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Bacteriophage PSP3 and φR73 Activator Proteins: Analysis of Promoter Specificities

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Transcription from the late promoters of bacteriophage P2 and its satellite phage P4 is activated by a unique class of small, zinc-binding proteins. Using plasmid expression systems, we compared activators from two P2-like (helper) phages with those encoded by two satellite phages. The helper phage activators have more activity on the P4 phage *sid* promoter. In contrast, the satellite phage activators function better on the four late P2 promoters and on the P4 late leftward promoter. We purified one activator encoded by a P2-like phage and an activator from a satellite phage and determined their binding sites within the P2 and P4 late promoters. Differences in activity levels correlate with binding specificities; promoters that function best with the satellite phage activators have only one activator binding site centered at −55, while the P4 *sid* promoter, which has more activity with helper phage activators, has a second binding site centered at −18. Surprisingly, DNase I footprinting revealed only very minor differences in promoter binding by the two activators reported here and the P4 activator reported previously. Thus, the differences in transcriptional activity are probably due to interactions between the activators and RNA polymerase, rather than interactions between the activators and DNA.

P2 is a temperate coliphage with a genome of 33.5 kb (36). Its late genes, which encode the proteins required for construction of the phage particle and its release from the host, are organized into four transcription units. Transcription from the four late promoters, which are designated P_F, P_V, P_O, and P_P, is activated by a small phage-encoded protein called Ogr (10, 11). The sequences of these late promoters are similar to those of σ^{70}-dependent promoters in their −10 regions, and they contain a region of partial dyad symmetry composed of a consensus sequence, $TGTN_{12}ACA$, centered at approximately −55 from the start site of transcription. Mutational analysis of the P2 *F* promoter has shown that only the upstream half of this partial dyad sequence is required for transcriptional activation (2, 19).

P4 is a satellite phage with an 11.6-kb genome that requires the presence of a helper phage, such as P2, to produce capsid and tail structures and to lyse the host. P4 can activate the late promoters of P2-like phages, using its δ protein, which is homologous to P2 Ogr (10, 11). In addition, δ and Ogr activate the two P4 late promoters, P_{sid} and P_{LL}, which show sequence similarity to the P2 late promoters (14, 15). Both halves of the upstream consensus sequence of P_{sid} are required for δ's transcriptional activity (52). P4 late promoters contain an additional copy of the partial dyad symmetry sequence, positioned at −18. Biochemical experiments indicate that transcription from the *sid* promoter requires the host RNA polymerase containing the σ^{70} subunit (31). Thus, Ogr and δ are not alternative sigma factors.

Ogr and δ are members of a growing family of related transcriptional activators, which currently includes six members: P2 Ogr (6, 12), B from phage 186 (29, 30), P4 δ (22), satellite phage φR73 δ (50), NucC from a cryptic prophage recently identified in *Serratia marcescens* (24), and Pag from *Salmonella* phage PSP3. Each contains a $CysX_2CysX_{22}CysX_4Cys$ motif, except for P4 δ, which contains two. This motif appears to be important for binding zinc (33).

Previous data demonstrated a difference in the activities of P4 δ and P2 Ogr; δ was able to activate the lysis genes in a P2-lysogenic strain, resulting in the lysis of the culture, whereas Ogr could not (21). We wanted to determine whether this difference is also found in other satellite and helper phage activators. We also wished to show whether the dimeric structure of P4 δ gives this special activity.

To address these issues, we studied another helper phage activator, Pag from phage PSP3, and another satellite phage activator, δ from φR73. Unlike P4 δ, φR73 δ is monomeric in structure. We report here the activities of these activators on the P2 and P4 late promoters in vivo. In addition, we determine their binding sites on these promoters, using purified proteins.

MATERIALS AND METHODS

Bacteria, plasmids, and phages. The *Escherichia coli* strains, previously described plasmids, and phages used are listed in Table 1.

Construction of *ogr*, *pag*, and φR73 δ expression plasmids. pBJ49, expressing *ogr*, was constructed in several steps. The *Kpn*I (65.8%)-to-*Bgl*II (75.4%) fragment from P2 *lg* was ligated into the *Kpn*I and *Bam*HI sites of pBluescriptSKII$^+$ to create pBJ34. pBJ34a was made by site-directed mutagenesis that created a *Bsp*HI site and a concomitant F2I mutation at the beginning of the *ogr* gene. Then, the 372-bp *ogr*-containing *Dde*I fragment of pBJ34a was ligated into the *Hin*dIII site of pBluescriptSKII$^+$, which had been treated with Klenow fragment, to construct pBJ39. pBJ49 was constructed by ligating the 273-bp *Bsp*HI-to-*Hin*cII fragment from pBJ39 into the *Nco*I and Klenow-treated *Pst*I sites of pUHE24-2Bf$^+$, which contains the strong T7A1 promoter repressed by two *Lac*I binding sites.

pBJ72, expressing *pag*, was constructed by amplifying the *pag* gene from PSP3 by PCR with primers containing engineered restriction sites. The pag2 primer (ATAATCATGATGCACTG), used for the upstream end of the gene, contained a *Bsp*HI restriction site at the start of translation, and the pag3 primer (CTGCGATGGTCGACAGA), used for the 3′ end, contained a *Sal*I restriction site. The amplified DNA was cleaved with *Bsp*HI and *Sal*I, and the 244-bp *pag*-containing fragment was ligated into the *Nco*I and *Sal*I sites of pUHE24-2Bf$^+$.

The φR73 δ expression plasmid pBJ17f was constructed in two steps. First, pBJ11 was made by isolating the 1,164-bp *Nsi*I fragment from pCl-23a and

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TABLE 1. *E. coli* strains, phages, and plasmids used

E. coli strain, phage, or plasmid	Characteristic(s)	Reference or source
E. coli C derivatives		
C-2322	F^- prototrophic (P2 *lg*)	18
C-2420	F^- prototrophic Δ(*argF-lac*)U169	26
C-2421	Prototrophic Δ(*argF-lac*)U169 (F′ *proAB lacI*^{q} *Z*ΔM15 Tn*10*)	26
C-2448	F^- prototrophic Δ(*argF-lac*)U169 *rna*::kan	26
E. coli K-12		
D1210	F^- *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44 lacI*^{q}	43
E. coli B		
BL21(DE3)	F^- *hsdS gal* λ *lacUV5* T7 gene1; expresses T7 RNA polymerase	49
Bacteriophages		
PSP3 *vir*	Makes clear plaques	8
P2 *del15*	*ogr* and unable to lysogenize	5
P4 *vir1*	Does not lysogenize	37
Plasmids		
pACYC177	Ap^r Kan^r p15A origin	9
pBluescriptSKII$^+$	Ap^r general cloning vector	Stratagene
pBluescriptSKIIN$^+$	pBluescriptSKII$^+$ containing a *Nsi*I linker inserted at the *Sma*I site	This work
pBJ47	Ap^r ColE1 origin; P4 δ gene ligated into pUHE24-2Bf$^+$	26
pBJ70	*pag* gene ligated into pBluescriptSKII$^+$	This work
pCl-23a	14.4-kb *Bgl*II-to-*Eco*RI fragment containing the entire ϕR73 genome ligated into pBR322	50
pEE672	PCR-amplified fragment containing the P4 P_{LL} promoter ligated into pUC118	Xiashan Wu
pFZa	Ap^r ColE1 origin; P2 P_F *lacZYA*	26
pFZb	Kn^r p15A origin; P2 P_F*lacZYA*	This work
pGP1-2	Kn^r p15A origin; allows temperature induction of T7 RNA polymerase synthesis	51
pGZ119EH	Cm^r ColD origin; expression vector	34
pMal-c2	Ap^r ColE1 origin; plasmid to construct fusion with *malE*	New England Biolabs
pRG1	Kn^r p15A origin; plasmid containing *lacI*^{q}	20
pRS415	Ap^r ColE1 origin; promoterless *lacZYA*	48
pSidZT	Ap^r ColE1 origin; P4 P_{sid} *lacZYA*	26
pT7-5	Ap^r ColE1 origin; expression vector using T7 ϕ10 promoter	51
pUCF1	P2 *F* promoter ligated into pUC9	19
pUCOP118	P2 *O* and *P* promoters in pUC118	2
pUHE24-2Bf$^+$	Ap^r ColE1 origin; expression vector using the T7A1 promoter	26

ligating it into the *Nsi*I site of pBluescriptSKIIN$^+$. Then, pBJ11 was cleaved with *Bsm*AI; the ends were treated with Klenow fragment, and the fragment was cleaved with *Bsp*HI. The 246-bp fragment containing δ was ligated into pUHE24-2Bf$^+$ that had been treated with *Bsp*HI and *Sal*I, after filling of the *Sal*I site with the Klenow fragment.

Plasmids were also constructed that expressed *pag* or ϕR73 δ from a T7 RNA polymerase promoter. pBJ18 was constructed by isolating the 280-bp *Eco*RI-to-*Hin*dIII fragment from pBJ17f, which was followed by ligation into the *Eco*RI and *Hin*dIII sites of pT7-5. pBJ79 was constructed by isolating the 279-bp *Eco*RI-to-*Sal*I fragment from pBJ72, which was followed by ligation into the *Eco*RI and *Sal*I sites of pT7-5.

Construction of *malE-pag* and *malE*-ϕR73 δ fusion plasmids. The *malE-pag* fusion plasmid, pBJ73, was constructed by isolating the 279-bp *Eco*RI-to-*Sal*I *pag*-containing fragment from pBJ72 and ligating it into the *Eco*RI and *Sal*I sites of pMalc-2. The *malE*-ϕR73 δ fusion plasmid, pBJ61, was constructed by isolating the 280-bp *Eco*RI-to-*Hin*dIII δ-containing fragment from pBJ17f and ligating it into the *Eco*RI and *Hin*dIII sites of pMalc-2. The origin of replication of pBJ73 and pBJ61 was changed from a ColE1 origin to a ColD origin for purposes of coexpression of proteins from compatible plasmids. pBJ84 was constructed by cleaving pBJ61 with *Pfm*I, treating the ends with Klenow fragment, and digesting with *Hin*dIII. The 3.0-kb fusion-containing fragment was ligated with the 2.3-kb fragment from pGZ119EH which had been cleaved with *Xho*I, treated with Klenow enzyme, and digested with *Hin*dIII. pBJ85 was constructed by cleaving pBJ73 with *Pfm*I, treating the ends with Klenow and digesting with *Pst*I. The 3.0-kb fusion-containing fragment was ligated with the 2.3-kb fragment from pGZ119EH which had been cleaved with *Xho*I, treated with Klenow enzyme, and digested with *Pst*I.

Construction of plasmids carrying fusions of *lacZ* and phage late promoters. pBJ31b contains a fusion of P_{sid} and *lacZ*. The fragment that contains this fusion was first cut out of pSidZT (26) by using *Sal*I, which was followed by Klenow filling and then cleavage with *Pst*I. pACYC177 was cleaved with *Bam*HI, filled, and then cleaved with *Pst*I. The fragment that contains the fusion was ligated into the cleaved pACYC177 vector to give pBJ31a. The 1.2-kb *lacI*^{q}-containing *Pst*I fragment was excised from pRG1 (20) and was ligated into the *Pst*I site of pBJ31a to give pBJ31b. pZa contains a fusion of the P2 *P* gene promoter and *lacZ*. It was constructed by ligation of the 208-bp *Eco*RI-*Bam*HI fragment from pUCOP118 (2) into the *Eco*RI and *Bam*HI sites of pRS415 (48), which contains ampicillin resistance and a ColE1 origin. pOZa has *lacZ* fused to the P2 *O* gene promoter. It was constructed by isolating the 208-bp *Eco*RI-*Bam*HI fragment of pUCOP118, filling the DNA ends, and ligating into the *Sma*I site of pRS415. pVZa has *lacZ* fused to the P2 *V* gene promoter. It was constructed by isolating the 255-bp *Nde*I-*Xho*I fragment containing the *V* promoter from pBJAV1N (26), filling the ends, and ligating into the *Sma*I site of pRS415. pLLZa contains *lacZ* fused to the P4 late leftward promoter. It was made by ligating the 266-bp *Eco*RI-*Bam*HI fragment from pEE672 into the *Eco*RI and *Bam*HI sites of pRS415. The pACYC177 versions of each of the late promoter fusion plasmids were made by using *Eco*RI and *Nco*I to excise the promoter fusions from the ColE1 versions and ligating them into the *Eco*RI and *Nco*I sites of pBJ31b.

Modification of repressor control in a plasmid that expresses T7 RNA polymerase. In pGP1-2, *lacI* controls the expression of λ*CI*ts repressor, which in turn controls expression of T7 RNA polymerase. In some experiments that employ this plasmid, we wished to control expression from the pMal-c2 plasmids using *lacI*. Thus, we chose to put λ*CI*ts expression under control of a constitutive promoter. λ*CI*ts under the control of one of its natural promoters, λP_{RM}, was excised from pRK248*cI*ts by using *Bgl*II, and the fragment was inserted into the *Bam*HI site of pUC118, making pBJCIts. The λ*CI*ts gene under the control of P_{RM} was excised from pBJCIts by using *Eco*RI and *Pst*I. pGP1-2 was cleaved with the same enzymes to remove λ*CI*ts under *lacI* control, and λ*CI*ts under P_{RM} control was inserted in its place, making pGP1-21.

Bacteria and phage growth. The medium used for the growth of bacteria and phage was Luria broth (44). The growth procedures and storage buffer for PSP3 were those used for P2 (28). D1210 was used for the growth of PSP3.

Enzymes, nucleotides, and DNA. Standard protocols were used for minipreps, cesium chloride-purified DNA, PCR, and end labeling of DNA (44). The Sequenase kit from U.S. Biochemicals was used for sequencing single- and double-stranded DNA. Single-stranded DNA was prepared as described elsewhere (53). Site-directed mutagenesis was performed as previously described (26).

S30 extract. An in vitro transcription-translation assay (S30 extract) was used

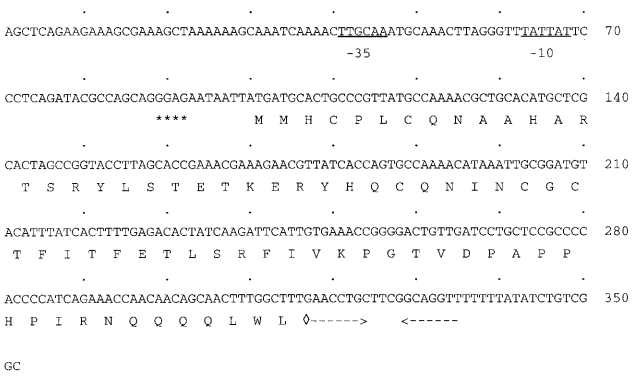

FIG. 1. The DNA sequence of the *pag* gene and its surrounding region. Amino acids are in one-letter code underneath the DNA sequence. Diamond, stop codon; asterisk, a potential Shine-Dalgarno sequence; inverted arrows, a potential rho-independent terminator; underlining, predicted −10 and −35 regions.

to monitor the transcriptional activities of Pag and ϕR73 δ. Extracts from C-2448 were prepared as described by Artz and Broach (3) and modified by Santero et al. (45). The medium used for growing C-2448 was MOPS (morpholinepropanesulfonic acid) supplemented with 0.2% Casamino Acids and 0.2 mM each of the following: uracil, thymine, adenine, guanine, and cytosine (41). Transcription-translation reaction mixtures were assembled in 50-μl volumes with 4 μg of reporter plasmid, and these were incubated with aeration for 60 min. Assays for β-galactosidase were performed according to Miller (40).

Overproduction and purification of the Pag and ϕR73 δ proteins. Luria broth supplemented with 5 μM $ZnSO_4$ was inoculated with C-2420 containing pBJ79, pBJ85, and pGP1-21 for Pag production or BL21(DE3) harboring pBJ18 and pBJ84 for ϕR73 δ. Cells with the plasmids pBJ79, pBJ85, and pGP1-21 were grown at 30°C to an A_{600} of 0.5. For induction of MalE-Pag, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 0.5 mM, and the culture was grown for 2 h. It was then shifted to 42°C for 30 min to induce the production of the unfused form of Pag and was subsequently incubated at 37°C for 1 h. BL21(DE3), containing pBJ18 and pBJ84, was grown at 37°C to an A_{600} of 0.9, IPTG was then added to 0.5 mM, and the culture was incubated for 3 h. The cells were then collected and stored at −70°C.

The purification procedures for Pag and ϕR73 δ were similar to those used for P4 δ, except that the buffer contained MOPS (20 mM MOPS [pH 7.1], 100 mM NaCl, 5% [vol/vol] glycerol, 20 μM EDTA, 1 mM β-mercaptoethanol) (26). Protein concentrations were determined by the Coomassie binding blue method (46).

DNase I footprinting. Pag and ϕR73 δ were footprinted on P2 and P4 late promoters in 20-μl reaction mixtures as previously described (26).

Nucleotide sequence accession number. The sequence presented has been assigned accession number U49731.

RESULTS

Cloning and sequencing of the PSP3 activator gene. PSP3 is a P2-like phage isolated from a strain of *Salmonella potsdam* (8). To clone its presumed P2 *ogr* homolog, a plasmid library was constructed by cleaving PSP3 DNA with *Pst*I and ligating the fragments into the *Pst*I site of pBluescriptIISK$^+$. This PSP3 plasmid library was screened for a DNA fragment containing the PSP3 activator gene, *pag*, by transforming the library into C-2420 containing the P4 P_{sid}-*lacZ* fusion plasmid pSidZT and plating cells on Luria broth plates containing ampicillin and X-Gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside). Any plasmid expressing *pag* should cause *lacZ* expression and make the colonies blue. Several blue colonies were obtained, and they all contained a plasmid with an inserted fragment of approximately 4 kb.

Sequencing of the *pag* gene was initiated by synthesizing a primer complementary to a conserved region in the *D* genes of phages P2 and 186, which are directly upstream of their respective late transcriptional activators. DNA sequence obtained for both strands of the *pag* gene region is shown in Fig. 1.

Analysis of this sequence reveals an open reading frame that would encode a 72-amino-acid protein, similar to P2 *ogr* and 186 *B* (Fig. 1). This open reading frame is preceded by a σ^{70}-dependent promoter sequence (38) and a good Shine-Dalgarno sequence. It is followed by a putative rho-independent terminator.

The organization of *pag* within the PSP3 genome is similar to those found for *ogr* in P2 and *B* in 186 (12, 29). For *ogr* and *B*, the σ^{70}-dependent promoters have been shown to be functional (7, 16, 42).

Analysis of the amino acid sequence of Pag using the BLASTP program (1) shows high homology to the P2 Ogr and the 186 B proteins. All three polypeptide chains are 72 amino acids long, and they share greater than 60% identity (Fig. 2). All other high-scoring proteins were members of the P2 activator family. Pag shares 40% identity with ϕR73 δ and 54% identity with NucC. P4 δ is a 166-amino-acid protein which is a covalently joined head-to-tail dimer of P2-like activators. Pag shares 35 and 24% identity with the first and second halves of P4 δ, respectively.

In vivo activity of Pag and ϕR73 δ proteins. Plasmids expressing *pag* (pBJ72) and ϕR73 δ (pBJ17f) were tested for the production of active protein by their ability to complement a P2 *ogr* phage, P2 *del15*. C-2421 was transformed with pBJ72 or pBJ17f, and P2 *del15* was plated on lawns of these strains with and without IPTG. In the absence of IPTG, P2 *del15* plated only on cells containing either pBJ72 or pBJ17f, and the efficiency of plating was the same as that for cells expressing *ogr* from plasmid pBJ49. Induction with 1 mM IPTG led to cell killing. Thus, pBJ72 and pBJ17f produce active Pag and ϕR73 δ, which can activate transcription from the late promoters of P2.

Activation of the lysis genes from a P2 prophage. When the P4 δ protein is induced from a plasmid in a P2 lysogenic strain, the cells lyse (21). This is due to the activation of the P_O

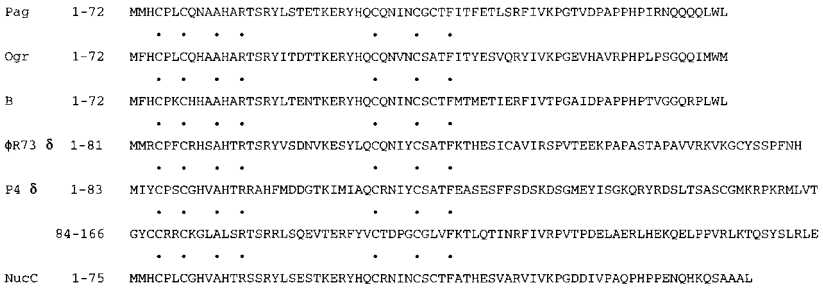

FIG. 2. Amino acid sequence comparison of Pag, Ogr, B, P4 δ, ϕR73 δ, and NucC. Dots, amino acids conserved in all six proteins. Protein sequences for Ogr (6, 12), B (29, 30), P4 δ (22), ϕR73 δ (50), and NucC (24) are given.

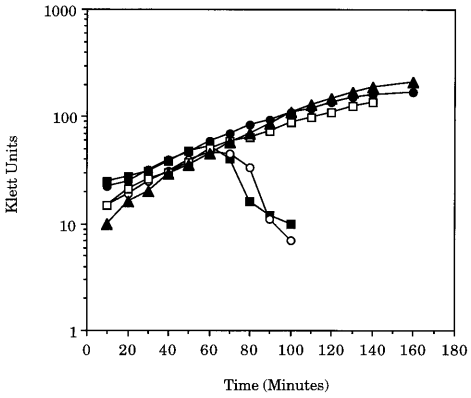

FIG. 3. Induction of lysis of a P2 lysogenic strain (C-2322) containing either the P4 δ plasmid pBJ47 (■), the φR73 δ plasmid pBJ17f (○), the *ogr* plasmid pBJ49 (●), the *pag* plasmid pBJ72 (▲), or no plasmid (□). The cells also carried the *lacI*^{q}-expressing plasmid pRG1. The cells were induced with 1 mM IPTG at 0 min, and the optical density of the cultures was monitored with a Klett-Summerson photoelectric colorimeter.

promoter, resulting in the production of all lysis proteins. However, when *ogr* is induced from a plasmid, the cells do not lyse. We used this lysis test to characterize Pag and φR73 δ. As shown in Fig. 3, expression of P4 δ and φR73 δ induces lysis of the culture 60 min after induction, whereas expression of Ogr and Pag does not. This difference in activities is not due to lower amounts of Ogr and Pag being produced, since they are synthesized at greater than twice the amounts of those of δ and φR73 δ from these plasmids (25). Thus, the two δ proteins are similar in activity, indicating that the covalent dimeric structure of P4 δ is not the important factor for predicting levels of transcriptional activity.

β-Galactosidase experiments with P2 and P4 late promoters fused to *lacZ*. To further investigate this difference in the activities of these four proteins, we fused each of the P2 and P4 late promoters to *lacZ* and assayed each promoter fusion with each activator. In the absence of an activator, these promoters show little activity (Fig. 4). In the presence of any one of these

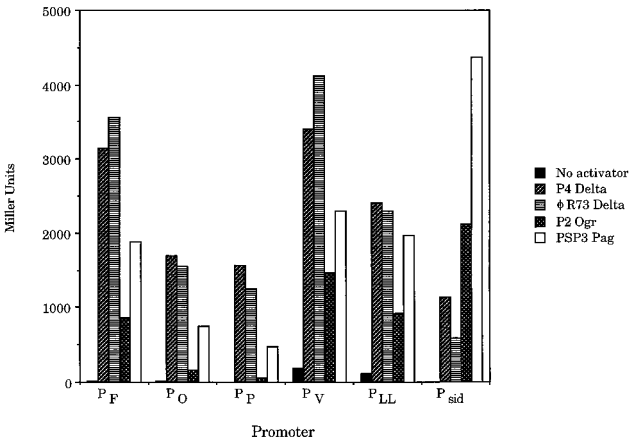

FIG. 4. Activation of P2 and P4 late promoters by helper and satellite phage activators. C-2420 containing a late promoter-*lacZ* fusion plasmid (either pFZb, pOZb, pPZb, pVZb, pPLLZb, or pBJ31b) was transformed with an activator plasmid (either pBJ17f, pBJ47, pBJ49, or pBJ72). Transformants were grown in M9 medium supplemented with 0.2% Casamino Acids to mid-log phase and then induced with 1 mM IPTG for 1 h. Aliquots were removed and assayed as described by Miller (40).

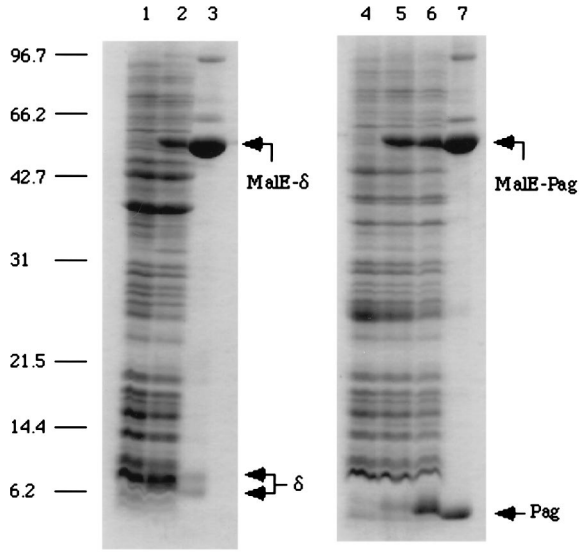

FIG. 5. Coomassie-stained SDS–12 to 19% polyacrylamide gel (17) of protein samples from the purification of Pag and φR73 δ proteins. Lanes: 1 and 2, cells uninduced and induced for φR73 δ, respectively; 3, the purified φR73 δ protein; 4, uninduced cells harboring the *pag*-expressing plasmids pBJ85 and pBJ79; 5, the same cells induced with IPTG; 6, cells induced with IPTG and a temperature shift to 42°C; 7, purified Pag protein. Molecular masses (in kilodaltons), which are shown at the left, correspond to those from the unstained ladder purchased from Life Technologies.

activators, transcription is induced. The satellite phage activators P4 δ and φR73 δ, activate transcription to a higher degree from the P2 and P4 P_{LL} late promoters and are less active with P4 *sid*. In contrast, helper phage activators P2 Ogr and PSP3 Pag cause more transcription from P_{sid} than from the other promoters. This pattern of activation has been seen with P4 δ and Ogr by using *cat* fusions to each P2 and P4 late promoter (2).

The unique activities seen with the *sid* promoter may be due to the presence of a second partial dyad sequence positioned between the −10 and −35 regions in P_{sid}. This second region has been shown to be protected in DNase I footprinting experiments with the P4 δ protein (26). To further investigate this difference, we purified the Pag and φR73 δ proteins and determined their binding sites within the P2 and P4 late promoters.

Purification of the Pag and φR73 δ proteins. We purified the Pag and φR73 δ proteins using the *malE* fusion system, the same procedure that was used to purify the P4 δ protein (26). This system was used because this group of proteins is difficult

TABLE 2. Summary of the purification of the Pag and φR73 δ proteins

Protein	Total U[a]	Amt of protein (mg)	Sp act (U/mg)	% Recovery
Pag				
Crude	1.6×10^7	512	3.1×10^4	
Amylose column	3.3×10^6	14	2.3×10^5	20
φR73 δ				
Crude	1×10^{11}	2,600	3.8×10^7	
Amylose column	1.5×10^9	22	6.6×10^7	1.5

[a] Units are defined as the amount of Pag or φR73 δ protein necessary to synthesize β-galactosidase from pSidZT in the S30 extract to convert 1 pmol of *o*-nitrophenyl-β-D-galactopyranosidase to *o*-nitrophenyl per min.

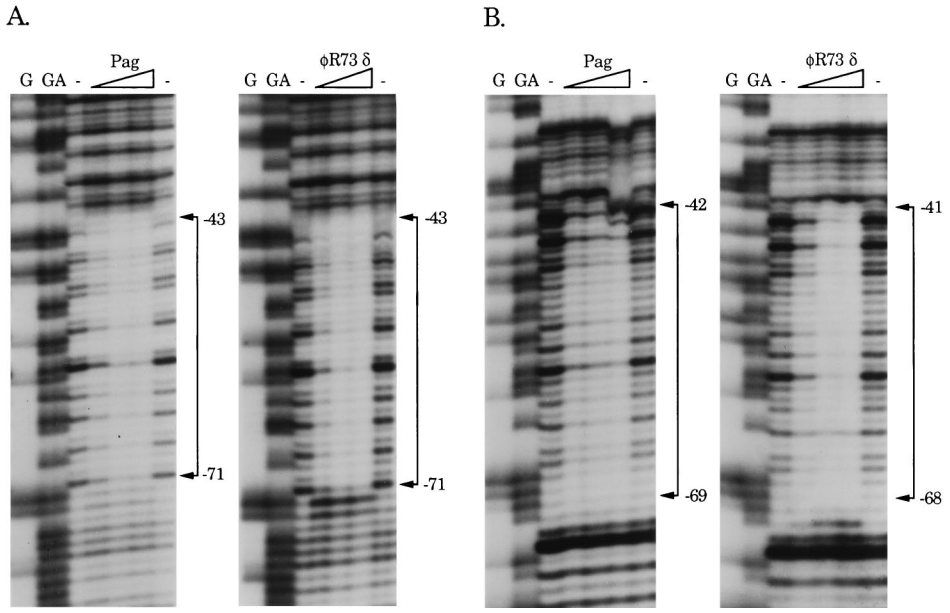

FIG. 6. DNase I footprints on the P2 *F* (A) and P2 *P* (B) promoters with Pag and ϕR73 δ proteins. The G and G+A lanes are the Maxam-Gilbert sequencing reactions. Minus sign, no protein added to the reactions. Pag (13.6, 20.4, or 27.2 μg) or ϕR73 δ protein (0.4, 0.8, or 1.5 μg) was used for protection. The protected regions are designated by two connected arrows on the right, and the regions where the protection occurs relative to the transcriptional start sites are indicated by the numbers beside the arrows.

to keep in solution, and MalE, the maltose-binding protein, aids in keeping them soluble. When we constructed the fusion plasmids, a Shine-Dalgarno sequence was included upstream of the translational start sites for *pag* and ϕR73 δ. As a result, when these plasmids were induced with IPTG, they produced the fusion form as well as an unfused form of Pag or the ϕR73 δ protein. This particular addition was also used in the P4 δ cloning strategy, and it was found that purification of the MalE-P4 δ fusion protein on an amylose column resulted in the copurification of the unfused form. Using the same strategy, we found that Pag and ϕR73 δ also copurify with their corresponding fusion proteins. Because the fusion proteins are produced in greater amounts than the unfused forms, and since it is the unfused forms of Pag and ϕR73 δ proteins which are transcriptionally active (data not shown), we constructed plasmids with *pag* or ϕR73 δ under the control of a T7 RNA polymerase promoter to increase the production of the unfused form of these proteins.

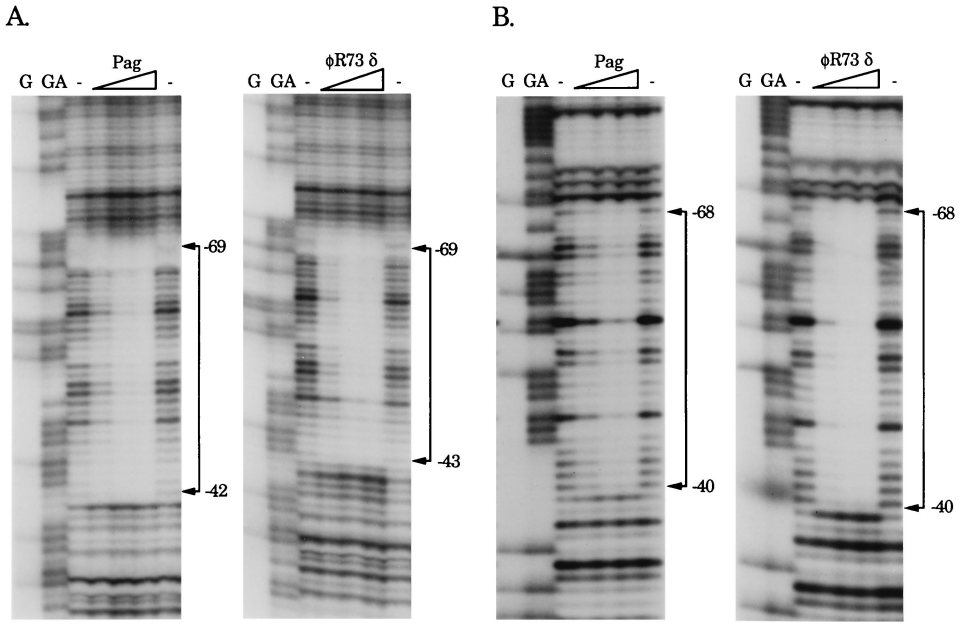

FIG. 7. DNase I footprints on the P2 *V* (A) and P4 P_{LL} (B) promoters with Pag and ϕR73 δ proteins. The amounts of proteins and notations are the same as those described in the legend to Fig. 6.

We purified the Pag and φR73 δ proteins using an amylose column. Figure 5 shows an electrophoretic analysis of the uninduced and induced cells expressing either φR73 δ or *pag*, as well as the purified proteins. Lane 3 shows φR73 δ eluted with maltose. The large protein migrating below the 66.2-kDa marker is the fusion, whereas the two proteins migrating near the 6.2-kDa standard are the unfused form.

Quantitation of the amounts of unfused Pag and φR73 δ were performed by scanning various dilutions of these proteins separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. The unfused forms of Pag and φR73 δ constitute approximately 70 and 50% of the purified molecules, respectively.

The purified φR73 δ protein shows two protein bands migrating at approximately 6.2 kDa. This has also been observed with the Ogr protein (33). However, it was concluded that it represented a reduced and an oxidized form of Ogr, and in the presence of a reducing agent, only one band appeared. This cannot be the case for φR73 δ, because dithiothreitol is present in the loading dyes, and the addition of excess β-mercaptoethanol to the protein before loading did not affect the ratio of the two bands (data not shown). Furthermore, this doublet is not observed with the Pag protein, which has one more cysteine than the φR73 δ protein. Western blot (immunoblot) analysis of purified φR73 δ protein with antibodies made against the P4 δ protein recognizes both proteins bands migrating at approximately 6.2 kDa (data not shown). Thus, the lower band in this doublet is probably a degradation product.

Because of the presence of a factor Xa recognition site just upstream of the fusion region of these proteins, we incubated small aliquots with factor Xa to determine whether the MalE portion of the fusion could be removed without loss of solubility and activity. As with the P4 δ protein (26), we were unable to keep the activators soluble without covalent attachment to MalE (data not shown). As a consequence, all of the following experiments were performed with uncleaved proteins.

Table 2 indicates the activities of the Pag and φR73 δ proteins and the percentages of recovered activity. The relative specific activities of these proteins differ dramatically, with the φR73 δ protein exhibiting a specific activity almost 300-fold higher than that of Pag. This is unexpected, since the proteins were assayed with the P_{sid}-*lacZ* fusion plasmid, which has been shown in vivo to activate transcription better with Pag. Their relative specific activities do not change if the proteins are assayed with the P_F-*lacZ* fusion plasmid (data not shown). The reason for this difference in specific activities is partially due to the differences in ability to bind promoter DNA, as observed in footprinting experiments.

DNase I footprinting experiments. To determine the binding sites of Pag and φR73 δ within the late promoters, we performed DNase I footprinting experiments. Figures 6 to 8 show the footprints for each protein with the P_F, P_P, P_V, P_{LL}, and the P_{sid} promoters, and a summary of the results is shown in Fig. 9. All of the promoters show a protected region over the partial dyad sequences centered at −55. In addition, there is a second binding region in the *sid* promoter for both the Pag and the φR73 δ proteins. Because there are two activator binding sites within the *sid* promoter, we refer to the one centered at −55 as site I and to the other as site II.

The P2 P_O and P_P are divergent promoters, and they share a common partial dyad sequence that activates transcription in both directions. Therefore, when these proteins bind to the promoter regions, they must have twofold symmetry.

Comparison of the protected regions for Pag and φR73 δ show nearly identical footprints, protecting between 27 and 31 nucleotides. There are only three minor observed differences. First, there are more hypersensitive sites on most of the late promoters with the φR73 δ protein. Second, the footprint on the *V* promoter is extended one nucleotide 3′ of the protected region in the presence of Pag. Finally, the footprint on the *P* promoter is shifted one nucleotide in the presence of φR73 δ.

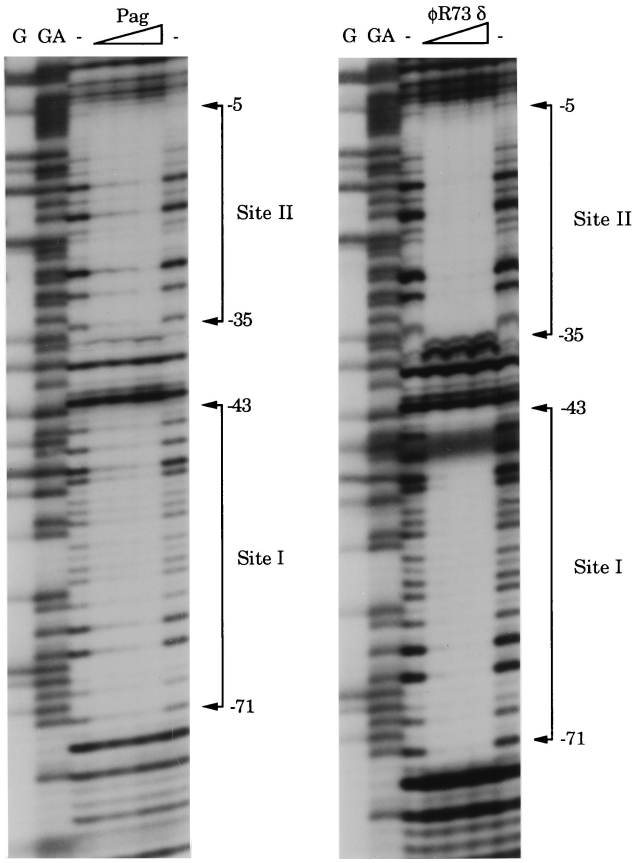

FIG. 8. DNase I footprints on the P4 P_{sid} promoter with Pag and φR73 δ proteins. The amounts of proteins and notations are the same as those described in the legend to Fig. 6.

As mentioned above, the Pag and φR73 δ proteins have different affinities for the binding sites within the late promoters. To get comparable protection with Pag and the φR73 δ protein, it is necessary to use between 25 and 30 times more Pag protein. This is consistent with the differences in the specific activities of Pag and φR73 δ proteins.

The footprint results obtained with the P4 δ protein are similar to those shown here (26). The P4 δ protein binds to the regions centered at approximately −55 in all of the late promoters, protecting between 27 and 31 nucleotides, and it also binds to the second site within the P4 P_{sid} promoter. The only difference is a one-nucleotide extension of the protected region within the P_{LL} promoter.

DISCUSSION

We have analyzed the promoter specificities of two helper phages and two satellite phage activators. In vivo, the helper phage activators cause higher levels of transcription on P4 P_{sid}, which contains two activator binding sites. The satellite phage activators function better on those promoters that contain a single activator site.

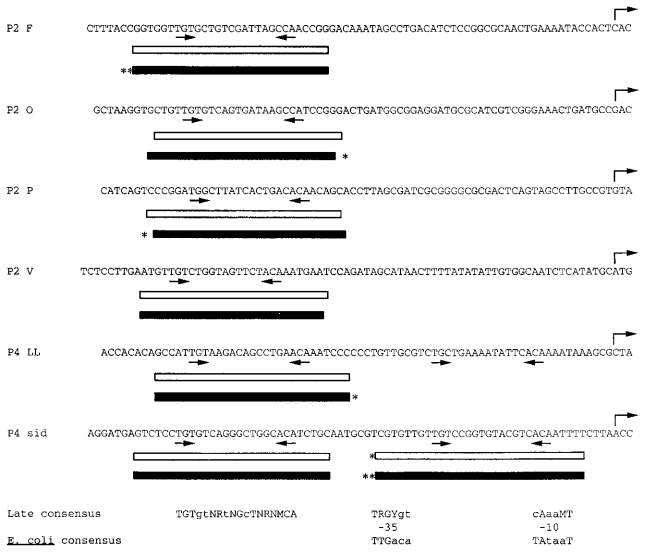

FIG. 9. Summary of the binding sites for Pag (white bars) and ϕR73 δ (black bars) on the P2 and P4 late promoters. The bottom strands of the *F*, *P*, and *sid* promoters and the top strands of the *O*, *V*, and LL promoters were analyzed. The *E. coli* σ^{70}-dependent promoter consensus sequence (38) is shown at the bottom, along with a consensus sequence for the P2 and P4 late promoters. Uppercase letters, nucleotides conserved in five or more promoters; lowercase letters, nucleotides conserved in three or more promoters; bent arrows, the transcriptional start site for each promoter (10, 11, 14, 15); inverted arrows, the partial dyad sequences; asterisks, sites that are hypersensitive to DNase I in the presence of the indicated activators.

What properties of these two classes of activators can explain this difference in promoter specificities? P4 δ appears to be a covalently joined dimer of our activator motif (Fig. 2). This dimeric structure cannot be a major factor in determining P4 δ activity, since ϕR73 δ, which is not a covalently joined dimer, has an activity pattern similar to that of P4 δ (Fig. 4). The difference in the specificities of helper and satellite phage activators is unlikely to be due to differences in the DNA-binding patterns of these activators, because the footprints are nearly identical for Pag, P4 δ, and ϕR73 δ (26) (Figs. 6 to 9).

The helper and satellite phage activators may interact differently with RNA polymerase holoenzyme. Genetic evidence suggests that these activators contact the α subunit of RNA polymerase. Two mutations near the C terminus of the α subunit, L289F and L290H, prevent the growth of P2, P4, and a P4 hybrid containing ϕR73 δ. Mutations in *ogr*, P4 δ, and ϕR73 δ restore the ability of these phages to grow in the mutant strains (4, 22, 27). Interaction with the α subunit is commonly observed for activators that bind upstream of RNA polymerase at positions similar to site I (23). The interactions of helper and satellite phage activators with the α subunit might be sufficiently different to give the different patterns of promoter specificity that we have observed.

In addition to the α subunit, the helper and satellite phage activators might interact with the σ^{70} subunit when bound at site II. This site is positioned between the −10 and −35 regions, where σ^{70} contacts DNA (47, 54). Recently, it has been shown that activators that bind near the −35 region interact with σ^{70} (32, 35, 39). When our phage activators are bound at site II, they might contact σ^{70} and alter the activity of polymerase at the *sid* promoter. The helper phage activators might increase transcription by polymerase when bound at site II. Alternatively, or in addition, the satellite phage activators might act as functional inhibitors of polymerase when bound at this position.

We are currently studying mutations of site II, in order to assess their effects on activator binding and on levels of transcription.

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