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Mutational Analysis of a Bacteriophage P4 Late Promoter

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Transcription from the late P_{sid} promoter of satellite bacteriophage P4 is dependent on the bacterial RNA polymerase carrying the σ^{70} subunit and is positively regulated by the product of the P4 δ gene or the *ogr* gene of helper bacteriophage P2. Through deletion and mutational analyses of the P_{sid} promoter, we identified mutations in the −10 region and in a region of hyphenated dyad symmetry centered around position −55 that inactivate P_{sid}. Most of these mutations alter base pairs that are highly conserved in the five other δ-activated P4 and P2 late promoters. We propose that the P4 δ and P2 *ogr* gene products bind the −55 region of the P4 and P2 late promoters.

P4 is a satellite bacteriophage that requires the products of the late genes of a helper phage such as P2 to carry out lytic multiplication (for reviews, see references 5 and 15a). The late genes of P2 code for the proteins involved in making the phage particle and lysing the bacterial host, *Escherichia coli* (49, 50). Transcription of the late genes of P2 is positively regulated by the product of the P4 δ gene (14, 15, 17, 18, 22, 51). The P4 δ gene is homologous to the P2 *ogr* gene, which also positively regulates transcription of the P2 late genes (7, 8, 16, 17, 54); each half of the 166-amino-acid residue δ protein is similar to the 72-residue *ogr* protein (24, 29). The P4 δ and P2 *ogr* genes also positively regulate transcription of the P4 late genes (17, 18). The *rpoA109* mutation of *E. coli*, which alters the α subunit of RNA polymerase, blocks transcription of the P2 and P4 late genes (14, 17, 20, 54). P2 and P4 mutants have been isolated that multiply in *rpoA109* hosts; the suppressor mutations occur in *ogr* and δ (24, 54). This is suggestive of direct interactions between the *ogr* or δ gene product and RNA polymerase at the P2 and P4 late promoters. We are interested in elucidating the mechanism by which the δ gene product activates transcription from the P2 and P4 late promoters.

The four P2 promoters and the two P4 promoters that are positively regulated by δ have been cloned and mapped at the nucleotide level (14, 15, 17, 18). The best characterized of these six late promoters is P4 P_{sid} (17, 18, 30). Experiments in vitro have demonstrated that δ-activated transcription from P_{sid} requires the bacterial core RNA polymerase and the σ^{70} subunit (30).

RNA polymerase holoenzyme containing the σ^{70} subunit recognizes two regions in the promoter, the −35 and −10 regions. The binding sites of transcriptional activators at their respective promoters have been located to at least four different positions relative to these promoter elements. (i) DNA between the −35 and −10 regions is recognized by MerR protein (43). (ii) Sequences flanking the −35 region are recognized by λ cII protein, which binds the opposite side of the DNA helix from RNA polymerase (26). (iii) DNA upstream of but near the −35 region is recognized by a large number of transcriptional activators, including λ cI protein (27), φ29 gene *4* protein (3), Mu *C* gene product (9, 37), AraC (33), catabolite activator protein (21, 47, 53), MalT (46), OmpR (35), OxyR (52), RhaR (57), and ToxR (40). (iv) Enhancerlike sequences more than 100 bp upstream of the −35 region are recognized by NtrC (2, 10, 41).

We believe that the δ gene product is a DNA-binding protein, but in the absence of direct evidence that a δ protein-DNA interactions occurs, it is not known where δ protein binds at the δ-activated promoters. In the work described here, our goal was to determine which regions of P4 P_{sid} are important for promoter function to identify potential binding sites for δ protein.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *E. coli* JM105 (60) was used as the host for all plasmid preparations. The *dut ung* strain RZ1032 (32) was used to make single-stranded dUTP-enriched template DNA for the mutagenesis experiments. MC1061 (Δ*lacZYA*) (12) was the bacterial host for β-galactosidase assays. Plasmid pUC119 (58) was used as the vector for the mutagenesis experiments. Plasmids pUC18 and pUC19 (42, 60) were used as vectors for sequencing. The δ expression plasmid, pGVB1, and the P_{sid}-*lacZ* fusion plasmids are described in detail below.

The growth medium used for DNA plasmid preparations was L broth supplemented with the appropriate antibiotic (36). The medium used during the preparation of single-stranded template DNA was 2× YT (58). Norit-treated M9 minimal medium supplemented with 1% Casamino Acids (Difco Laboratories, Detroit, Mich.) was used for the β-galactosidase assays. Ampicillin and kanamycin were used at a final concentration of 50 μg/ml each.

Recombinant DNA methodology and DNA sequencing. Restriction enzymes, DNA linkers, T4 DNA ligase, and Klenow fragment of DNA polymerase I were supplied by New England BioLabs, Inc. (Beverly, Mass.). A potassium-glutamate-based buffer was used for all restriction digests, primer extension reactions, and ligations (38). *Bal* 31 exonuclease and T4 polynucleotide kinase were purchased from

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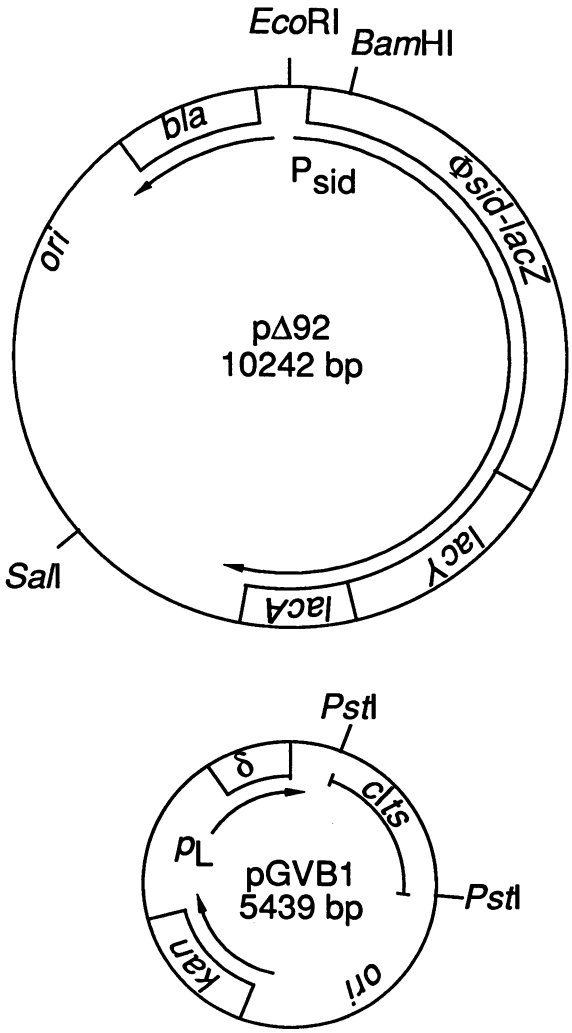

FIG. 1. Maps of the P_{sid}-*lacZ* fusion plasmid pΔ92 and the δ expression plasmid pGVB1. At 30°C, transcription of δ from the p_L promoter is repressed by temperature-sensitive λ repressor. At 42°C, δ is expressed and activates transcription of *lacZ* from the P_{sid} promoter of pΔ92.

Bethesda Research Laboratories. Other recombinant DNA techniques were as described previously (36).

Double-stranded DNA was sequenced by the method of Kraft et al. (31) with modified T7 DNA polymerase (55, 56) from United States Biochemical Corp. (Cleveland, Ohio) and 5′-[α-^{35}S]thio-dATP (6) (>600 Ci/mmol) from Amersham Corp. (Arlington Heights, Ill.).

The δ expression plasmid. Plasmid pGVB1 (Fig. 1) carries the P4 δ gene downstream of the λ p_L promoter and a temperature-sensitive allele of the λ repressor gene. pGVB1 was constructed as follows. (i) The 2,392-bp *Bgl*II fragment of pRK248cIts (4) containing the temperature-sensitive λ repressor allele was ligated into the *Bam*HI site of pUC19 (42, 60); (ii) the daughter plasmids were screened to identify one in which the inserted *Bgl*II fragment was oriented in such a way that the λ repressor allele could be isolated on a 1,120-bp *Pst*I restriction fragment; (iii) this *Pst*I fragment was ligated into the unique *Pst*I site of pNB58, a derivative of pACYC177 (13, 48) that carries the P4 δ gene downstream of the λ p_L promoter (21a).

Construction of P_{sid}-*lacZ* fusion plasmids. The P_{sid}-*lacZ* fusion plasmids are derivatives of pMC1403, which contains a *lacZ* gene that is missing transcriptional and translational initiation signals (11). In the original P_{sid}-*lacZ* fusion plasmid, pSidZ, the P_{sid} promoter with upstream sequence to position −451 and a portion of the *sid* gene was fused in frame to the *lacZ* gene of pMC1403 (30). The 702-bp *Eco*RI-*Bam*HI fragment of pSidZ containing P_{sid} and the upstream portion of the *sid-lacZ* fusion was ligated into pUC19 digested with *Eco*RI and *Bam*HI to create pUC-Δ451. (In the names of this and subsequent plasmids, the number following Δ indicates the number of bases present upstream of the start point of transcription.)

The first deletion, Δ92, was created by digesting pUC-Δ451 with *Bst*NI, end filling the single-base overhangs with Klenow fragment, and then ligating on *Kpn*I linkers. A 342-bp *Kpn*I-*Bam*HI fragment containing P_{sid} was isolated and ligated into the *Kpn*I-*Bam*HI sites of pUC19 to create pUC-Δ92. Subsequent upstream promoter deletions were created by digesting pUC-Δ92 with *Kpn*I, treating with *Bal* 31 exonuclease, ligating on *Kpn*I linkers, digesting the DNA with *Kpn*I and *Bam*HI, isolating the promoter-containing *Kpn*I-*Bam*HI fragments, and ligating them into pUC19 digested with *Kpn*I and *Bam*HI. Plasmids isolated from transformants of JM105 were sequenced, and a collection of promoter deletion mutations was identified.

Other P_{sid} promoter mutations were constructed by the method of oligonucleotide-directed mutagenesis devised by Kunkel (32). We modified the Kunkel method by using a potassium-glutamate buffer system instead of a NaCl-based buffer and by using Sequenase version 2.0, which contains no 3′-5′ exonuclease activity (56). These modifications resulted in a greater level of mutagenic efficiency than when Klenow fragment was used. Extension and ligation reactions were performed at room temperature. Single-stranded template DNA was packaged by using helper phage M13K07 (58). Oligonucleotides were designed according to the recommendations of Zoller and Smith (61). All other aspects of the mutagenesis system were as described previously (32).

The template for construction of deletions Δ64 and Δ63 contained the *Eco*RI-*Bam*HI fragment from pUC-Δ67 cloned in pUC119. The template used for the mutational scan across P_{sid} contained the *Eco*RI-*Bam*HI fragment from pUC-Δ92 cloned in pUC119. The *Eco*RI-*Bam*HI fragment containing each of the deletions or other mutations was transferred from the parent pUC derivative into the *Eco*RI and *Bam*HI sites of pMC1403 to generate each P_{sid}-*lacZ* fusion plasmid. The structure of pΔ92 is shown in Fig. 1; the other P_{sid}-*lacZ* fusion plasmids have a similar structure.

Assays for β-galactosidase activity. MC1061 was transformed to kanamycin resistance with pGVB1 and subsequently to ampicillin resistance with the P_{sid}-*lacZ* fusion plasmids. pGVB1, a derivative of pACYC177 (13), is replicatively compatible with the P_{sid}-*lacZ* fusion plasmids. The transformants were grown at 30°C on a low-speed shaker to the early log phase. The cells were then shifted to 37°C until the cultures reached a density of A_{600} = 0.3. The cells were induced for 1 h at 42°C and placed on ice for 30 min before the assays were performed. β-Galactosidase activity was assayed as described by Miller (39).

Nucleotide sequence accession numbers. The DNA sequences of the promoters used in this study are available from GenBank under the following accession numbers: J02474 (P_O and P_P), X02301 (P_V), X02300 (P_F), and X51522 (P_{LL} and P_{sid}). The GenBank accession number for P4 nucleotide positions 9468 to 9562 is X51522 (see Fig. 3) (23).

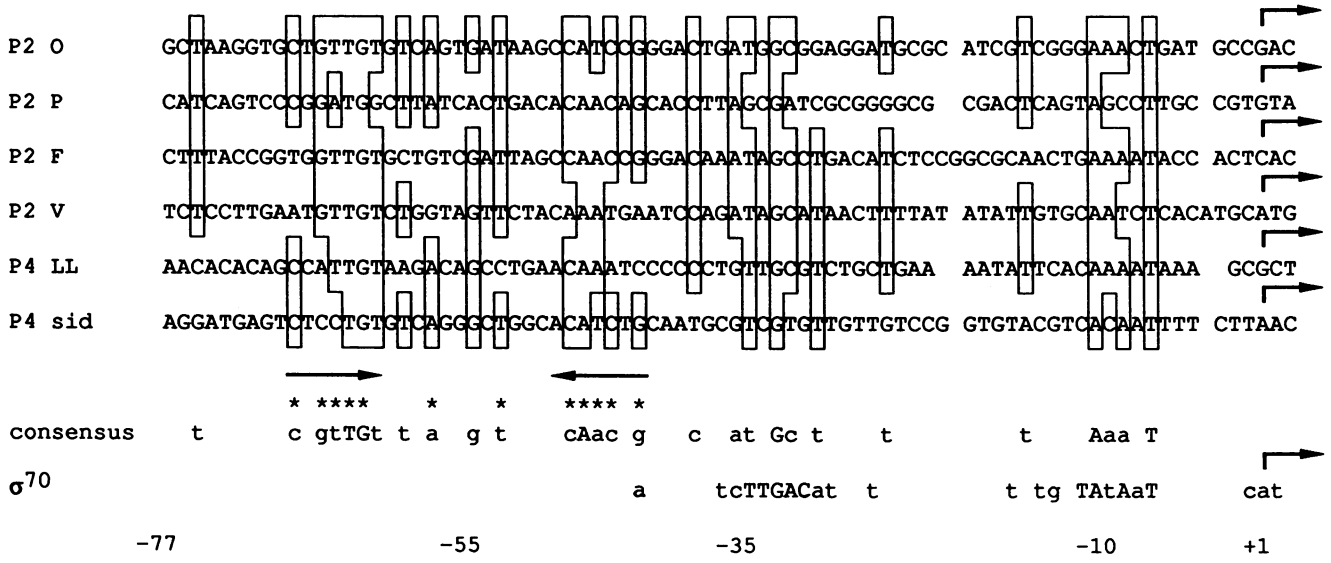

FIG. 2. Alignment and comparison of the sequences of the six late promoters of P4 and P2 (14, 15, 17, 18). Each promoter except the P4 left late promoter (P_{LL}) is named for the first gene in the transcript. The boxes around the nucleotides indicate positions at which the same nucleotide occurs in at least four of the six promoters. The arrows at the right indicate the point of initiation of transcription. The consensus sequence for the P2 and P4 late promoters is given. The convergent arrows above this consensus sequence indicate the position of the hyphenated dyad symmetry centered at −55, and the asterisks mark the nucleotides that define the dyad. The consensus sequence for *E. coli* σ^{70} promoters (25) is given for comparison. At the bottom, the positions relative to the start of transcription at P_{sid} are indicated.

RESULTS

Similarity of the P4 and P2 late promoters. Two P4 promoters and four P2 promoters are positively regulated by the P4 δ gene (14, 15, 17, 18). It is likely that the DNA sequences of these promoters differentiate them from other promoters and allow their recognition by the δ gene product and RNA polymerase. We aligned the sequences of these six promoters to reveal conserved sequences that might be involved in positive regulation (Fig. 2). Since it has been shown that transcription from the P4 late promoter P_{sid} requires the bacterial RNA polymerase utilizing the σ^{70} subunit (30), we maximized the similarities with the consensus sequence for *E. coli* σ^{70} promoters, TTGACA-N_{15-19}-TATAAT-N_{5-7}-start (25), by adjusting the spacing between the −35 and −10 regions and between the −10 region and the transcription start. In our alignments, shown in Fig. 2, the spacing between the −10 region and +1 was 5 to 7 bp, and the spacing between the −35 and −10 regions was held conservatively to 17 ± 1 bp, the most common distances (25).

Positively regulated σ^{70} promoters usually have −10 regions that are similar to the −10 consensus sequence but −35 regions that are a poor match to the −35 consensus (45); this proved to be true for the P2 and P4 late promoters. The −10 regions match the consensus sequence at four (P_F, P_{sid}, and P_{LL}), three (P_O and P_P), or two (P_V) positions, and the highly conserved A and T of the TATAAT consensus are present in all six promoters (Fig. 2). The −35 regions show less similarity to the consensus sequence, with three matches for P_F and only two matches for the other five promoters. Of the highly conserved T and G in the TTGACA consensus, the T is present in five of the promoters and the G in all six promoters. These similarities in the −35 regions may not be significant since the variable spacing between the −35 and −10 regions usually makes it possible to align at least one or two nucleotides in the −35 region with the consensus, even for promoters with a poor −35 region (45).

We were able to identify a region of conserved sequence in the six promoters extending from approximately −68 to −43 and centered around −55 (Fig. 2). This −55 region, which occurs at a constant distance upstream of what we have defined as the −35 region of each promoter, has an imperfect hyphenated dyad symmetry (indicated by the arrows in Fig. 2). The left arm of the hyphenated dyad contains an invariant TG sequence, and the right arm contains an invariant A. Remarkably, the divergent P2 P_P and P_O promoters (14) are spaced such that they share the same −55 region.

As explained in detail below, we used the results of these alignments to make a number of predictions about the sequences important for activity of a δ-regulated promoter, and we tested these predictions by performing a deletion and mutational analysis of the P4 late P_{sid} promoter. The strength of the mutant P_{sid} promoters was determined by using P_{sid}-*lacZ* fusion plasmids. In previous experiments, a reporter plasmid, pSidZ, was constructed in which P_{sid} and the first part of the *sid* gene was fused to the *lacZ* gene (30). In cells containing pSidZ or other fusion plasmids, transcription of *lacZ* from P_{sid} is activated by δ protein produced at 42°C from the compatible plasmid pGVB1 (Fig. 1). The strength of P_{sid} is measured indirectly by assaying the production of β-galactosidase in the Δ*lacZ* strain MC1061 (30).

Upstream deletions of P_{sid}. The six δ-regulated promoters display no similarity upstream of the left boundary of the −55 region. Furthermore, the divergent P_O and P_P promoters share the same −55 region. These observations suggest that the −55 region, but not DNA to the left of approximately position −68, is important for positive regulation by δ. We tested this hypothesis by examining the effects of progressive left-to-right deletions of the P4 P_{sid} promoter.

The P4 DNA in pSidZ extends to position −451 upstream of the start of transcription. We took advantage of a *Bst*NI site to remove DNA upstream of position −92 to generate

TABLE 1. Strength of P_{sid} promoters with upstream deletions[a]

P_{sid} promoter	β-Galactosidase activity (U)
Δ92	14,500 ± 5,200
Δ68	3,500 ± 1,200
Δ66	4,500 ± 300
Δ63	1,500 ± 200
Δ62	44 ± 4
Δ61	54 ± 4
Δ57	72 ± 12
Δ51	61 ± 11
Δ44	67 ± 19
Δ31	56 ± 11
Δ14	70 ± 4
Δ4	62 ± 14

[a] Cultures of MC1061 transformed with both pGVB1 and the appropriate P_{sid}-*lacZ* fusion plasmid were thermally induced and assayed for β-galactosidase activity as described in Materials and Methods. β-Galactosidase units are given as described by Miller (39). The value for Δ92 is the average of four independent determinations; the other values are the averages of two independent determinations.

the P_{sid}-*lacZ* fusion plasmid pΔ92 (Fig. 1). The strength of the P_{sid} promoter carried on pΔ92 was equivalent to that of the promoter carried on pSidZ (data not shown). Hence, the DNA upstream of position −92 is not required for full promoter strength. pΔ92 was used as the reference plasmid in subsequent experiments, and the P_{sid} promoter carried by this plasmid is referred to as wild type.

We created a series of plasmids similar to pΔ92 in which the upstream region of P_{sid} was progressively deleted from left to right. The results of β-galactosidase assays of cells containing the P_{sid} plasmids are given in Table 1. The relative strengths of the mutant P_{sid} promoters are presented in Fig. 3A, using the assumption that promoter strength is proportional to production of β-galactosidase. Deletion to −68 or −66 reduced the strength of the mutant promoters to 31% or less of the strength of wild-type P_{sid}, and deletion to −63 reduced the promoter strength to 10% of wild type. The strength of P_{sid} declined sharply when the deletions extended beyond position −63; such deletions reduced the strength of P_{sid} to a very low level of 0.59% or less of the strength of wild-type P_{sid}. These results indicate that the left boundary of P_{sid} lies near −63 in the left arm of the dyad. None of the deletions, including the deletion extending to −4, eliminated transcriptional activity. We believe that the low level of background activity was due to small amounts of transcription originating in the vector. When a mutation reduced the activity of P_{sid} to a level not significantly different from the background activity, we concluded that the promoter had been inactivated.

Mutational scan. The left boundary of P_{sid} near −63 determined by deletion analysis may not be accurate since the deleted promoter DNA is replaced by vector DNA that might contribute to or interfere with the activity of the mutant promoter. Furthermore, the deletion analysis tells little about which specific base pairs between −63 and +1 are important for promoter function.

To more accurately determine which regions of P_{sid} are important for promoter function, we performed a mutational scan across the promoter. As a simple approach, mutations were constructed in which a block of three or five contiguous base pairs was changed from the wild type to the complementary sequence; this scan extended from −79 to −12. The results of the β-galactosidase assays are presented in Table 2, and the relative strengths of the mutant promoters are summarized in Fig. 3B. Substitutions of complementary base pairs at positions −64 to −62, −55 to −53, or −49 to −47 inactivated P_{sid}, reducing its strength to less than 0.3% of wild type, a level comparable to that produced by deletions extending rightward beyond −63 (Fig. 3A). Mutation of base pairs from −74 to −70 reduced the strength of the mutant promoter to less than 7% of wild type, and mutation of most of the bases of the −35 region (from −36 to −32) reduced the strength to less than 8% of wild type. The effects of other mutations were less severe; the other down mutations gave mutant promoters with strengths varying from 14 to 79% of wild type. Mutation of base pairs at positions −61 to −59, −46 to −42, or −41 to −37 had no significant effect on promoter strength.

These results demonstrate that P_{sid} can be inactivated by mutation of certain base pairs in the −55 region. These base pairs include the innermost base pairs of the arms of the hyphenated dyad and a block of three base pairs just to the right of center of the −55 region. The 5-bp block from −74 to −70, which lies to the left of the −55 region, and the −35 region, which is examined in greater detail below, are also important for maximum promoter activity. There is a 10-bp region from −46 to −37 in which our mutations had no effect; part of this region overlaps most of the right arm of the hyphenated dyad.

Point mutations in the −55 region. In the mutational scan, P_{sid} was inactivated when any of three 3-bp blocks of the −55 region were altered (Fig. 3B). Since mutation of a single base pair in a 3-bp block was potentially responsible for the inactivation of P_{sid}, we conducted a search for such point mutations by constructing and analyzing seven mutant promoters containing complementary base pairs at single positions. The results of the β-galactosidase assays are given in Table 2, and the strengths of the mutant promoters are summarized in Fig. 3C.

Replacement of all three base pairs with complementary base pairs in the block from −64 to −62 inactivated P_{sid} (Fig. 3B). Individual changes at two of the positions in the block were tested. The G at position −63 in P_{sid} is found in all six δ-activated promoters (Fig. 2). When this G was replaced by a C, the strength of the promoter was reduced to 2.3% of wild type (Fig. 3C). The T at position −62 in P_{sid} is present in five of the six δ-regulated promoters (Fig. 2). When this T was changed to A, P_{sid} was inactivated (Fig. 3C).

P_{sid} was inactivated when all three base pairs of the block from −55 to −53 were replaced with complementary base

FIG. 3. Strengths of P_{sid} promoters as assayed by β-galactosidase production after thermal induction of transformants of MC1061 containing pGVB1 and P_{sid}-*lacZ* fusion plasmids. The bars indicate the range of promoter strength relative to the mean for Δ92 (100% is equivalent to production of 14,500 Miller units of β-galactosidase). The consensus sequence for the P2 and P4 late promoters, taken from Fig. 2, is presented above the sequence of P_{sid}. The numbering is relative to the start point of transcription. The −35 and −10 regions of P_{sid} are underlined. (A) P_{sid} promoter deletions. The base of the dotted bar lies to the immediate left of the last promoter base pair present in a particular deletion mutant. (B) P_{sid} complementary replacement mutations; (C) P_{sid} point mutations. The sequences below the bars indicate the base pair changes in the mutant promoters.

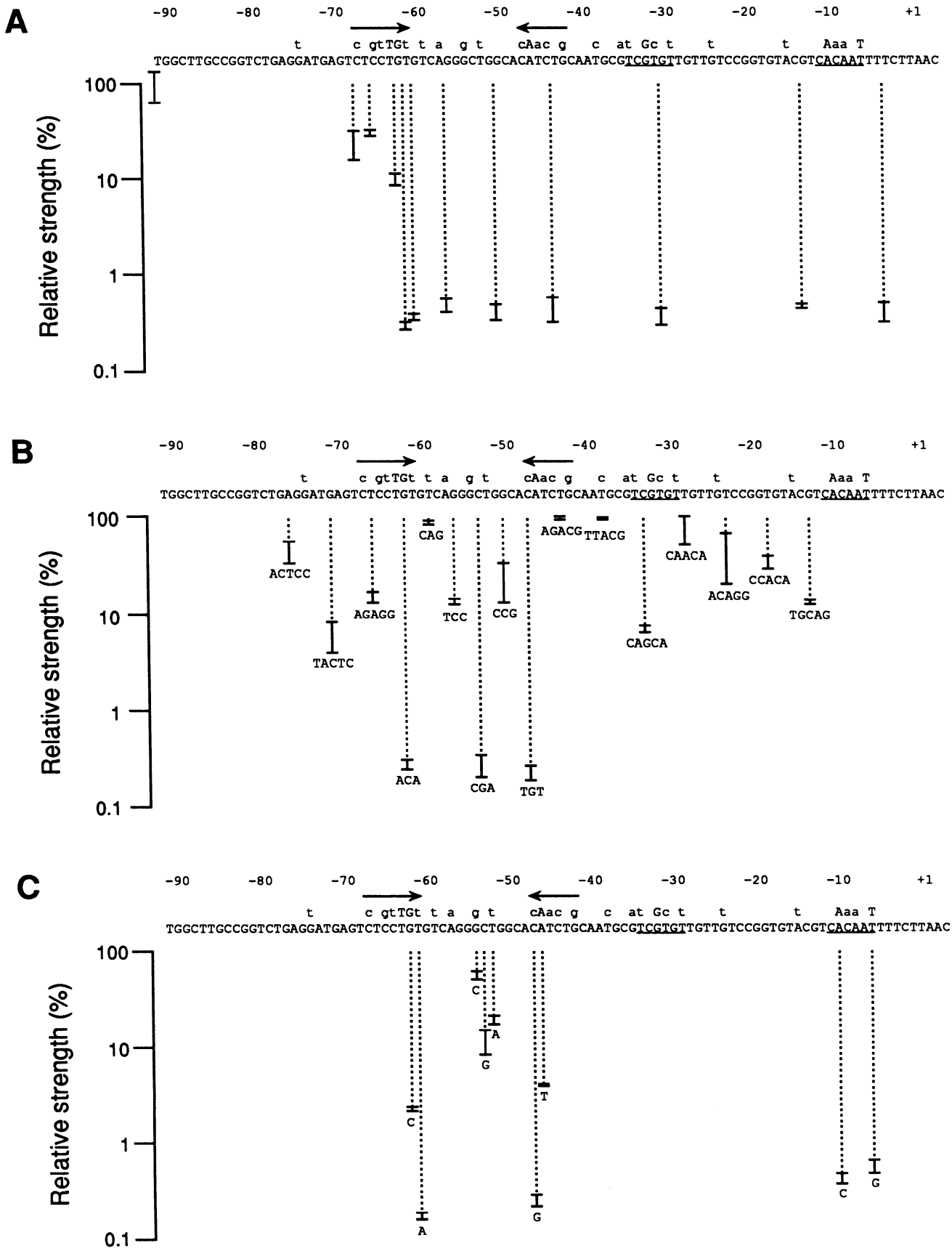

FIG. 3—*Continued*

TABLE 2. Strength of mutant P_{sid} promoters[a]

P_{sid} promoter	Sequence change	β-Galactosidase activity (U)
Δ92	None	14,500 ± 5,200
Complementary replacements		
cr79-75	TGAGG→ACTCC	6,800 ± 1,700
cr74-70	ATGAG→TACTC	940 ± 330
cr69-65	TCTCC→AGAGG	2,300 ± 300
cr64-62	TGT→ACA	42 ± 5
cr61-59	GTC→CAG	13,100 ± 600
cr58-56	AGG→TCC	2,040 ± 140
cr55-53	GCT→CGA	42 ± 11
cr52-50	GGC→CCG	3,600 ± 1,600
cr49-47	ACA→TGT	35 ± 6
cr46-42	TCTGC→AGACG	14,700 ± 300
cr41-37	AATGC→TTACG	14,600 ± 300
cr36-32	GTCGT→CAGCA	1,070 ± 90
cr31-27	GTTGT→CAACA	11,500 ± 3,600
cr26-22	TGTCC→ACAGG	6,700 ± 3,600
cr21-17	GGTGT→CCACA	5,300 ± 800
cr16-12	ACGTC→TGCAG	2,030 ± 130
Point mutations		
pm63	G→C	330 ± 20
pm62	T→A	25 ± 2
pm55	G→C	8,200 ± 700
pm54	C→G	1,700 ± 500
pm53	T→A	2,800 ± 300
pm48	C→G	36 ± 5
pm47	A→T	600 ± 10
pm11	A→C	59 ± 7
pm7	T→G	79 ± 12

[a] Assays and units were as described in Table 1, footnote *a*. Each value is the average of two independent determinations except the value for Δ92 (taken from Table 1), which is the average of four independent determinations.

pairs (Fig. 3B). Individual mutations at all three positions of this block were tested. The G at position −55 is present in five of the six promoters (Fig. 2), but changing it to a C had little effect, reducing the strength of the mutant promoter to 57% of wild type (Fig. 3C). The C position −54 is not conserved, appearing in three of the six promoters (Fig. 2). When the C was changed to G, the strength of the mutant promoter was 12% of wild type (Fig. 3C). The T at position −53 is present in five of the six promoters (Fig. 2), and changing it to an A reduced the activity of the mutant promoter to 19% of wild type (Fig. 3C). Hence, inactivation of P_{sid} by substitution of complementary base pairs at positions −55 through −53 cannot be attributed to a substitution at a particular base pair.

Replacement of the three base pairs in the block from −49 to −47 with complementary base pairs also inactivated P_{sid} (Fig. 3B). Point mutations were tested at two of these positions. The C at position −48 is present in five of the six promoters. When this C was mutated to a G, P_{sid} was inactivated (Fig. 3C). The A at position −47 is conserved in all six promoters. Mutation of this A to a T did not inactivate P_{sid} but reduced its strength to 4.1% of wild type (Fig. 3C).

Analysis of the −35 region. Mutation of the −35 region (−36 to −32) reduced the strength of the promoter to 7% of the wild-type P_{sid} promoter (Fig. 3B; Table 2), suggesting that the −35 region is important for activity of P_{sid}. The consensus sequence for the −35 region of σ^{70} promoters is TTGACA, and the average spacing between the −35 and −10 regions is 17 ± 1 bp (25). The −35 region of P_{sid}, which we defined as TCGTGT, shows little similarity to the consensus sequence, matching at only two of the six positions. This is in accord with the general observation that the −35 regions of positively regulated promoters show little or no similarity to the consensus sequence (45).

TABLE 3. Strength of P_{sid} promoters with altered −35 regions[a]

P_{sid} promoter	−35 region	β-Galactosidase activity (U)	
		−δ	+δ
Δ92	GTCGTGT	37 ± 10	14,500 ± 5,200[b]
cr36-32	CAGCAGT	ND[c]	1,070 ± 90[d]
Con35-30	GTTGACA	690 ± 130	29,100 ± 200

[a] Production of β-galactosidase in MC1061 transformed with only the appropriate P_{sid}-*lacZ* plasmid (−δ) or with both the P_{sid}-*lacZ* plasmid and pGVB1 (+δ) was assayed as described in Materials and Methods. The activity is expressed in the units defined by Miller (39). Each value is the average of two independent determinations except Δ92 (+δ), which is the average of four independent determinations.
[b] This value is from Table 1.
[c] ND, Not determined.
[d] This value is from Table 2.

Significant levels of transcription from P_{sid} were observed only when δ protein was produced in the cell (Table 3). It was possible that P_{sid} required activation by δ solely because of its poor −35 region, which might prevent recognition by RNA polymerase bearing the σ^{70} subunit. A mutant P_{sid} promoter with a consensus −35 region might be readily utilized by σ^{70} RNA polymerase in the absence of δ. A consensus −35 region might even strengthen the promoter to the point that it would no longer be positively regulated by δ. We tested these possibilities by creating a mutant P_{sid} promoter that contained a consensus −35 region located 17 bp upstream of its −10 region. We found that in the absence of δ, the mutant promoter had 18 times the activity of the wild-type promoter, yet this activity was less than 5% of the activity of a wild-type promoter activated by δ (Table 3). When activated by δ, the mutant promoter was twice as strong as the wild-type promoter (Table 3). Hence, the consensus −35 region increased the strength of the promoter, but the promoter was still dependent on δ for maximum activity.

Point mutations in the −10 region. P_{sid} is recognized by RNA polymerase carrying the σ^{70} subunit (30). The consensus sequence for the −10 region of σ^{70} promoters is TATAAT, with the underlined bases being the most highly conserved. The −10 region of P_{sid} has the sequence CAC AAT and is therefore similar to the consensus sequence at four of the six positions. We predicted that mutation of the A at −11 and the T at −7 of P_{sid}, which correspond to the two most highly conserved nucleotides of the consensus sequence, would greatly reduce the strength of the promoter. Indeed, we found that an A-to-C mutation at −11 or a T-to-G mutation at −7 inactivated P_{sid} (Fig. 3C).

DISCUSSION

Comparison of the δ-activated promoters of P2 and P4 revealed a conserved region of hyphenated dyad symmetry extending from positions −68 to −43 (the −55 region). In our deletion analysis of P_{sid}, we found that base pairs from −63 to the right are critical for promoter activity. In a mutational analysis, we identified some critical base pairs in the −55 region. Point mutations in P_{sid} at −62 and −48 inactivated P_{sid}, and point mutations at −63 and −47 greatly reduced

promoter activity. Mutation of three contiguous base pairs from −55 through −53 inactivated P_{sid}. Because RNA polymerase is not believed to contact DNA in the −55 region, these mutations may define critical contacts between the DNA and a transcription factor. We infer that δ protein is this transcription factor since our data and previously published data (30) show that transcription from P_{sid} occurs only when P4 δ protein is present. Assuming that δ protein binds the −55 region, it appears that δ protein is a member of the class of transcriptional activators that binds upstream of and close to the −35 region of the promoter. However, we have as yet no direct evidence that δ protein binds DNA.

The conserved base pairs of the dyad arms in P_{sid} are located at positions −68 through −63 and −48 through −43 (Fig. 2). Deletion of most of the left arm inactivates P_{sid}, and mutation of either one of the two innermost base pairs of each arm is sufficient to greatly reduce the strength of P_{sid}. These results suggest that the arms of the dyad are important for function of P_{sid}. This conclusion is supported by the results of deletion analysis of the P2 P_F promoter. Deletion to −69 does not affect P_F, but deletion to −64, well within the left arm of the dyad, inactivates the promoter (21a).

The centers of the dyad arms are separated by 20 bp, or two helical turns of B-form DNA. On the same side of the DNA helix between the dyad arms is a block of three base pairs from −55 through −53 which the mutational scan revealed is also critical for function of P_{sid}. We propose that δ protein acts as a dimer to contact three adjacent major grooves of the helix from approximately −68 through −48.

The P4 δ and P2 *ogr* proteins, which positively regulate the P2 and P4 late promoters, are comparable in size (166 and 72 residues, respectively) to the helix-turn-helix proteins, which have been shown through X-ray crystallography to contact two adjacent major grooves of the DNA helix (1, 28, 44, 59). The helix-turn-helix proteins have considerable α-helical character and are more or less globular in structure. If δ protein and *ogr* protein had less α-helical structure than the helix-turn-helix proteins and were consequently more elongated, a dimer of each could easily contact three adjacent major grooves of the DNA helix. δ protein, *ogr* protein, and the product of the *B* gene of the P2-related phage 186 are homologous and show no trace of the helix-turn-helix DNA-binding motif. Rather, they contain conserved cysteine residues reminiscent of the DNA-binding zinc-finger proteins (19, 24). Recently, *ogr* protein has been shown to bind zinc in vitro (34).

A simple model for the activation of transcription by these proteins follows the paradigm established for catabolite activator protein and λ *cI* protein (21, 27, 47, 53). δ protein, acting as a dimer, binds promoter DNA at the −55 region. The bound protein then acts, through direct protein-protein interactions or by altering the conformation of the DNA, to increase promoter recognition and use by RNA polymerase. It is possible that the *rpoA109* mutation, which alters the α subunit of RNA polymerase and prevents δ- or *ogr*-activated transcription, causes a disruption of the favorable protein-protein contacts between activator and polymerase.

Two mutations upstream of the −55 region were identified that also significantly decreased the activity of P_{sid}. Deletion of P_{sid} to −68 reduced the strength of the promoter to 25% of wild type. Substitution of the block of base pairs from −74 through −70 with complementary base pairs reduced the strength of the promoter to 6.5% of wild type. Perhaps these mutations define an additional site upstream of the −55 region at which δ protein or by an unidentified transcription factor binds.

In the mutational analysis of P_{sid} upstream of the −10 region, base pairs were changed to their complements. When blocks of contiguous base pairs were changed, it is possible that mutation of what would ordinarily be a critical base pair was compensated by the alteration of nearby base pairs. It is more likely that some base pairs critical for promoter function were not identified since mutation to the complementary base may confer no phenotype, whereas mutation to one of the remaining two possible bases might significantly reduce promoter strength. Thus, it is unlikely that we have identified all base pairs that are essential for function of P_{sid}.

Transcription from P_{sid} is initiated by RNA polymerase carrying the σ^{70} subunit (30). The −10 region of P_{sid}, CACAAT, matches the TATAAT consensus sequence for σ^{70} promoters at four of six positions. Not surprisingly, P_{sid} is inactivated by mutation of the highly conserved A at −11 or the highly conserved T at −7. The phenotype conferred by these mutations, which probably disrupt binding by the polymerase, confirms that the −10 region is important for promoter function.

Positively regulated promoters usually have −35 regions that show little similarity to the consensus sequence for the −35 regions of σ^{70} promoters (45), and this is true for P_{sid}. Mutation of the −35 region reduced the strength of P_{sid} but did not inactivate it. Conversion of the −35 region of P_{sid} to the consensus sequence strengthened the promoter but did not make it independent of activation by δ protein. Hence, the weakness of P_{sid} in the absence of δ protein cannot be attributed solely to the poor −35 region.

Currently, we are attempting to purify δ protein so that interactions between the protein and P_{sid} can be investigated. The promoter mutants characterized in this report potentially can be used to isolate compensatory mutations in the δ protein, which will provide clues as to specific interactions between amino acid residues of the protein and nucleotides of the promoter DNA.

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