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Analysis of Early Promoters of the *Bacillus* Bacteriophage GA-1

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Bacteriophage GA-1, which infects *Bacillus* sp. strain G1R, is evolutionarily related to phage ϕ29, which infects *Bacillus subtilis*. We report the characterization of several GA-1 promoters located at either end of its linear genome. Some of them are unique for GA-1 and drive the expression of open reading frames that have no counterparts in the genome of ϕ29 or related phages. These unique promoters are active at early infection times and are repressed at late times. In vitro transcription reactions revealed that the purified GA-1-encoded protein p6 represses the activity of these promoters, although the amount of p6 required to repress transcription was different for each promoter. The level of protein p6 produced in vivo increases rapidly during the first stage of the infection cycle. The protein p6 concentration may serve to modulate the expression of these early promoters as infection proceeds.

A large variety of phages that infect bacteria of the genus *Bacillus* have been characterized. Particular attention has been given to the so-called ϕ29 family of phages that infect different *Bacillus* species. The genome of these lytic phages consists of a small linear double-stranded DNA of about 20 kbp, with a terminal protein covalently linked to the 5′ ends that plays a key role in the initiation of phage DNA replication. On the basis of serological properties, DNA physical maps, peptide maps, and partial or complete DNA sequences (26, 36, 37), the ϕ29 family of phages has been classified into three groups. The first group includes phages ϕ29, PZA, ϕ15, and BS32; the second one includes B103, Nf, and M2Y; and the third group has phage GA-1 as its sole member. Among them, phage ϕ29 has been extensively characterized, being one of the best-studied bacteriophages of gram-positive bacteria. Its mechanism of DNA replication and its regulation of transcription have been reviewed previously (19, 28, 29). Within this family of phages, GA-1 is the one most distantly related to ϕ29; this has stimulated the study of its mechanisms of DNA replication (6, 7, 8, 12) and transcription regulation (11).

The DNA sequences of the complete genomes of phages ϕ29 (34), PZA (23), B103 (25), and GA-1 (19) have been determined. The GA-1 genome has a size of 21,129 bp, which is larger than those of ϕ29 (19,285 bp), PZA (19,366 bp), and B103 (18,630 bp). In most aspects, the genomes of phages ϕ29, B103, and GA-1 are similarly organized. In ϕ29 (group I) and B103 (group II), the genes expressed soon after infection (early genes) are clustered in two operons located at each end of the genome. The late genes are located in a single operon that is positioned at the central part of the genome. As shown schematically in Fig. 1, the late genes of GA-1 (genes 7 through 16) are also present in a single operon located in the central part of the genome. As in ϕ29 and B103, the late GA-1 operon is flanked on its left side by an early operon that contains genes necessary for DNA replication and for transcriptional regulation (genes 6 through 2). These genes are expressed from the early promoters A2b and A2c (11). The right region of the GA-1 genome contains open reading frames (ORFs) whose deduced protein sequences are homologous to those of the ϕ29 early genes 17 and 16.7, which are involved in DNA replication (5, 17, 18). However, both ends of the GA-1 genome contain a number of sequences and ORFs that have no counterparts in ϕ29 or in any of the other related phages characterized (19). Therefore, the proteins that are probably encoded by these ORFs are unique for GA-1. Several putative promoters that could be responsible for the expression of these unique ORFs were identified. In this work we characterized these promoters, analyzing their expression patterns throughout the infection cycle. We also analyzed the role of GA-1 protein p6 in the regulation of the early promoters in vitro.

Identification of the early promoters A1a, A1b, A1c, C2, and C1b. Analysis of the 2.8-kb region on the left side of the GA-1 genome led to the identification of at least three possible promoters (Fig. 1), all of which contain typical −35 and −10 boxes for the σ^A RNA polymerase. Promoter A1b is homologous to the ϕ29 A1 promoter, which is responsible for the expression of a small RNA (named pRNA) required for the encapsidation of the viral genome into the proheads (2, 9). The two other promoters, A1a and A1c, are not present in the genomes of other related phages whose sequences are known. Promoter A1a is located upstream of a putative operon containing ORFs M, N, and O, and promoter A1c is located upstream of another putative operon containing ORFs P, Q, R, S, and T. These ORFs account for part of the difference in size of GA-1 DNA and ϕ29 (GA-1 DNA ca. 2 kb larger than ϕ29).

Two other promoters, named C2 and C1b, can be predicted in the right region of the GA-1 genome. Promoter C2 is homologous to the ϕ29 C2 promoter that drives the expression of the operon containing genes 17 and 16.7. In ϕ29, an additional weak promoter, named C1, is present within gene 16.7. In the case of GA-1, the second predicted promoter in this region maps upstream of gene 16.7. Therefore, this promoter is not equivalent to ϕ29 C1, and we have named it C1b.

To investigate whether the predicted GA-1 promoters cor-

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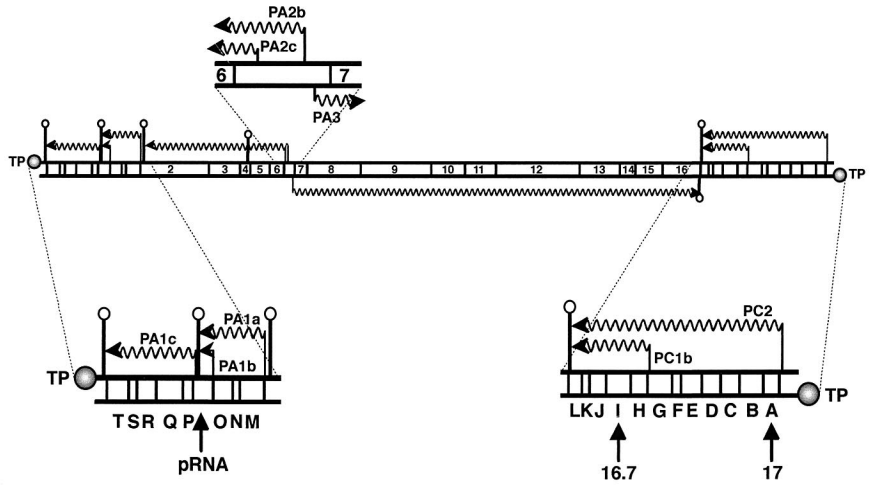

FIG. 1. Genetic and transcriptional map of phage GA-1 genome. The terminal protein (TP) is shown attached to the 5′ ends of the DNA. Promoters are indicated; the start sites of promoters A1c, A1a, C1, and C2 correspond to those determined in this work. Genes with known function, or for which counterparts are present in phage ϕ29, are indicated with numbers. ORFs at both ends of the genome are indicated with letters. Except for ORFs A and I, which are homologous to ϕ29 proteins 17 and 16.7, respectively, all other ORFs correspond to polypeptides of unknown function. Predicted transcription termination sites are indicated with stem-loops. The complete sequence of the GA-1 genome is in the EMBL/GenBank/DDBJ database under accession number X96987 (19).

respond to in vivo promoters, total RNA was purified from infected cells at different times after infection. Cells of *Bacillus* sp. strain G1R, the host for phage GA-1 (1), were grown in Luria-Bertani medium (30) supplemented with 5 mM $MgSO_4$ at 37°C to a density of about 1×10^8 cells/ml and infected with phage GA-1 at a multiplicity of infection of 5. At different times, samples were taken and total RNA was purified as described previously (20). Viral transcripts were detected by primer extension analysis (20) using primers hybridizing at distinct positions downstream of the predicted transcription start sites. Promoter A1b, responsible for the expression of pRNA (2), was not studied because it is homologous to the ϕ29 A1 promoter, which is known to be actively expressed throughout the infection cycle (20). The expression patterns of the early A2b and A2c promoters and the late A3 promoter have been characterized before (11). Analysis of the A3 promoter was included in the primer extension reactions to serve as an internal control to mark the transition from the early to the late stage of transcription. The results of the primer extension assays are shown in Fig. 2A. The relative amounts of the various transcripts, taking into account the number of adenines, are presented in Fig. 2B. Whereas relatively high levels of transcripts corresponding to the predicted promoters A1c and A1a were present early after infection (5 and 10 min), these levels were lower at late times. This indicates that the A1a and A1c promoters are negatively regulated at later infection times. Weaker signals were detected for the transcripts originating from the right end of the genome, corresponding to the predicted C1b and C2 promoters. Although these promoters are also negatively regulated, their expression patterns are different. Whereas the transcripts of the A1a and A1c promoters reached their maximum levels at 10 min postinfection and decreased gradually at later postinfection times, the maximum level of transcripts for promoters C2 and C1b was found at 5 min postinfection and decreased quickly at later postinfection times.

The sequences of the newly identified promoters and their transcription start sites are shown in Fig. 3, together with those of the previously characterized GA-1 promoters. All of the newly identified promoters, A1a, A1b, A1c, C2, and C1b, have sequences at their −35 and −10 regions that are very similar to the consensus promoter sequences recognized by the vegetative RNA polymerase (σ^A RNA polymerase) (16, 21). The A1b, A1c, and C2 promoters contain a TG dinucleotide located 1 bp upstream of the −10 region. The presence of this so-called "extended −10" motif increases the strength of *Escherichia coli* promoters (14, 15, 27) and of *Bacillus subtilis* phage ϕ29 (4). The distance between the −10 and −35 boxes (referred to as the spacer) in each of these promoters is within the standard 17 bp ± 1 bp (16). The absence of the extended −10 motif in promoter C1b may, at least in part, account for its low level of activity. For the *B. subtilis* phage ϕ29, it has been shown that the −35 region is important for promoter activity even in the presence of an extended −10 motif (4). In addition to the extended −10 motif, the sequence at the −16 region has been shown to be important for promoter strength in some *B. subtilis* promoters (35). The observation that many gram-positive promoters contain the sequence TRTG (where R is purine) at the −16 region suggests that this motif also contributes to promoter strength. This motif is present at the GA-1 promoters A1c, A3, and C2. Inspection of Fig. 3 shows, however, that a correlation between the presence or absence of the −10, extended −10, −16, and −35 motifs and promoter strength is not always straightforward. It is known that both DNA sequences and DNA structure are important for recognition by the RNA polymerase.

Repression of GA-1 early promoters. Although the ϕ29 protein p6 binds DNA with a low sequence specificity, it has a

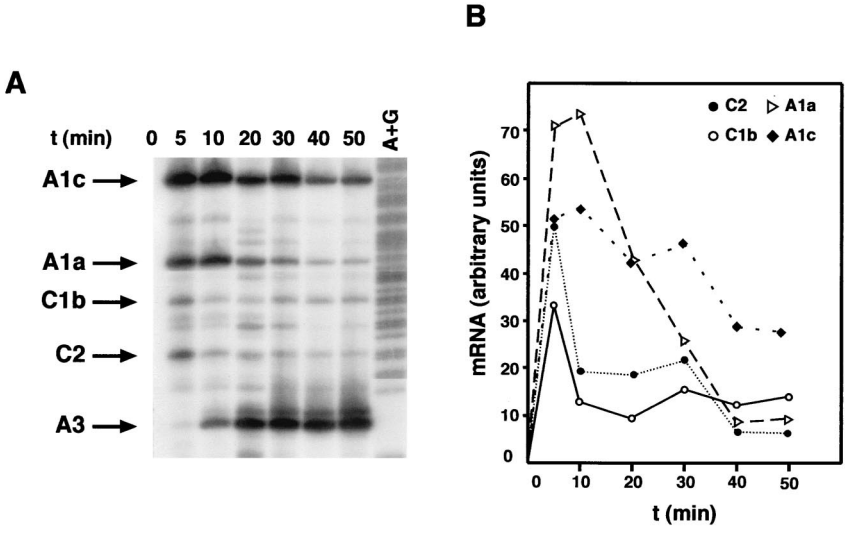

FIG. 2. Characterization of the GA-1 promoters located at both ends of the genome. (A) Expression of promoters A1c, A1a, C1b, C2, and A3 throughout the infection cycle. Total RNA was purified from cells at different times after infection; the transcripts originating from the indicated promoters were analyzed by primer extension using primers designed to obtain a cDNA of a distinct length for each promoter. The A3 promoter, known to be expressed at late times postinfection (11), was analyzed as a control. Lane A+G corresponds to a DNA size ladder obtained by chemical sequencing (30). (B) Quantitative analysis of the transcripts produced from promoters A1c, A1a, C1b, and C2. The signals obtained for each promoter in the primer extension reactions (panel A) were quantitated using a laser scanning densitometer. Since transcripts were detected by the incorporation of [α-^{32}P]dATP into the cDNA, the signals obtained were corrected for the number of adenines incorporated into the corresponding cDNA and normalized relative to an internal control. The graph shows the amount of mRNA observed through the infection cycle for each promoter.

clear preference for certain DNA regions showing anisotropic bendability (31). Moreover, under conditions favoring protein-DNA interactions (high protein concentrations and low ionic strength), protein p6 can bind, at least in vitro, to the entire ϕ29 genome (10). The binding of p6 to the ends of the ϕ29 genome leads to the activation of the initiation of DNA replication and to repression of the promoters of the right DNA end, C2 and C1 (3, 31). Thus, it was possible that the GA-1 C1b and C2 promoters could be repressed similarly by GA-1 protein p6. Taking into account that the A1a and A1c promoters are also repressed from minute 10 after infection (Fig. 2), we considered that GA-1 protein p6 might also repress the two

		Spacer	TGN-10	Strength	Expression
A1a	GTTTCTATTGACTTCTTTTTTATTTCATAGTATACTAGAGATAGTTAA	17	-	+++	Early
A1b	ATAATTGTTGACTTGGAGTTTAAATAATGCTATAATAATATTATAAGG	17	+	+++	Early
A1c	ATAATAGTTGTAAATGATTTCACTATGTGATATAATAAAGATAGAAAG	17	+	+++	Early
A2b	AAAAGGGTTGTGTTTTTGTTCTAGTGGTGCTATTATTTAATTAACGCA	17	+	+++	Early
A2c	AACAAAACTAGCATTTAATAAAGAGTGTGTAAAAGAATGTGTTTCCTA	16	-	+++	Early
A3	TCTTCCTATTATAATGCGTCAAAATATGATATAATTAATCTATAGTAA	-	+	+++	Late
C1b	TGTTAGGTTGACAGAAGAGAAATAATATAGTATACTAAAGATAGTCAA	17	-	+	Early
C2	TTTTAGCTTGACTCTCATTGTTAATCTATGTTATACTATAATTAGTTA	18	+	++	Early

FIG. 3. Sequence and transcription start sites of phage GA-1 promoters. Promoters are aligned relative to their −35 boxes. Arrows indicate the transcription start sites. Those of promoters A1b, A2b, A2c, and A3 have been reported before (2, 11). The start site of promoters A1a, A1c, C1b, and C2 were deduced from primer extension assays of RNA obtained from infected cells. The following are also indicated: the length (in nucleotides) of the spacer between the −35 and −10 boxes (− denotes that a −35 sequence is not detected), the presence (+) or absence (−) of the TG dinucleotide 1-bp upstream from the −10 box (TGN-10), the relative strength of each promoter (+++, strong; ++, medium; + weak), and whether the promoter is active at early or late times postinfection.

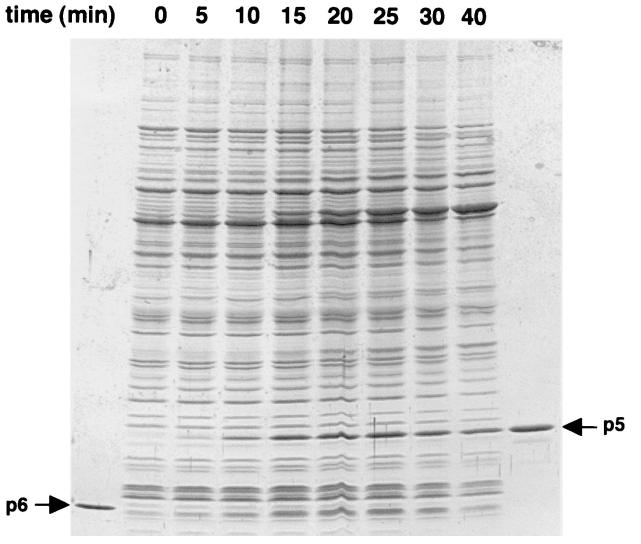

FIG. 4. Production of viral proteins during the infection cycle. Culture samples were obtained at different times after infection of *Bacillus* sp. strain G1R with phage GA-1, and proteins were resolved in sodium dodecyl sulfate-polyacrylamide gels in parallel with purified GA-1 proteins p5 and p6. The positions of GA-1 proteins p6 and p5 are indicated.

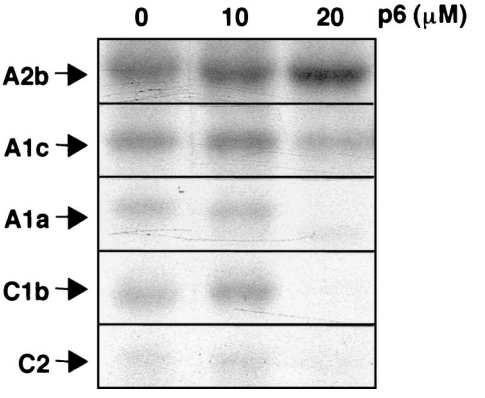

FIG. 5. Effect of GA-1 protein p6 on the expression of GA-1 promoters A2b, A1c, A1a, C1b, and C2 in vitro. Transcription reactions were performed using the complete GA-1 genome as a template and the indicated concentrations of GA-1 protein p6. Samples were preincubated with protein p6 for 10 min at 37°C prior to the addition of RNA polymerase and nucleoside triphosphates. Transcripts which originated from each promoter were analyzed by primer extension.

latter promoters. Therefore, it was of interest to determine whether the expression pattern of GA-1 protein p6 was similar to that of ϕ29. To this end, *Bacillus* sp. strain G1R was grown in Luria-Bertani medium (30) supplemented with 5 mM $MgSO_4$ at 37°C to a density of about 1×10^8 cells/ml and infected with phage GA-1 at a multiplicity of infection of 5. At different times, samples of 1.5 ml were taken and centrifuged. The resuspended pellets were sonicated, and the proteins were resolved in sodium dodecyl sulfate–10 to 20% polyacrylamide gel electrophoresis gels. As shown in Fig. 4, the amounts of the most abundant early proteins, p6 and p5, increased during the first 20 min after infection with GA-1, as occurs in the case of ϕ29. To test the possible role of protein p6 in transcriptional repression, GA-1 protein p6 was purified as described previously (6). The activities of the promoters under study were analyzed by in vitro transcription assays in the absence or presence of increasing amounts of purified GA-1 protein p6 and the *B. subtilis* σ^A RNA polymerase, purified as described previously (32). In these experiments, the complete GA-1 genome was used as a template to facilitate the formation of multimeric protein p6-DNA complexes. Each reaction mixture contained, in a 25-μl volume, 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM dithiothreitol, a 200 μM concentration of each nucleoside triphosphate, 7.5 U of RNasin, 0.2 nM genomic GA-1 DNA (purified as described in reference 13), and 200 nM σ^A RNA polymerase. Transcripts were analyzed by primer extension assays (20). Figure 5 shows that promoters A1c, A1a, C1b, and C2 were repressed in the presence of 20 μM protein p6, while the early promoter A2b, used as a control, was not; in fact, some activation was observed. The level of repression was different for each promoter. The activities of the A1c, A1b, C1b, and C2 promoters decreased to 32, 12, 6, and 5%, respectively. Interestingly, the level of p6-dependent repression shows a correlation with the expression profiles observed in vivo (Fig. 2), since transcripts arising from the A1c promoter were still abundant at late times postinfection, which is consistent with poor repression. Similarly, transcripts arising from the A1a promoter were scarce at late times postinfection, which agrees with efficient repression by protein p6. The parallelism between the behavior of the promoters in vivo and their response to protein p6 in vitro suggests that protein p6 represses these promoters in vivo. Considering that the amounts of protein p6 produced in vivo increase as infection proceeds, reaching very high concentrations, it is tempting to speculate that the protein p6 concentration serves to differentially modulate the expression of these early promoters during the infection cycle.

To further analyze the behavior of GA-1 protein p6 as a repressor, its ability to repress the GA-1 C2 promoter was studied under different ionic strength conditions. It had been demonstrated that although ϕ29 protein p6 can form a nucleoprotein complex with the DNA of the right end of the GA-1 genome, this complex did not stimulate initiation of DNA replication in assays containing the terminal protein and the DNA polymerase of GA-1 (6). Therefore, it was also interesting to study the effect of this heterologous DNA-protein p6 complex on the activity of promoter C2, located within this region of the genome. The effects of ϕ29 and GA-1 protein p6 on the GA-1 C2 promoter were studied in in vitro runoff transcription assays. Each reaction mixture contained, in a 25-μl volume, 25 mM Tris-HCl (pH 7.5); 10 mM $MgCl_2$; 2 mM dithiothreitol; 200 μM (each) CTP, GTP, and ATP; 100 μM [α^{32}-P]UTP (1 μCi); 2 μg of poly[d(I-C)]; 7.5 U of RNasin; 20 nM template DNA; and KCl and protein p6 at the concentrations indicated in the figure legends. The DNA used as a template was a 246-bp fragment containing the GA-1 C2 promoter (positions −165 to +81, with respect to the C2 promoter start site) and was obtained by PCR from the viral genome with primers 5′-AAATAGATTCCCCATGAACAAGCG-3′ and 5′-GAATAAGGCTAGATAGATATATTTAGG-3′. Phage ϕ29 protein p6 was purified from *B. subtilis* 110NA (22) as described previously (24). The reaction mixtures were incubated

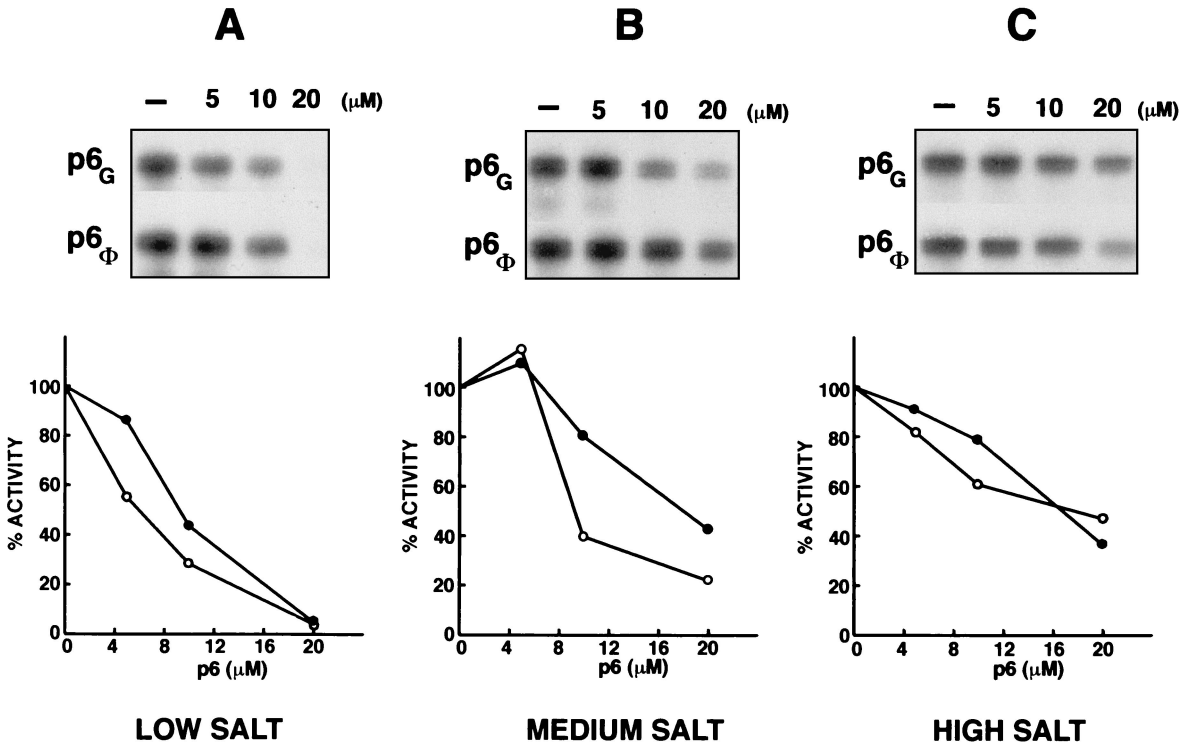

FIG. 6. Capacity of GA-1 and ϕ29 protein p6 to repress the GA-1 early C2 promoter in vitro. Transcription reactions were carried out under low (20 mM) (A), medium (100 mM) (B), or high (200 mM) (C) KCl concentrations. The graphs show promoter activity in the presence of increasing amounts of protein p6 from either GA-1 ($p6_G$ [open circles]) or ϕ29 ($p6_\phi$ [filled circles]).

for 5 min at 37°C, and reactions were initiated by the addition of 70 nM σ^A RNA polymerase. After an additional incubation for 15 min at 37°C, reactions were stopped and further processing was carried out as described previously (20). Transcripts were resolved on denaturing 6% (wt/vol) polyacrylamide gels and quantified using a Fuji BAS-IIIs image analyzer. As shown in Fig. 6, whereas the GA-1 C2 promoter was repressed by either phage-encoded p6 protein, repression by GA-1 protein p6 was in general more efficient than repression by ϕ29 protein p6. In addition, as predicted, repression of the GA-1 C2 promoter was more efficient under conditions of low ionic strength, but it was also evident at conditions of high ionic strength.

In phage ϕ29, the early genes are involved in DNA replication and transcription regulation. As shown in this work, the GA-1 ORFs that are absent in ϕ29 are transcribed from early promoters that are repressed at late infection times. This suggests that the putative proteins encoded by these ORFs may have functions related to DNA replication and/or transcription regulation. However, since both DNA replication and control of transcription occur without these ORFs in ϕ29 and related phages, this could imply that these putative proteins are involved in as yet unknown aspects of these processes. Alternatively, the possibility that these ORFs may have a role in interaction with the infected host cannot be excluded. Phage ϕ29 infects *B. subtilis*, while GA-1 infects the poorly characterized *Bacillus* sp. strain G1R, being unable to infect intact *B. subtilis* cells (1). Analysis of the 16S rRNA of *Bacillus* sp. strain G1R (performed by MIDI LABS Company, Newark, Del.) showed that it is more than 99% identical to that of *Bacillus pumilus*, an evolutionary distance that is small enough to consider them two strains of the same species (33). For comparison, the similarity with the *B. subtilis* 16S rRNA was 94.7%. Therefore, the ϕ29 and GA-1 hosts are different bacterial species. This may justify the need for additional functions in phage GA-1.

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