Repression of Bacteriophage φ29 Early Promoter C2 by Viral Protein p6 Is Due to Impairment of Closed Complex*

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Bacteriophage φ29 gene expression is regulated at the transcription initiation step either by repressing strong early promoters or by activating the otherwise poorly expressed late promoter (Ref. 1 and see Fig. 1). The transition between early and late transcription occurs 15–20 min postinfection when the late promoter A3 is activated, whereas early promoters A2b, A2c, and C2 become repressed. Two phage-encoded transcription regulators, proteins p4 and p6, are responsible for this transcriptional switch. In nonsuppressor bacteria infected with a nonsense mutant in gene 4, transcription from promoters A2b and A2c (7). At the complex the DNA adopts a right-handed toroidal conformation winding around a core of protein p6 in which the DNA is remarkably curved, ~66° every 12 bp1 (5), condensing the DNA up to 6-fold; this architectural alteration is likely to influence promoter C2 expression. On the other hand, repression of promoter activity depends on the rates at which repressor and RNA polymerase (RNAP) bind to their respective binding sites (8). Then competition between p6 and the RNAP for the sequence corresponding to promoter C2 might be crucial in the repression. We were interested to find out at what precise stage during transcription the observed inhibition occurs and what the underlying mechanism might be. Although nothing is known about the molecular mechanism by which p6 represses promoter C2, several alternative, although not mutually exclusive, models can be envisioned to explain the effect of p6 on promoter C2 activity: (i) the binding of p6 to the genome right end might occlude the promoter in the nucleoprotein complex; (ii) the p6-induced modification of promoter architecture might impair the RNAP bending needed for the formation of a stable transcription complex; (iii) protein p6 bound at the upstream sequence of the promoter might help to block transcription by impeding a postbinding step. In this work, we aimed to gain insight into the molecular bases of the repressive function of protein p6 on the C2 promoter. We show that the p6-DNA nucleoprotein complex does not seem to occlude the C2 promoter sequence to the RNAP but rather affects the stability of the closed complex.

EXPERIMENTAL PROCEDURES

Proteins and Nucleotides—Bacillus subtilis RNAP was purified as described previously (9). Protein p6 was purified from bacteria infected with φ29 (10). Unlabeled NTPs and dNTPs, [γ-32P]ATP (3000 Ci/mmol), and [α-32P]UTP (3000 Ci/mmol) were purchased from Amersham Pharmacia Biotech.

DNA Substrate—The 267-bp right terminal φ29 DNA fragment containing promoter C2 was obtained by polymerase chain reaction amplification from full-length DNA with the synthetic primers (Isogen Bio...
Impairment of Promoter C2 Closed Complex by Protein p6

RESULTS

Expression of Promoter C2 Depends on Salt Concentration—

Transcription from promoter C2 is salt-dependent. In the truncated transcription assay shown in Fig. 2, there was a stimulation of transcript production when the concentration of KCl increased. 12-fold more transcription was obtained when 200 mM KCl was added than in the absence of salt or when only 50 mM KCl was present. This salt-mediated activation seems to be favored by K⁺ ions because when NaCl was used instead only a 4-fold activation was observed, and the presence of ammonium sulfate did not affect the level of transcription. To gain insight into the step of the transcription favored by the K⁺ ions, closed and open complex formations were analyzed by band shift assays with the addition 50 or 200 mM KCl both in the reaction and in the gel solution. As shown in Fig. 3, no effect of the KCl was observed on closed complex formation at 4 °C. However, the RNAP-DNA complexes formed at 37 °C were favored by the increase of KCl concentration. At 200 mM KCl, up to 4-fold less RNAP was needed to form the same amount of RNAP-DNA complex; a similar result was obtained when 100 mM KCl was used (not shown).

Protein p6 Inhibits Transcription from Promoter C2 in Vitro

in a Salt-dependent Manner—Both low temperature (0 °C) and low salt concentrations greatly favor p6-DNA interaction (11), but these are not the best conditions for transcription at promoter C2 (see Fig. 2). Hence it was necessary to achieve conditions in which both transcription complexes and p6-DNA complexes could be formed. To this end, we analyzed the effect of KCl on the p6-mediated repression of promoter C2. In agreement with the data described above, up to 5-fold more truncated transcripts were obtained when the concentration of KCl was increased from 50 to 100 mM, and 15-fold more were obtained if the increase was up to 200 mM salt (Fig. 4). On the other hand, the inhibition mediated by p6 was affected by the concentration of KCl. Transcription from promoter C2 was inhibited with 3.5 mM p6 in an assay containing 50 mM KCl, but 7 mM p6 was needed if the assay contained 100 mM KCl, and with 14 mM p6 only 2-fold inhibition was achieved in the presence of 200 mM salt.

Because cations compete with proteins for DNA, the increased concentration of salt and more specifically the presence of KCl could affect the p6-mediated repression of promoter C2 by altering the binding capacity of the protein to DNA. Thus, the effect of KCl on the binding of both p6 and RNAP to the promoter was assayed by DNase I footprinting (Fig. 5). Protein p6 does not recognize a specific sequence on the viral DNA but includes promoter C2 with its sequence of the genome right end used in this study, which includes promoter C2 with its +1 position located at 160 nucleotides, located between nucleotides 40–125 and 46–68 at the right and left phage genome ends, respectively (12). The results showed that protein p6 cooperatively forms a nucleoprotein complex that could cover the 267-bp strand of promoter C2 protecting the sequence between positions −56 to +22 (Fig. 5, lanes d and h). RNA polymerase interacts with the late strand of promoter C2 protecting the DNA strand with a characteristic pattern of strong hypersensitive bands every 24 bp. The pattern was identical at 50 and 100 mM KCl (Fig. 5, lanes d and h). Quantitative data obtained from the Bas-lls image analyzer are indicated at the bottom of each lane as relative transcription.
KCl, only the pattern corresponding to bound p6 was observed, whereas at 100 mM salt a mixed pattern of p6 and RNAP was present (Fig. 5, compare lane c with g). The RNAP bands at positions 237 and 229 could be observed over the regular pattern of bands between positions 114 and 2132 because of p6 binding. It should be pointed out also the loss of the p6 pattern downstream position 114 (Fig. 5, compare lanes g and h). Taking into account these data and those described above, we used 100 mM KCl to study the effect of p6 on the transcriptional complex formation.

Transcription Complexes Formed at Promoter C2 in the Presence of Protein p6—

We have analyzed the effect of the p6-derived nucleoprotein complex on the formation of transcription complexes at 4 °C and 37 °C. At 4 °C, only closed complex formation could be achieved, whereas at 37 °C the complex has been structurally characterized as open complex by probing with KMnO4 (data not shown). The DNase I footprints of the closed complexes indicated that RNAP contacts the late strand from approximately positions −37 to −5 and the early strand from positions −19 to +10 (Fig. 6, lanes b and k). At 37 °C in the open complex, the RNAP binds both DNA strands also with a footprint of protections more extended toward the downstream promoter sequence (Fig. 6, compare lane b with f and lane k with o). Independently of the type of transcription complex formed, RNAP binding produced a specific hypersensitive band at position −37 in the late strand and at position −19 in the early strand, although it was more intense in the open complex footprint of the early strand. Protein p6 protected either DNA strand with the characteristic pattern of hypersensitivities every 24 bp similarly at either temperature (Fig. 6, lanes d, h, i, and m). When RNAP and p6 were added together and the assay was carried out at 37 °C, the RNAP-derived hypersensitivity at position −37 (late strand) and at position −19 (early strand) could be identified over the footprint pattern corresponding to p6 (Fig. 6, lanes g and n). This result suggests that RNAP is bound to the promoter with p6 attached to the DNA around the bound enzyme. However, at 4 °C, the RNAP-derived hypersensitive band at position −19 was greatly decreased, and the one located at −37 was almost undetectable (Fig. 6, lanes f and c), suggesting that p6 affects the closed complex but not the open complex.

DNase I footprinting shows the binding positions of proteins to the sequence under study, but the bands in the footprints could potentially derive from a mixed population and therefore might not prove that both proteins are present on the same
DNA molecule. To find out whether p6 affects the transcriptional closed complex, we analyzed the binding of RNAP to the DNA fragment containing the C2 promoter in the presence of increasing amounts of p6 by gel retardation assays at 4 °C. As shown in Fig. 7, independently of the concentration of p6 assayed, only a retarded DNA-protein complex can be observed, but the increase on protein concentration was reflected by differences on the relative mobility of the complexes (Fig. 7, lanes f–h). This result indicates that p6 will cooperatively bind along the fragment, and the differences in mobility might be due to the progressive occupation of the DNA fragment by the protein. However, because in this and other experiments always a unique band was obtained for each amount of protein used, most probably the architectural modification of the DNA upon binding of p6 is an additional factor responsible for the decreased mobility of the complex. RNAP, upon interaction with the C2 promoter, yields a retarded band corresponding to the transcriptional closed complex (Fig. 7, lane b). With increasing concentration of p6 the amount of closed complex formed was altered. At the lower concentration of p6, the reduced mobility of the transcriptional complex indicated additional binding of p6. However, increasing the amount of p6 from 7 to 10 μM results in the reduction of the intensity and mobility of the band containing RNAP-p6-DNA. Concomitant
A number of different regulatory functions have been assigned to protein p6. It has been characterized as an activator of the phage DNA initiation of replication and as a transcriptional regulator able to both activate late transcription and repress early promoters A2b and A2c in the presence of protein p4. In addition, protein p6 is the only factor required for repression of early promoter C2. For all these functions we are far from a molecular understanding of the mechanisms involved. Here we were interested in exploring some of the properties that make p6 an efficient transcriptional repressor of promoter C2.

Protein p6 binds along the φ29 DNA molecule but exhibits preference for sequences at the genome ends called nucleation sites (10). Promoter C2 is located close to the genome right end with its −35 box adjacent to the nucleation site. Considering that the p6-DNA binding unit is formed by a protein dimer bound every 24 bp, the center of binding of the two monomers is flanked by DNase I-hypersensitive sites that are best explained as strong DNA bends (Fig. 5). Indeed it was demonstrated that the DNA in the p6-DNA complex is strongly bent (66° every 12 bp), untwisted (11.5 bp/turn), and compacted (5). From its resistance to nuclease, we know that p6 binds to DNA immediately if enough protein is present; however, the apparent affinity constant (10^3 M⁻¹) of p6 for DNA and its cooperativity parameter (α = 100) indicate that the complex is dynamic (4). All these characteristics are likely to influence the function of p6 as a transcriptional repressor of promoter C2.

We have shown, by DNase I protection assays, the positions occupied when protein p6 is bound to promoter C2 (Figs. 5 and 6). To ensure the formation of the p6-nucleoprotein complex on every DNA molecule we had taken into account that the p6 units (dimers) are uniformly distributed along the 267-bp fragment containing the promoter, hence, that each DNA molecule should be saturated by about 12 dimers. Consequently in this study, we have used more than a 200-fold excess of p6 molecules with respect to DNA.

Under these conditions the DNase I data indicate that p6 does not hide the promoter to the RNAP because a mixed footprint could be detected when the ternary complex RNAP-p6-DNA is allowed to form. Closed inspection (see Fig. 5, lane g) reveals that, when both RNAP and p6 are present, the footprint pattern obtained is not the average of the individual patterns. In fact, the hypersensitive bands at positions +14, -10, -29, -35, -37, and -59 are identical to the bands corresponding to the footprints of each of the proteins. Furthermore, RNAP seems to be able to displace p6 from the main core promoter and bind and form a transcriptional complex surrounded by p6 (Fig. 8). These results agree with the existence of the ternary p6-RNAP-DNA complex detected by gel retardation assay (Fig. 7). However, when the amount of p6 was increased, the RNAP was displaced from the transcriptional closed complex. This result is surprising if one considers that (i) the closed complex at promoter C2 is stable because at 4 °C it has a t½ of 9 min (not shown), and (ii) the p6-DNA complex is unstable and has to be dynamic to cope with the DNA replication process occurring from this end of the genome. In fact, the low DNA binding constant of p6, the sensitivity of the p6-DNA complex to salt and temperature, and the high amount of protein p6 needed to form the complexes indicate unstable complexes. Therefore, we are tempted to speculate that it is not only the binding of p6 but also the p6-mediated bend at the promoter sequence that represses transcription. Recognition of promoter C2 by RNAP could occur as in the case of the lac UV5 promoter (13) where only the –35 region is contacted, leading

**DISCUSSION**

with the decrease of the p6-RNAP-DNA complexes, a new band most probably corresponding to p6-DNA complex appeared. Hence, we infer that progressive p6-DNA complex formation impairs the stability of the transcriptional closed complex.

**Competition of p6 and RNAP for the Promoter Sequence**—To get further insight into the RNAP and p6 mutual interaction, competition experiments varying either the relative amounts of p6/RNAP or the incubation time were carried out (Fig. 8). In the absence of p6, 50 nM RNAP was sufficient to produce the RNAP/p6, and aliquots of 10 μl were taken after 2, 4, 8, and 10 min of incubation. In lanes g–i, increasing amounts of RNAP (50, 70, or 100 nM, respectively) were incubated with 4 nM DNA and 7 μM p6 for 20 min at 37 °C. Lanes a, b, and j are controls of DNA without proteins added (a) or in which only 100 nM RNAP (b) or 7 μM of p6 (j) was incubated with DNA. The right-pointing arrow indicates DNase I hypersensitivity produced upon RNAP binding, and the left-pointing arrows indicate the bands due to p6 binding. Each reaction contained 100 mM KCl.

**FIG. 8.** Competition between RNAP and p6 for the DNA sequence containing promoter C2. For the competition experiment of lanes c–f, 280 nM RNAP and 16 nM DNA were incubated at 37 °C with 24 μM p6, and aliquots of 10 μl were taken after 2, 4, 8, and 10 min of incubation. In lanes g–i, increasing amounts of RNAP (50, 70, or 100 nM, respectively) were incubated with 4 nM DNA and 7 μM p6 for 20 min at 37 °C. Lanes a, b, and j are controls of DNA without proteins added (a) or in which only 100 nM RNAP (b) or 7 μM of p6 (j) was incubated with DNA. The right-pointing arrow indicates DNase I hypersensitivity produced upon RNAP binding, and the left-pointing arrows indicate the bands due to p6 binding. Each reaction contained 100 mM KCl.

**2 A. Camacho, unpublished results.**
to a stressed intermediate. The latter is relaxed when the extended closed complex footprint is formed by transferring the torsion stress to the DNA. Protein p6 could repress transcription by impeding this stress transference when the promoter is highly occupied by the repressor.

The in vitro expression of promoter C2 is remarkably stimulated by the presence of salt, especially K\(^+\). Salt seems to favor specifically open complex formation at this promoter. On the other hand, the inhibitory effect of protein p6 was also affected by the presence of K\(^+\). These results imply two possible effects of K\(^+\) on promoter C2 transcription: activation of the promoter transcription per se and modulation of the inhibitory effect of p6. A parallel case of K\(^+\) ion-dependent regulation has been described for the H-NS-dependent repression of the Escherichia coli promoter proV of the proVWX operon (14).

Prokaryotic transcription repression generally involves a sequence-specific DNA-binding protein whose binding site is close to a promoter sequence (15). Intimate contacts between the repressor and RNAP occur in many cases. In recent years, however, the role of nonsequence-specific DNA-binding proteins in repression of transcription has been well documented (16). Protein p6 belongs to these so-called prokaryotic histone-like proteins, such as the E. coli proteins H-NS and HU (17). Protein p6 is small (103 amino acids), very abundant, binds DNA in a nonsequence-specific manner preferentially to regions containing intrinsic curvature, produces the twist of the sequence, and generates high order DNA-multiprotein complexes (5, 18). Similarities between the mechanisms of HU, H-NS, and p6 actions are evident. Protein p6 acts as cofactor of the transcriptional regulator p4 in repression of the A2c promoter (7), whereas HU represses GalR-mediated gal operon transcription (19). Protein p6 is sufficient for repression of promoter C2, whereas H-NS represses proV promoter transcription initiation through its nonspecific binding to the upstream promoter sequence (14). Recent results on the regulation of rRNA transcription modulated by FIS and H-NS indicate than in the absence of FIS, H-NS bound to the main promoter core sequence does not occlude the promoter to RNAP but impedes the transcriptional open complex (20). In this study we went a step further, demonstrating that the only element responsible for the repression of promoter C2, the multimeric p6nucleoprotein complex, does not occlude the promoter sequence to RNAP but affects the transcriptional complex stability. We propose that a DNA domain with a precise topology is formed when p6 binds to certain regions of the phage DNA. In turn, p6-dependent changes in local DNA topology play a crucial role in transcription regulation.

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