analysis (Borowiec & Gralla, 1986).

The cells harboring the plasmid containing the *mom* regulatory region (pBM2) were treated with DMS under conditions of C protein expression as described in Materials and Methods. The DNA was cleaved with piperidine to display the contacts with the N7 group of guanine residues in the top and bottom strands (Figure 2(a) and (b)). The pattern of DNA methylation of naked DNA by DMS under identical conditions served as the control. The −35 and −36 Gs in the bottom strand were protected completely against methylation, whereas the −52 G in the top strand was protected to a lesser extent. Thus, the protected region extends beyond the interrupted palindromic sequence (Figure 1). The Gs at positions −33, −43 and −53 in the bottom strand and the adenine at the −37 position in the top strand were hyper-methylated. This enhanced activity could be a result of a more exposed site caused by protein-induced structural alterations. The ten base-pair periodicity of hypersites (−33 G, −43 G and −53 G) in the bottom strand could reflect the protein-induced bending. Densitometric scanning (Figure 3) of protected and hypersensitive Gs indicate different intensities of reactivity. While the −35 and −36 Gs show complete protection (unlike the −52 G), the −53 G located adjacent to the 5′ half site exhibits maximum hyper-reactivity amongst the three hyper-reactive Gs. Thus, the overall interaction pattern seems to be asymmetric despite having an internal palindromic sequence in the C binding site. This point is further supported by an *in vitro* A > G DMS reaction wherein the −37 adenine in the top strand is hypersensitive upon C protein binding (Figure 2(c)). This "A" is localized at the 3′ half site and is the only hypersensitive adenine amongst the eight "A"s of the internal palindromic sequence (5′-TTAT—ATAA-3′) found in the C recognition site. In this A > G analysis, the −33 and −43 Gs (bottom strand) also showed hyper-reactivity to some extent. The overall protein contacts mapped by *in vivo* experiments are in agreement with those obtained by *in vitro* DMS-protection analysis with double-stranded DNA fragments containing the C binding site (Ramesh & Nagaraja, 1996; Sun *et al.*, 1997). Based on the G-protection analysis described here and earlier, the minimal target site of C at P_{mom} is 5′-GATTATgccccaATAACC-3′.

$(OP)_2Cu$ footprinting analysis of the *mom* regulatory region

In vivo footprinting and the *in vitro* A > G DMS reaction indicated asymmetric distortion of the C binding site upon protein binding. Complete protection of the −35 and −36 Gs (as opposed to the

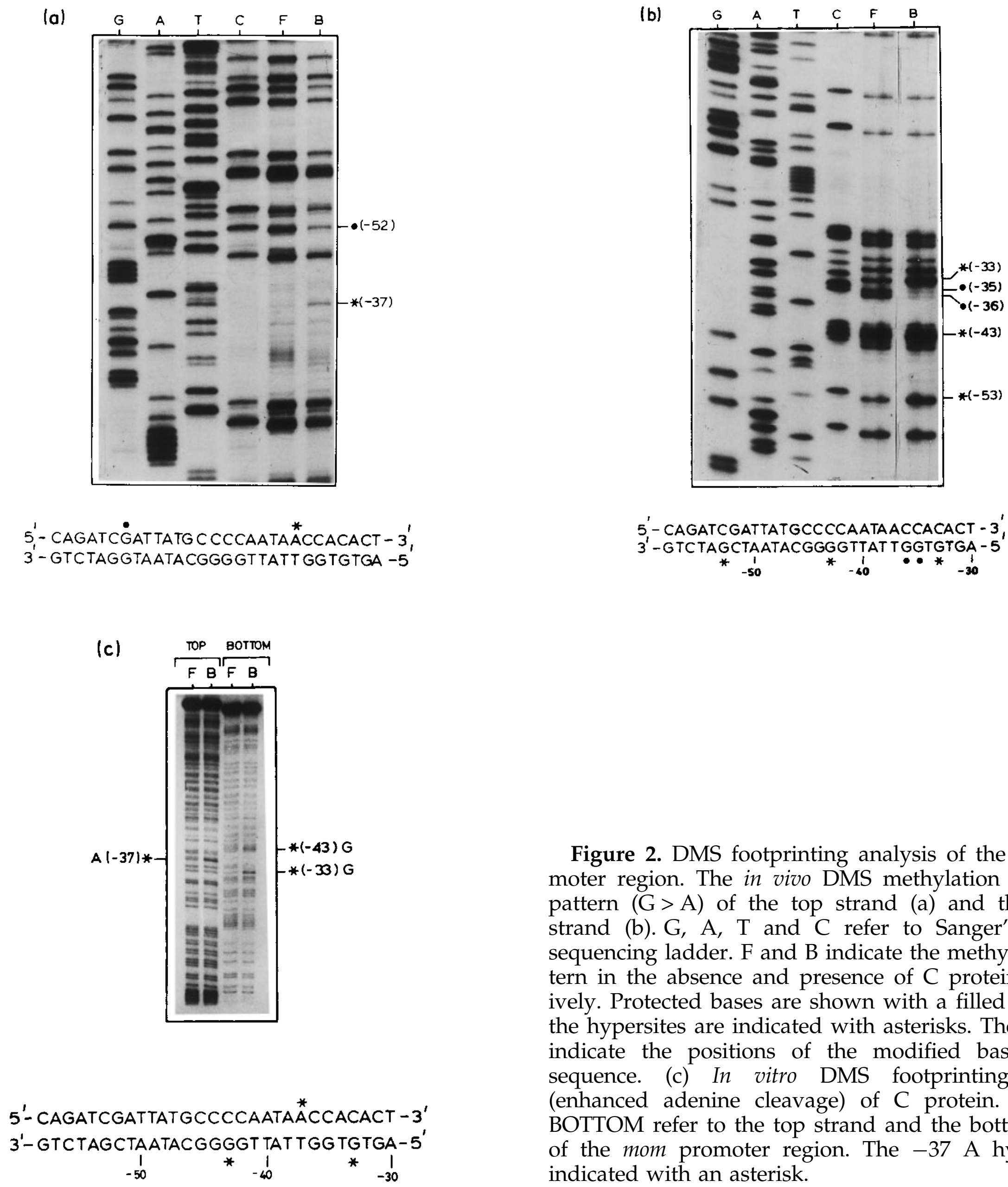

Figure 2. DMS footprinting analysis of the *mom* promoter region. The *in vivo* DMS methylation protection pattern (G > A) of the top strand (a) and the bottom strand (b). G, A, T and C refer to Sanger's dideoxy sequencing ladder. F and B indicate the methylation pattern in the absence and presence of C protein, respectively. Protected bases are shown with a filled circle and the hypersites are indicated with asterisks. The numbers indicate the positions of the modified bases in the sequence. (c) *In vitro* DMS footprinting analysis (enhanced adenine cleavage) of C protein. TOP and BOTTOM refer to the top strand and the bottom strand of the *mom* promoter region. The −37 A hypersite is indicated with an asterisk.

−52 G) and the hypersensitivity of the −37 A reflects the asymmetric interaction of C with the target site (Figures 2 and 3; see also Figure 5). This could also be inferred from the pattern obtained with hydroxy-radical footprinting (Ramesh & Nagaraja, 1996; Sun *et al.*, 1997). The region towards the end of the 3′ half site in the top strand was more accessible to free radicals compared to the other two protected regions, while the bottom strand showed three protected regions of near equal intensity. The chemical nuclease 1,10-phenanthroline-copper ($(OP)_2Cu$), which has the ability to detect protein-induced conformational changes in DNA (Spassky & Sigman, 1985), was used to address this aspect further. This minor-groove-specific ligand is also known to intercalate into underwound DNA (Sigman *et al.*, 1991). A supercoiled plasmid containing the *mom* promoter region was used for the footprinting analysis, as it allows a more realistic assessment of interactions as compared to a linear fragment. Figure 4 shows the protection pattern on the top strand of the *mom* promoter region. While C-mediated protection was observed, there are two hypersensitive residues at positions −31 and −32 (C and A), which lie in the region flanking the 3′ half site of the C-binding site. The bottom strand footprint does not show any such hypersensitive sites upon protein binding (results not shown). A summary of the footprinting data is shown in Figure 5. $(OP)_2Cu$ footprinting carried out with a linear fragment also showed hypersensitivity in the three bases (CAC) at positions −31, −32 and −33 of the top strand (Ramesh & Nagaraja, 1996). The minor difference

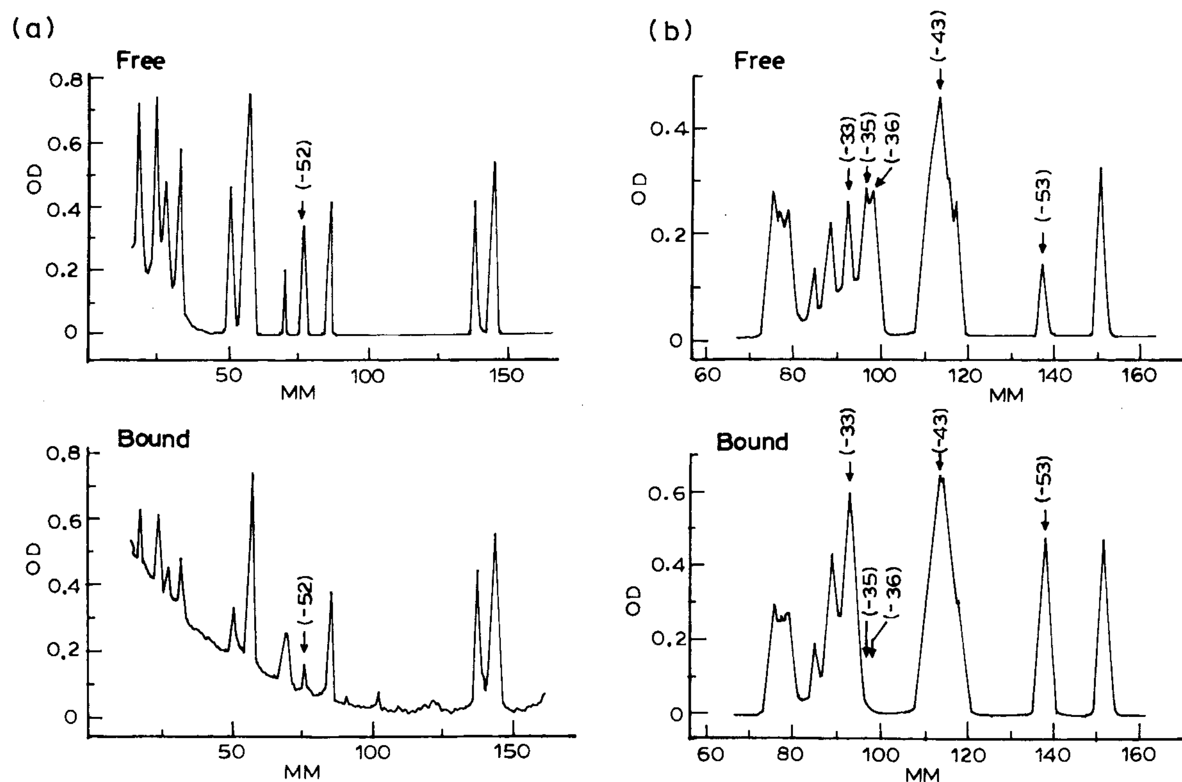

Figure 3. Densitometric scan of Figure 2(a) and (b): (a) top and (b) bottom strand of the *mom* promoter region. The protected and the hypersensitive bases are indicated.

in the hypersensitivity pattern in these different sets of experiments could be due to the choice of templates having different topological forms. The enhanced cleavage observed above could reflect widening of the minor groove as a result of C binding. Widening of the minor groove could also be a consequence of the untwisting of DNA. Alternatively, a change in groove width could also be interpreted as the result of the protein-induced bending of DNA.

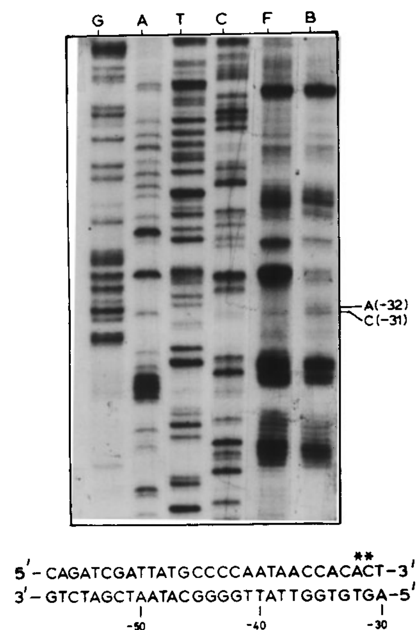

Figure 4. $(OP)_2Cu$ footprinting of the *mom* promoter region. The cleavage pattern of the top strand was analysed in the absence (F) and presence (B) of C protein. G, A, T and C refer to Sanger's dideoxy sequencing ladder of the region. Hyper-reactive residues are indicated.

C protein-induced untwisting of DNA

A coupled topoisomerase assay was used to test whether C protein mediates the untwisting of the DNA. The topoisomer distribution would change when the DNA relaxation is carried out in the presence of a ligand which untwists or overtwists DNA. After removal of the ligand, the distribution of the topoisomer species would still conform to a Gaussian curve, yet differing from the preceding one by its median, and in some cases by its width. The change in the position of the median between

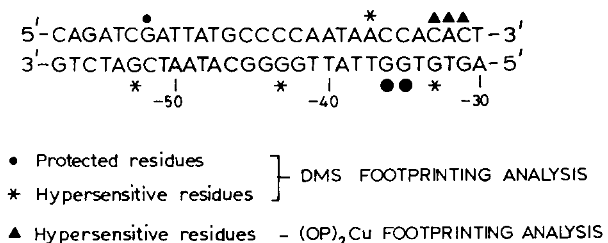

Figure 5. Summary of the footprinting data.

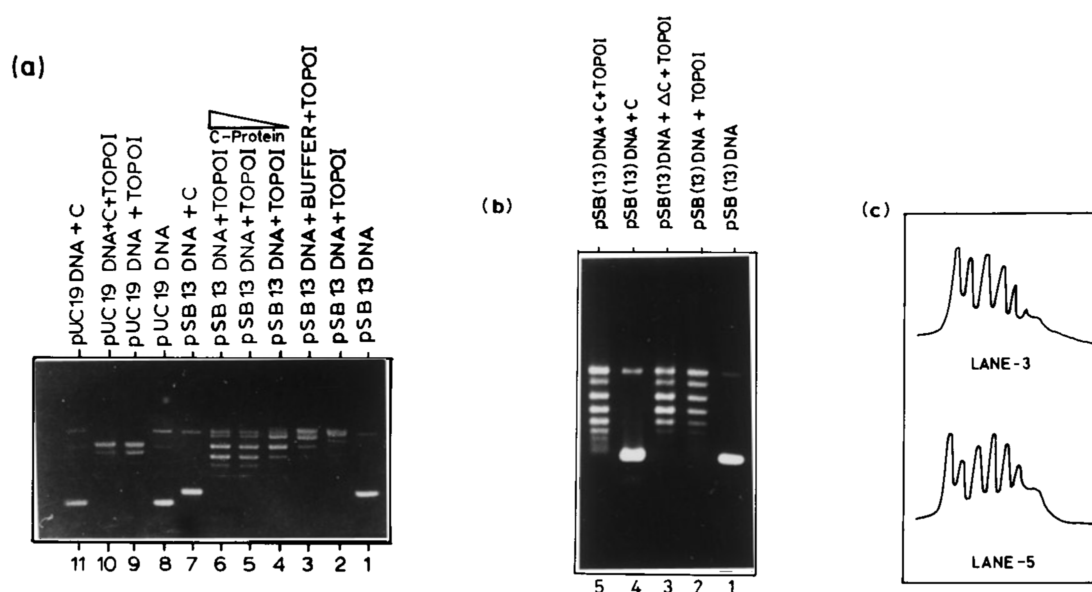

Figure 6. Coupled topoisomerase assay. (a) Supercoiled pSB13 (0.3 pmol, lane 1) was relaxed with 0.5 unit of *E. coli* topoisomerase I (lane 2) in the presence of C protein storage buffer (lane 3) and 2.0, 4.0 and 6.5 pmol of purified C protein (lanes 4, 5 and 6) at 37°C. Topoisomerase I was omitted in lane 7. Supercoiled pUC19 DNA (0.3 pmol, lane 8) was similarly relaxed with topoisomerase I alone (lane 9) or in the presence of 6.5 pmol of C protein (lane 10). C protein incubated with pUC19 is shown in lane 11. (b) Supercoiled pSB13 DNA (0.6 pmol, lane 1) was relaxed with 0.5 unit of *E. coli* topoisomerase I (lane 2) in the presence of 8.4 pmol of heat-denatured C protein (lane 3) or native C protein (lane 5). Topoisomerase I was omitted in lane 4. (c) Densitometric scan of lanes 3 and 5 from (b).

the two experiments characterizes the change in the linking number at the time of ring closure and therefore the topological winding of the DNA induced by the ligand (Kolb & Buc, 1982). The results of such an experiment with C protein and negatively supercoiled pSB13 DNA (see Materials and Methods) are presented in Figure 6(a). Preincubation with increasing amounts of C protein (lanes 4, 5 and 6) results in shifting the topoisomer distribution to a greater mobility relative to the control samples (lanes 2 and 3). Further, such an increase in C concentration did not alter the topoisomer pattern. Under these saturating amounts of C, pUC19 DNA does not show any change in the topoisomer distribution (compare lanes 6 and 10, Figure 6(a)). Heat-inactivated C protein does not form a specific complex with DNA, and hence the topoisomer distribution remains unaltered (Figure 6(b), lanes 2 and 3). The C protein-mediated change in the linking number of pSB13 could be due to either the overtwisting or untwisting of DNA. In order to distinguish between these two possibilities, the reaction products in lane 6, Figure 6(a) were further incubated with *E. coli* topoisomerase I, which can relax only negatively supercoiled DNA. The complete relaxation of topoisomers in this experiment confirmed that C protein induces the untwisting and not the overtwisting of DNA. The experiment depicted in Figure 6(b) was carried out to obtain a Gaussian distribution of the topoisomers in order to determine the C protein-mediated untwisting angle. Since writhe is likely to make a negligible contribution to the linking number of a fully relaxed topoisomer, the δLk values predominantly reflect changes in twist. The densitometric scan of the control lane (lane 3) and the test lane (lane 5) of Figure 6(b) are shown in Figure 6(c). The change in the average linking number was determined using the method of Kolb & Buc (1982). The δLk value for the C protein was calculated to be 1.04 turns of DNA or 28.8° untwisting per C binding site.

Localized unwinding of DNA by C protein

$(OP)_2Cu$ footprinting with the *mom* promoter region showed hypersensitivity at AC bases located at the 3′ half site of the C recognition sequence, reflecting the C protein-induced untwisting of the DNA. Though the protein binds to DNA as a dimer (De *et al.*, 1997) and interacts with a sequence having an internal palindrome, it seems to untwist the DNA only at the 3′ half site (at CAC; Ramesh & Nagaraja, 1996). In order to determine whether this increased reactivity is the property of the sequence *per se*, we carried out $(OP)_2Cu$ footprinting with the supercoiled plasmid pSB3 (containing a trimeric repeat of the C binding site), but having a different sequence in the hypersite region (CGA instead of CAC in the positions corresponding to −31, −32 and −33). The results of such an analysis showed an increased reactivity only towards the 3′ end of the third C binding site. No major hypersites were seen at the 5′ end of the first C binding site. The hypersites at the 3′ end in this case were offset by 2 bp (Figure 7) as compared to the hypersite position at P_{mom}. This shift in the position of the hypersites in the case of pSB3 could be because of the

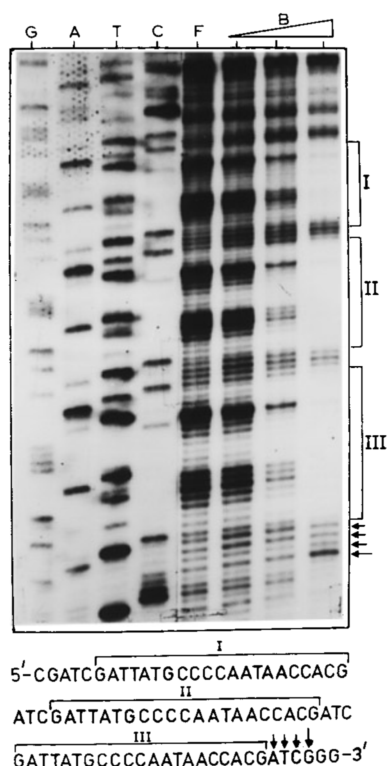

Figure 7. $(OP)_2Cu$ footprinting with the multiple C binding sites. G, A, T and C indicate Sanger's dideoxy sequencing ladder. F indicates the cleavage pattern in the absence of C protein and B indicates the pattern in the presence of increasing amounts (200, 250 and 300 ng) of C protein. Arrows indicate the hypersensitive bases on the gel and in the nucleotide sequence. I, II and III represent C sites protected upon C binding. The sequencing lane is of the complementary strand of the sequence shown below the gel.

binding of C protein to three closely placed sites oriented in the same direction. This point can also account for the absence of any major hyper-reactive bands between the three C binding sites. Hypersensitivity in the same position (corresponding to −31, −32 and −33), but with a different sequence (CGG or CGA instead of CAC) was observed when a single C binding site was used for $(OP)_2Cu$ analysis (results not shown). The following conclusions could be drawn from these experiments: (1) untwisting is localized only in the 3′ half site of the C recognition sequence; and (2) the untwisting activity of C protein is independent of the flanking sequences.

Discussion

Here, we have addressed the role of the sequence-specific binding of bacteriophage Mu C protein in *mom* gene transactivation. We demonstrate site-specific, yet asymmetric, interaction of bacteriophage Mu C protein at its recognition sequence. While this small protein makes an extended contact, it distorts the DNA. The binding of C leads to a localized untwisting of the DNA towards the distal region downstream of the 3′ half site.

The C protein binding site is located upstream of and overlapping the −35 element of the *mom* promoter. Thus, C binding may result in the bending of the DNA, which could help in protein-protein interaction to influence transcription. DMS-footprinting experiments suggest such axial flexibility of the DNA. These data represent a realistic picture as the experiments were carried out with supercoiled plasmid under *in vivo* conditions. The data are in agreement with our *in vitro* footprinting data (Ramesh & Nagaraja, 1996) and those of Sun *et al.* (1997). Bending was also observed when a circular permutation assay was carried out with a 50 bp fragment from the *mom* promoter region having the C recognition sequence by Sun *et al.* (1997). A single TGTG motif is found in their sequence and in the footprinting substrates used here. It should be noted that the TGTG motif is kink prone and could be contributing to the protein-induced bending of DNA. The CAP protein recognition sequence has this motif on either side of its binding site, resulting in a symmetric bending/distortion of the DNA (Kolb *et al.*, 1993). The presence of TGTG at only one side of the C recognition site could be contributing to the axial flexibility and asymmetric distortion induced by C protein binding. The dimeric C protein (De *et al.*, 1997) seems to bind to its site in a symmetric fashion, based on the DNase I protection pattern (Gindlesperger & Hattman, 1994; Ramesh, 1997).

C protein joins the growing family of DNA-binding proteins that exert their effect by unwinding DNA. MerR, DnaA protein, histone H1, and DNA ligase (Ansari *et al.*, 1995; Mizushima *et al.*, 1996; Ivanchenko *et al.*, 1996a,b) are the well-characterized examples. Amongst these proteins, MerR serves as a classic example of a transcription factor which mediates the unwinding of its target site to bring about transcription activation of the *mer*T promoter. MerR protein binds at the 19 bp spacer region of the *mer*T promoter and allosterically regulates the expression of a Hg^{2+}-resistance operon in response to mercury by controlling the DNA conformation (Frantz & O'Halloran, 1990). The activation of its target gene takes place by compensating for the overtwisting between the −35 and −10 elements (Ansari *et al.*, 1992, 1995). The protein-DNA interactions in MerR and C protein are, however, distinct, though the mechanisms of their action seem to be the same. The interaction is different because their target sequences and their locations with respect to the promoters are different. The MerR binding site consists of a 7 bp inverted repeat interrupted by 4 bp (Summers, 1992), whereas the C protein binding site in P_{mom} (5′-GATTATgccccaATAACC-3′) has an internal tetranucleotide palindromic sequence separated by a 6 bp spacer. The binding site of MerR is located between the −10 and −35 boxes of the *mer*T promoter. It has been proposed that mercury-depen-

dent activation of the *mer*T promoter brings the −10 and −35 elements into better helical alignment through a MerR-mediated DNA untwisting effect at the center of its binding site, resulting in a symmetric DNA distortion. This is in contrast to the asymmetric unwinding mediated by C protein. The asymmetric interaction of the C protein could be necessary because of its binding upstream of and overlapping the −35 element, unlike that of MerR. The protein-DNA interactions in these two similar yet different systems could also be influenced by the respective protein structures. A parallel could be drawn for their response to metal ions. C protein binds to its target site only in the presence of Mg^{2+} (Ramesh *et al.*, 1994a). The presence of Mg^{2+} results in conformational changes leading to more α-helicity and DNA binding (De *et al.*, 1998). MerR is also subjected to Hg^{2+}-mediated conformational changes (Frantz & O'Halloran, 1990).

We envision the following scenario for C-mediated transcription activation of the *mom* promoter (Figure 8). In the absence of C protein, RNAP is unable to bind to P_{mom}, probably due to unfavorable interactions with the −35 and −10 elements of the promoter. The *mom* promoter (Figure 1) has a 19 bp spacer separating the −10 and −35 regions, as compared to the optimal 17 (±1) bp spacing seen in a large number of the promoters. The 19 bp spacer adds an additional twist angle of at least 34°, and the two elements may be out of phase with respect to each other. The C protein-induced torsional change in DNA unwinding (by ∼30°) could compensate adequately for this difference. Thus, upon binding to its cognate site, C could untwist the DNA so as to reorient the promoter elements. Reorientation of the promoter elements would enable RNAP to recognize the *mom* promoter. It remains to be demonstrated whether RNAP recognizes only the −10 or both the promoter elements in the presence of C protein. It is possible that alternate and/or additional explanations could account for C-mediated *mom* transactivation. C-induced bending (Sun *et al.*, 1997) or overall distortion (Ramesh & Nagaraja, 1996; Sun *et al.*, 1997; present study) of DNA could play an important role in promoter occupancy by RNAP. In addition, protein-protein interactions between C and RNAP could also contribute in the initiation of *mom* transcription. The majority of the transcription activators studied so far interact with one or other of the subunits of RNAP (Ishihama, 1992, 1993; Niu *et al.*, 1996). A recent report shows that C protein does not interact with the α or σ subunits of RNAP (Sun *et al.*, 1998). While the interaction of C with the β or β′ subunits of RNAP is yet to be assessed, DNA distortion and/or untwisting seems to be a major mechanism operating for *mom* activation.

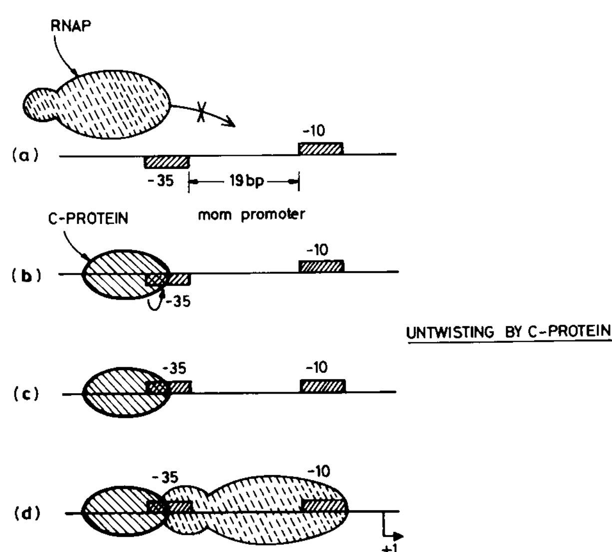

Figure 8. A model for C protein-mediated *mom* gene transactivation. (a) In the absence of C protein, RNAP is unable to bind to P_{mom}. (b) C protein binds to its cognate site in P_{mom} and unwinds the DNA. (c) Reorientation of the two promoter elements. (d) RNAP is now able to make favorable contacts with P_{mom}.

The complexity of *mom* gene regulation is beginning to be understood. Dam methylation of adenine residues in three closely spaced GATCs located immediately upstream of the C recognition site is required for *mom* gene expression (Seiler *et al.*, 1986). Dam methylation blocks the binding of another host protein, OxyR, which represses *mom* transcription in dam^{-} strains (Bolker & Kahmann, 1989). The situation becomes more complex with the observation that the reduced form of OxyR does not inhibit C protein binding to its site (Sun & Hattman, 1996). However, prior binding of the reduced form of OxyR prevents RNAP binding at P2 as well as C-activated RNAP binding at P1. In circumstances where OxyR is not bound to its site, RNAP would bind to P2 (Balke *et al.*, 1992). C protein binding to its site would then displace RNAP from P2. It is still unclear to us as to how, with the interplay of these factors, RNAP is recruited to P1 to bring about *mom* gene expression inside the cell.

Materials and Methods

Strains, plasmids, enzymes and chemicals

E. coli DH10B was used for generating different plasmid constructs. *E. coli* BL26(DE3), having a C overexpressing plasmid, was employed for both *in vivo* DMS footprinting and for C protein purification. Table 1 shows the list of plasmids used here. The various restriction enzymes and modifying enzymes were purchased from Pharmacia, New England Biolabs, Amersham and Boehringer Mannheim, and were used according to the suppliers', recommendations. Chemicals and other reagents were purchased from Sigma, Gibco BRL and High Media. Primers and DNA oligonucleotides were synthesized by Bangalore Genei (Pvt.) Ltd, Bangalore, India. [α-^{32}P]dATP and [γ-^{32}P]ATP were purchased from Amersham Corp. C protein was purified from *E. coli*

Table 1. Plasmids used

Plasmids	Description	Reference
pCSM1	Single C binding site (5′-AGATCGATTATGCCCCAATAACCAC-3′) cloned into the *Sma*I site of pUC19	B. D. Paul and V. N., unpublished results
pSB13	13 C binding sites in the same orientation cloned into the *Sma*I site of pUC19	This study
pSB3	Three C-binding sites in the same orientation cloned into the *Sma*I site of pUC19	This study
pUW4	*mom* promoter region (260 bp *Eco*RI-*Hin*dIII fragment from pLW4; Balke *et al.*, 1992) cloned into the *Eco*RI-*Bam*HI site of pUC19	Ramesh *et al.* (1994a)
pBM2	220 bp *Eco*RI/*Bam*HI fragment from pUW4 containing the *mom* promoter region cloned into the *Ban*II site of the pLysS vector	B. D. Paul and V. N., unpublished results
pVR7	C-gene overexpressing plasmid	Ramesh *et al.* (1994a)
pUC19	Laboratory stock	Sambrook *et al.* (1989)

BL26(DE3) carrying plasmid pVR7 as described by Ramesh *et al.* (1994a,b). Most of the standard procedures were carried out as described by Sambrook *et al.* (1989).

Construction of pSB13 and pSB3 plasmids

Two oligonucleotides (5′-CGATCGATTATGCCC-CAATAACCA-3′ and 5′-GATCGTGGTTATTGGGGCA-TAATC-3′) containing the C binding site were designed in such a way that annealing and subsequent ligation would result in multimerization of the C binding sites only in one orientation. Samples (150 pmoles) of the above oligonucleotides were annealed in a buffer containing 20 mM Tris-HCl (pH 7.2), 1 mM EDTA, 5 mM $MgCl_2$, and 50 mM NaCl, phosphorylated using T4 polynucleotide kinase, and then subjected to self ligation using T4 DNA ligase. This mixture of the multimerized C binding sites were blunted and then cloned into the *Sma*I site of the pUC19 vector. Clones having different numbers of the single C binding sites were obtained. Of these, two clones were used here: pSB13 contains 13 C binding sites and pSB3 has three C binding sites, all in the same orientation. Dideoxy sequencing was employed to confirm the sequences (Sambrook *et al.*, 1989).

In vivo DMS protection analysis

E. coli BL26(DE3) cells harboring pVR7 (a C gene overexpressing plasmid) and pBM2 (a plasmid containing the *mom* promoter region) were grown to A_{600} 0.6 in 20 ml of Luria-Bertani medium. Cultures were induced with 0.3 mM IPTG for the expression of C protein, and were further grown for one hour. Cells were treated with 6 mM dimethyl sulfate (DMS) for five minutes. The reaction with DMS was stopped by transferring the cultures to pre-chilled tubes. The cells were harvested and the plasmids isolated by the alkaline-lysis method (Sambrook *et al.*, 1989). The DMS-modified plasmid DNA was subjected to cleavage by the addition of 10 μl of piperidine to 90 μl of methylated DNA and heating to 90°C for 30 minutes followed by freeze drying. The DNA was resuspended in 100 μl of sterile distilled water and again freeze dried. A final round of freeze drying was performed after resuspending the DNA in 20 μl of distilled water. The DNA was purified by passing it through Sephadex G-50 columns. The *mom* forward primer (5′-GAAACGAGCGCATATA-3′) and *mom* reverse primer (5′-TGTTATTTAAGGCGGATTCC-3′) which anneal upstream of the −96 position and downstream of the +56 position, respectively (with respect to the +1 (transcription start site) position of the *mom* gene), were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and purified by passing through Sephadex G-25 columns. End-labeled primer (2×10^6 cpm), 1–2 μg of piperidine-cleaved plasmid DNA and 1.0 mM NaOH were mixed to a total volume of 40 μl, heated to 80°C for two minutes and then immediately chilled followed by the addition of 5 μl of primer-extension buffer (0.5 mM Tris-HCl (pH 7.2), 100 mM $MgSO_4$, 2.0 mM DTT). After 40°C incubation for three minutes, 1 mM dNTP mix was added and the extension reaction was carried out with one unit of Klenow enzyme at 45°C for ten minutes. Reactions were stopped by adding 17 μl of Klenow stop mix (4.0 M ammonium acetate, 20 mM EDTA). The DNA was precipitated by adding 2.5 × volume of 100% (v/v) ethanol and then loaded onto a denaturing 6% (w/v) polyacrylamide gel. Dideoxy sequencing reactions with the same labeled primers were also carried out with the pBM2 plasmid template and loaded onto the gel. The gel was then exposed to Kodak X-ray film to obtain the autoradiogram. The autoradiograms were scanned on a BioRad (Hercules, CA) model GS-700 densitometer using the Molecular Analyst program.

In vitro A>G DMS reaction

The cleavage at methylated purine nucleotides (A > G) was performed as described by Maxam & Gilbert (1977). The methylated DNA was suspended in 20 μl of sterile distilled water, chilled on ice, 5 μl of 0.5 M HCl was added and the sample was incubated for two hours on ice with occasional mixing. The DNA was precipitated, dissolved in 100 μl of 0.1 M NaOH, 1 mM EDTA, heated at 90°C for 30 minutes for strand cleavage, and recovered by precipitation.

$(OP)_2Cu$ footprinting

C protein-DNA (7.8:1 pmol) complexes were formed in 180 μl of binding buffer (20 mM Tris-HCl (pH 7.2), 1 mM EDTA, 5 mM $MgCl_2$, 50 mM NaCl) by incubating on ice for ten minutes. A 10 μl sample of 4 mM 1,10-phenanthroline/0.3 mM $CuSO_4$ and 10 μl of 58 mM 3-mercaptopropionic acid were added and incubated on ice for one minute. Digests were quenched by adding 7 μl of 100 mM 2,9-dimethyl-1,10-phenanthroline, deproteinised by phenol/chloroform extractions and the DNA was precipitated with ethanol. The DNA was purified by passing through Sephadex G-50 columns. pUC19 forward and reverse primers were end-labeled with [γ-^{32}P]ATP and used for primer extension of the DNA as described above. The extended products were electrophoresed on a denaturing 6% polyacrylamide gel. The gel was dried and exposed to X-ray film obtain the autoradiogram.

Coupled topoisomerase assay

A supercoiled plasmid, pSB13 (with 13 C binding sites) was constructed, as described above, for carrying out the coupled topoisomerase assay. This assay was performed as follows: C protein complexes were formed by incubating varying amounts of C protein with 0.6 pmol of supercoiled pSB13 plasmid or 0.4 pmol of pUC19 plasmid (for the control) in buffer containing 20 mM Tris-HCl (pH 7.2), 1 mM EDTA, 5 mM $MgCl_2$ and 50 mM NaCl, on ice, for ten minutes. These complexes were incubated with *E. coli* topoisomerase I at 37°C for 15 minutes. The reactions were stopped by the addition of SDS to a final concentration of 1.5% (w/v) and heat inactivation at 65°C for 20 minutes. The reaction mixes were electrophoresed on 1.2% (w/v) agarose slab gel at 25°C in 1 × TAE buffer at 3 V/cm for 16 hours. Gels were stained with ethidium bromide and photographed. The negatives were analyzed on a BioRad (Hercules, CA) model GS-700 densitometer using the Molecular Analyst program.

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References

Ansari, A. Z., Chael, M. L. & O'Halloran, T. V. (1992). Allosteric underwinding of DNA is a critical step in positive control of transcription by Hg-MerR. *Nature*, **355**, 87–89.

Ansari, A. Z., Bradner, J. E. & O'Halloran, T. V. (1995). DNA-bend modulation in a repressor-to-activator switching mechanism. *Nature*, **374**, 371–375.

Balke, V., Nagaraja, V., Gindlesperger, T. & Hattman, S. (1992). Functionally distinct RNA polymerase binding sites in the phage Mu *mom* promoter region. *Nucl. Acids Res.* **20**, 2777–2784.

Bolker, M. & Kahmann, R. (1989). The *Escherichia coli* regulatory protein OxyR discriminates between methylated and unmethylated states of the phage Mu *mom* promoter. *EMBO J.* **8**, 2403–2410.

Bolker, M., Wulczyn, F. G. & Kahmann, R. (1989). Role of bacteriophage Mu C protein in activation of the *mom* gene promoter. *J. Bacteriol.* **171**, 2019–2027.

Borowiec, J. A. & Gralla, J. D. (1986). High resolution analysis of Lac transcription complexes inside cells. *Biochemistry*, **25**, 5051–5057.

Busby, S. & Ebright, R. (1994). Promoter structure, promoter recognition, and transcription activation in prokaryotes. *Cell*, **79**, 743–746.

Chiang, L. W. & Howe, M. M. (1993). Mutational analysis of a C-dependent late promoter of bacteriophage Mu. *Genetics*, **135**, 619–629.

Collado-Vides, J., Magasanik, B. & Gralla, J. D. (1991). Control site location and transcriptional regulation in *Escherichia coli*. *Microbiol. Rev.* **55**, 371–394.

De, A., Paul, B. D., Ramesh, V. & Nagaraja, V. (1997). Use of protein A gene fusions for the analysis of structure-function relationship of the transactivator protein C of bacteriophage Mu. *Protein Eng.* **10**, 935–941.

De, A., Ramesh, V., Mahadevan, S. & Nagaraja, V. (1998). Mg^{2+} mediated sequence specific binding of transcriptional activator protein C of bacteriophage Mu to DNA. *Biochemistry*, **37**, 3831–3838.

Frantz, B. & O'Halloran, T. V. (1990). DNA distortion accompanies transcriptional activation by the metal repressor gene-regulatory protein MerR. *Biochemistry*, **29**, 4747–4751.

Gindlesperger, T. L. & Hattman, S. (1994). *In vitro* transcriptional activation of the phage Mu *mom* promoter by C protein. *J. Bacteriol.* **176**, 2885–2891.

Hattman, S. (1982). DNA methyltransferase dependent transcription of the phage Mu *mom* gene. *Proc. Natl Acad. Sci. USA*, **79**, 5518–5521.

Hattman, S., Ives, J., Margolin, W. & Howe, M. M. (1985). Regulation of the bacteriophage Mu *mom* gene: mapping of the transactivation (Dad) function to the C-region. *Gene*, **39**, 71–76.

Hattman, S., Newman, L., Krishna, Murthy H. M. & Nagaraja, V. (1991). Com, the phage Mu *mom* translational activator, is a zinc binding protein that binds specifically to its cognate mRNA. *Proc. Natl Acad. Sci. USA*, **88**, 10027–10031.

Heisig, P. & Kahmann, R. (1986). The sequence and *mom* transactivation function of the C gene of bacteriophage Mu. *Gene*, **43**, 59–67.

Ishihama, A. (1992). Role of RNA polymerase α subunit in transcription activation. *Mol. Microbiol.* **6**, 3283–3288.

Ishihama, A. (1993). Protein-protein communication within the transcription apparatus. *J. Bacteriol.* **179**, 2483–2489.

Ivanchenko, M., Hassan, A., van Holde, K. & Zlatanova, J. (1996a). H1 binding unwinds DNA. Evidence from topological assays. *J. Biol. Chem.* **271**, 32580–32585.

Ivanchenko, M., van Holde, K. & Zlatanova, J. (1996b). Procaryotic DNA ligases unwind superhelical DNA. *Biochem. Biophys. Res. Commun.* **226**, 498–505.

Kahmann, R. & Hattman, S. (1987). Regulation and expression of the *mom* gene. In *Phage Mu* (Symonds, N., Toussaint, A., Van de Putte, P. & Howe, M. M., eds), pp. 93–109, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Kolb, A. & Buc, H. (1982). Is DNA unwound by the cyclic AMP receptor protein?. *Nucl. Acids Res.* **10**, 473–485.

Kolb, A., Busby, S., Buc, H., Garges, S. & Adhya, S. (1993). Transcriptional regulation by cAMP and its receptor protein. *Annu. Rev. Biochem.* **62**, 749–795.

Maxam, A. M. & Gilbert, W. (1977). A new method for sequencing DNA. *Proc. Natl Acad. Sci. USA*, **74**, 560–565.

Mizushima, T., Katayama, T. & Sekimizu, K. (1996). Effect on DNA topology by DnaA protein, the initiation factor of chromosomal DNA replication in *Escherichia coli*. *Biochemistry*, **35**, 11512–11516.

Nagaraja, V. (1993). Control of transcription initiation. *J. Biosci.* **18**, 13–25.

Niu, W., Kim, Y., Tau, G., Heyduk, T. & Ebright, R. (1996). Transcription activation at class II CAP-dependent promoters: two interactions between CAP and RNA polymerase. *Cell*, **87**, 1123–1134.

Raibaud, O. & Schwartz, M. (1984). Positive control of transcription initiation in bacteria. *Annu. Rev. Genet.* **18**, 173–206.

Ramesh, V. (1997). DNA binding studies with the transcriptional activator protein C of bacteriophage Mu. PhD thesis, Indian Institute of Science, Bangalore, India.

Ramesh, V. & Nagaraja, V. (1996). DNA binding of the phage Mu C protein: footprinting analysis reveals altered DNA conformation upon protein binding. *J. Mol. Biol.* **260**, 22–33.

Ramesh, V., De A., & Nagaraja, V. (1994a). Engineering hyperexpression of bacteriophage Mu C protein by removal of secondary structure at the translational initiation region. *Protein Eng.* **7**, 1053–1057.

Ramesh, V., De A., & Nagaraja, V. (1994b). Overproduction and purification of C-protein, the late gene transcription activator from phage Mu. *Protein Expt. Purif.* **5**, 379–384.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edit., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Seiler, A., Blocker, H., Frank, R. & Kahmann, R. (1986). The *mom* gene of bacteriophage Mu: the mechanism of methylation dependent expression. *EMBO J.* **5**, 2719–2728.

Sigman, D. S., Kuwabara, M. D., Chen, C.-H. B. & Bruice, T. W. (1991). Nuclease activity of 1,10-phenanthroline-copper in study of protein-DNA interactions. *Methods Enzymol.* **208**, 414–433.

Spassky, A. & Sigman, D. (1985). Nuclease activity of 1,10-phenanthroline-ion. Conformational analysis and footprinting of the lac operon. *Biochemistry*, **24**, 8050–8056.

Summers, A. O. (1992). Untwist and shout: a heavy metal-responsive transcriptional regulator. *J. Bacteriol.* **174**, 3097–3101.

Sun, W. & Hattman, S. (1996). *Escherichia coli* OxyR protein represses the unmethylated bacteriophage Mu *mom* operon without blocking binding of the transcriptional activator C. *Nucl. Acids Res.* **24**, 4042–4049.

Sun, W., Hattman, S. & Kool, E. (1997). Interaction of the bacteriophage Mu transcriptional activator protein, C, with its target site in the *mom* promoter. *J. Mol. Biol.* **273**, 765–774.

Sun, W., Hattman, S., Fujita, N. & Ishihama, A. (1998). Activation of bacteriophage Mu *mom* transcription by the C protein does not require specific interaction with the carboxy terminal region of the α or σ^{70} subunit of *Escherichia coli* RNA polymerase. *J. Bacteriol.* **180**, 3257–3259.

Wulczyn, F. G. & Kahmann, R. (1991). Translational stimulation: RNA sequence and structure requirements for binding of Com protein. *Cell,* **65**, 259–269.

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