JOURNAL OF BACTERIOLOGY, July 1995, p. 3743–3751
0021-9193/95/$04.00+0

Vol. 177, No. 13

Purification and Characterization of the Bacteriophage P4 δ Protein

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Received 13 January 1995/Accepted 13 April 1995

The bacteriophage P4 δ protein is a transcriptional activator of the late genes of P4 as well as the late genes of its helpers, such as bacteriophage P2. δ was purified, using a variation of the MalE fusion system. With this method we purified two forms of δ: a fusion of MalE and δ and an unfused form. The fusion by itself is not active in vivo or in vitro, but the mixture of the fusion and the unfused δ is active in both. Using nitrocellulose filtration and gel mobility shift assays, we show that δ binds DNA, and using DNase I footprinting, we show that δ binds to sequences centered at approximately −55 in the two late promoters of P4 as well as the four late promoters of its helper P2. In addition, the P4 *sid* promoter contains a second δ binding site centered at −18.

P4 is a bacteriophage that requires the presence of a helper phage, such as P2, to grow lytically (for reviews, see references 4, 10, and 30). Because P4 does not code for the necessary proteins needed to build the phage particle or lyse the host, it must recruit them from the helper. The late genes of P2, which encode the morphopoietic and lysis proteins, are activated by a small P2-encoded protein called Ogr (8, 9). In the absence of Ogr, P4 δ is able to activate the late genes of P2 (20). In addition, transcription of the late genes of P4 requires δ or Ogr (13, 14).

Ogr and δ are members of a unique class of transcriptional activators. Ogr is made up of 72 amino acids, while δ is made up of 166 amino acids (5, 11, 20). δ can be aligned such that it resembles a covalent head-to-tail dimer of Ogr. All members of this class of protein contain a structural motif, $CysX_2CysX_{22}CysX_4Cys$, which does not resemble any known zinc binding motifs. However, it has been shown by the zinc-blotting technique that Ogr binds zinc, and it is presumed that all members do (27).

Analysis of the four P2 and two P4 late promoters identifies a conserved region of partial dyad symmetery, centered at approximately −55. In addition, the P4 promoters contain the same partial dyad sequence centered at −18 (15). Mutational analysis of the P2 *F* promoter and the upstream dyad in the P4 *sid* promoter showed that deletions into or base substitutions in this dyad sequence disrupt activation of these promoters (1, 19, 49). From these analyses, it is proposed that these partial dyad sequences serve as binding sites for δ or Ogr to activate transcription. Even though Ogr has been purified, there are no biochemical data for this model, because of its low specific activity (27). In the work presented here, our goal was to purify the P4 δ protein and determine if it binds to these partial dyad sequences in the late promoters of P2 and P4.

MATERIALS AND METHODS

Bacterial and bacteriophage strains. The strains and plasmids used are listed in Table 1. The strain C-2421 was constructed by first transducing the Tn*10*-linked Δ(*argF-lac*)U169 mutation from SH210 (41) into strain C-1a to create C-2418. A tetracycline-sensitive derivative was isolated on Bochner medium (6) to construct C-2420. Finally, the F′ from XL1-Blue was mated into C-2420 to create C-2421. C-2448 was constructed by transducing the *rna*::*kan* from DK533 into C-2420.

Plasmids. The expression vector pUHE24-2Bf$^+$ was constructed by first removing the *Bsm*I fragment in the *cat* gene. Next, the *Rsa*I fragment from pUC119, which contains the f1 packaging region, was ligated into the Klenow fragment-filled-in *Aat*II site of pUHE24-2Bf. pUHE24-2Bf$^+$ packages the coding strand of the nonfunctional *cat* gene. Plasmid pBJ47 was constructed by ligating the δ containing *Bsp*HI-to-*Hpa*I fragment from P4 *vir1* DNA into the *Nco*I and Klenow fragment-filled-in *Sal*I site of pUHE24-2Bf$^+$. pBJ60 was constructed by ligating the δ containing *Eco*RI-to-*Pst*I fragment from pBJ47 into pMal-c2. pSidZT was constructed by replacing the *Eco*RI-to-*Sal*I fragment of pSidZ with the *Eco*RI-to-*Sal*I fragment from pRS475. This places four copies of the *rrnB1* terminator upstream of P_{sid}. pFZa was constructed by ligating the Klenow fragment-filled-in *Bam*HI-to-*Fok*I fragment from pUCF1 into the *Sma*I site of pRS415. pBJAV1N was constructed by introducing an *Nde*I site, by site-directed mutagenesis, in pBJAV1 at positions 10136 to 10141 according to the P2 map. pBJ67 was used for making single-stranded DNA for site-directed mutagenesis of the δ gene. It contains the δ gene from pBJ47 ligated into pUC118. The δ gene is oriented such that it is transcribed from the *lac* promoter.

Enzymes, nucleotides, and DNA. Restriction enzymes, exonuclease III, factor Xa, and Klenow fragment were purchased from New England Biolabs. T4 DNA ligase and DNase I were purchased from Boehringer Mannheim. [α-^{32}P]dATP was purchased from Amersham. Standard protocols were used for minipreps, cesium chloride-purified DNA, end labeling of DNA, and Maxam-Gilbert sequencing reactions (37). Sequencing was performed using the Sequenase kit from United States Biochemical.

Site-directed mutagenesis. The method used for generating mutations is a modification of the method of Taylor et al. (48). Briefly, the phosphorylated primer was annealed to the single-stranded template, using 5 μg of DNA and 4 pmol of primer. The oligonucleotide was extended and ligated by adding 10 mM $MgCl_2$; 0.25 mM (each) dTTP, dGTP, dATP, and dCTPαS; 6 U of Klenow fragment; 1 mM ATP; and 1 U of T4 DNA ligase. The reaction mixture was incubated at room temperature overnight. The next day, any remaining single-stranded DNA was removed by centrifugation through a nitrocellulose disposable microfilter (Schleicher & Schuell). The DNA was precipitated and dissolved in 65 μl of *Nci*I buffer (30 mM NaCl, 12 mM Tris [pH 8.0]), and the wild-type strand was cleaved with 5 U of *Nci*I for 90 min. To digest the wild-type strand, 16 μl of exonuclease buffer (333.75 mM NaCl, 253.5 mM Tris [pH 8.0], 30 mM $MgCl_2$, 50 mM dithiothreitol) was added to the reaction mixture along with 2 U of exonuclease III. The reaction was stopped after 15 min by heating to 70°C for 15 min. The digested strand was resynthesized by adding 10 mM $MgCl_2$, 0.25 mM (each) of the deoxynucleotides, 1 mM ATP, 3 U of DNA polymerase I, and 2 U of T4 DNA ligase. After 3 h, an aliquot was transformed. Transformants were then sequenced to confirm the presence of the mutation.

Phage growth and transduction. Growth and storage of P4 were as described previously (23). P1 stocks were grown and P1 transductions were performed as described previously (46). M13K07 was used for making single-stranded DNA (50).

S30 extract. An S30 extract was used to monitor the transcriptional activity of δ. The strain used for making extracts was C-2448, and the extracts were prepared as previously described by Artz and Broach (2) and modified by Santero et al. (38). The medium used for growing C-2448 was MOPS (morpholinepropane-

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TABLE 1. *Escherichia coli* strains, phages, and plasmids

E. coli strain, phage, or plasmid	Characteristic(s)	Reference or source
C strains		
C-1a	F^- prototrophic	39
C-2322	F^- prototrophic (P2 *lg*)	18
C-2418	C-1a made *zai-736*::Tn*10* Δ(*argF-lac*)U169	This work
C-2420	C-2418 made Tet^s	This work
C-2421	C-2420 F′ *proAB lacI*^{q} *ZΔM15* Tn*10*	This work
C-2448	C-2420 *rna*::*kan*	This work
K-12 strains		
DK533	HfrC, *proC19 metB* Str^r *rna*::*kan*	45
SH210	HfrC, *phoA8 glpD3 glpR2 relA1 tonA22* (λ) *zai-736*::Tn*10* Δ(*argF-lac*)U169	41
XL1-Blue	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lac* (F′ *proAB lacI*^{q} *ZΔM15* Tn*10*)	7
Bacteriophages		
P4 *vir1*	Does not lysogenize	31
P2 *lg* Δ*ogr17*	Unable to activate late genes	20
P1 *clm clr*	Forms chloramphenicol-resistant, temperature-inducible lysogens	Lab stock
M13K07	Helper phage	50
Plasmids		
pBluescriptSKII$^+$	General cloning vector	Stratagene
pBJAV1	P2 *V* promoter cloned into pBluescriptSKII$^+$	29
pBJAV1N	An *Nde*I site was introduced into *orf-30*	This work
pBJ47	P4 δ cloned into pUHE24-2Bf$^+$	This work
pBJ60	P4 δ from pBJ47 cloned into pMal-c2	This work
pBJ67	P4 δ cloned into pUC118	This work
pBJ74	pBJ60 with the initiating methionine for δ mutated to a glycine; produces only the MalE-δ fusion	This work
pBJ76	P4 P_{LL} promoter cloned into pUC118	This work
pEE672	PCR-amplified fragment containing the P4 P_{LL} promoter	Xiashan Wu
pFZa	P2 P_F cloned upstream of *lacZYA*	This work
pMal-c2	Plasmid to construct fusions with *malE*	New England Biolabs
pRS415	Promoterless *lacZYA*	44
pRS475	*lacUV5 lacZYA*	44
pSidZ	P4 P_{sid} cloned upstream of *lacZYA*	24
pSidZT	Same as pSidZ, except there are 4 tandem copies of the *rrnB1* terminator before P_{sid}	This work
pUHE24-2	Expression vector using the T7A1 promoter	H. Bujard
pUHE24-2B	pUHE24-2 which has the *Bsm*I fragment removed; contains only one *Nco*I site	This work
pUHE24-2Bf$^+$	pUHE24-2B which has the f1 packaging region on an *Rsa*I fragment from pUC118 cloned into the *Aat*II site; packages the coding strand	This work
pUC118	General cloning vector	50
pUCF1	P2 *F* promoter cloned into pUC9	19
pUCOP118	P2 *O* and *P* promoters cloned into pUC118	1
pΔ48	*sid* promoter deletion that removes site I	49
pΔ93	*sid* promoter deletion that removes DNA upstream of −93; functions as wild type	49

sulfonic acid) supplemented with 0.2% Casamino Acids and 0.2 mM (each) uracil, thymine, adenine, guanine, and cytosine (35).

Overproduction and purification of δ protein. Luria broth (1 to 10 l), supplemented with 1 μM $ZnSO_4$ was inoculated with pBJ60 or pBJ74. The cells were grown to an optical density at 600 nm of 0.4 to 0.5, at which time IPTG (isopropyl-β-D-thiogalactopyranoside) was added to 0.5 mM and the cells were grown for an additional 2 to 3 h. The cells were collected and frozen at −70°C.

δ was purified by resuspending the frozen cells in 10 ml of buffer (50 mM Tris [pH 8.0] at 4°C, 175 mM NaCl, 5% [vol/vol] glycerol, 20 μM EDTA, 1 mM β-mercaptoethanol) for every gram of cells. The cells were passed through an ice-cold French press at 10,000 lb/in^2. The lysate was centrifuged at 16,000 rpm in an SS-34 rotor for 30 min. The supernatant was measured and applied to an amylose column at a flow rate of 15 ml/h. The column was washed with 5 column volumes of buffer, and the protein was eluted with buffer containing 10 mM maltose. The fractions containing δ were pooled and dialyzed against buffer containing 20% glycerol and stored at −70°C. Protein concentrations were determined by using the Coomassie blue binding assay (42).

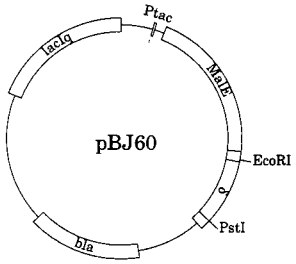
Cleavage of the MalE-δ fusion with factor Xa. The conditions used were those recommended by the manufacturer. Protein was diluted 1:1 in cleavage buffer (50 mM Tris [pH 8.0] at 4°C, 100 mM NaCl, 10% glycerol, 20 μM EDTA, 1 mM β-mercaptoethanol, 2 mM $CaCl_2$), and 1 μg of factor Xa was added for every 50 μg of fusion protein. The reaction mixture was incubated at room temperature (20°C), and aliquots were removed at various times and assayed for activity.

Nitrocellulose filter binding. The fragments used were isolated from plasmids. The *F* promoter fragment was isolated by cleaving pUCF1 with *Eco*RI and *Bam*HI. The −55-centered site from P_{sid} was isolated by cleaving pΔ93 with *Eco*RI and *Brs*DI. This fragment contains only the upstream site. The −18-centered site from P_{sid} was isolated by cleaving pΔ48 with *Eco*RI and *Eco*57I. pΔ48 is missing the upstream site.

For the experiment, approximately 50,000 cpm of labeled DNA (100 fmol) was incubated in binding buffer (50 mM Tris, 100 mM NaCl, 10% glycerol, 20 μM EDTA, 1 mM β-mercaptoethanol [pH 8.0] at 4°C) with purified protein for 10 min at 37°C. The reaction mixtures were filtered through nitrocellulose filters presoaked in binding buffer and washed three times with 500 μl of binding buffer. The filters were dried, put into EcoLite scintillation fluid, and counted.

Gel mobility shift assay. The fragments used were isolated from plasmids. The *F* promoter fragment was isolated by cleaving pUCF1 with *Eco*RI and *Bam*HI. The *OP* promoter fragment was isolated by cleaving pUCOP118 with *Eco*RI and *Bam*HI. The *V* promoter fragment was isolated by cleaving pBJAV1N with *Nde*I and *Hin*dIII. The *sid* promoter fragment was isolated by cleaving pΔ93 with *Eco*RI and *Eco*57I. Finally, the P_{LL} promoter fragment was isolated by cleaving pBJ75 with *Hin*dIII and *Eco*RI.

For the gel mobility shift experiment, 10,000 to 20,000 cpm of labeled DNA (50 fmol) was incubated in binding buffer (see above) with 2 μg of poly(dI-dC), purified protein, and double-distilled water to 15 μl. The reaction mixture was incubated at 37°C for 15 min. The samples were loaded onto a 1.8% agarose gel



pBJ60

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                                                    ---->
ATC GAG GGA AGG ATT TCA GAA TTC ATT AAA GAG GAG AAA TTA ACC ATG
 ile  glu  gly  arg  ile  ser  glu  phe  ile  lys  glu  glu  lys  leu  thr  Met
                  ^
```

pBJ74

```
                                                    AgeI
ATC GAG GGA AGG ATT TCA GAA TTC ATT AAA GAG GAG AAA TTA ACC GGT
 ile  glu  gly  arg  ile  ser  glu  phe  ile  lys  glu  glu  lys  leu  thr  gly
                  ^
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FIG. 1. Plasmid diagram of pBJ60 and pBJ74. The sequence shows the junction between MalE and δ. The Shine-Dalgarno sequence is underlined, and the initiating methionine for δ is designated with an arrow. ^, factor Xa cleavage site.

with 90 mM Tris, 90 mM boric acid, and 20 μM EDTA running buffer and run at 4°C at 200 V for 2 to 3 h until the bromophenol blue dye had reached the bottom. The gel was dried on Whatman 3MM filter paper at 70°C for 90 min.

DNase I footprinting. The fragments used for footprinting were the same as those used for the gel mobility shift assay, except the *sid* promoter and the *OP* promoter fragments were isolated with different restriction enzymes. The *sid* promoter was isolated by cleaving pΔ93 with *Eco*RI and *Bam*HI. The *OP* promoter was isolated by cleaving pUCOP118 with *Afl*III and *Bam*HI.

For the footprinting reactions, 100,000 cpm of labeled DNA (100 fmol) was added to buffer (50 mM Tris, 100 mM NaCl, 10% glycerol, 20 mM EDTA, 1 mM β-mercaptoethanol, 10 mM $MgCl_2$, 1.5 mM $CaCl_2$ [pH 8.0] at 4°C), 2 μg of poly(dI-dC), and purified protein in a total volume of 20 μl. The reaction mixture was incubated at 37°C for 10 min to initiate the binding. DNase I (0.5 ng) was added, and the reaction mixture was incubated for an additional 2 min. Then 5 μl of DNase I stop solution (2 M ammonium acetate, 250 mM EDTA, 0.1 mg of tRNA per ml) was added, and the tube was placed on ice. The reaction mixture was brought to a volume of 100 μl with TE (10 mM Tris, 1 mM EDTA [pH 7.6]), phenol chloroform extracted to remove excess protein, and then precipitated. Loading dyes were added to the pellet at 1 μl for every 5,000 cpm. The reactions were run on a 6% sequencing gel.

RESULTS

Purification of δ. Many attempts at purifying δ were made, using conventional methods. However, when δ is overproduced, the vast majority of the protein pellets with the cellular debris after lysis and centrifugation. This has also been shown to occur when the P2 Ogr protein is overproduced. Previous attempts to unfold and renature Ogr from the pellet fraction showed that little activity was obtained, and the protein was unable to bind DNA or activate transcription in a purified system (27). Since fusions made to MalE, the maltose-binding protein (MBP), have been shown to make insoluble proteins more soluble (26), we constructed a *malE*-δ fusion. This construct is unique, in that the δ gene was cloned into the pMal-c2 vector with its own Shine-Dalgarno sequence. Figure 1 shows a diagram of pBJ60 with the sequence of the junction between *malE* and the δ gene. This construct will produce a fusion, and because of the Shine-Dalgarno sequence correctly positioned upstream of the δ gene, an unfused form of δ may be produced. When induced, pBJ60 produces active protein in vivo, as shown by plating P2 *lg* Δ*ogr17*. In the absence of the plasmid, the phage was unable to form plaques, but in the presence of pBJ60 the phage formed plaques with the same efficiency as that of cells expressing *ogr* from a plasmid. In addition, another

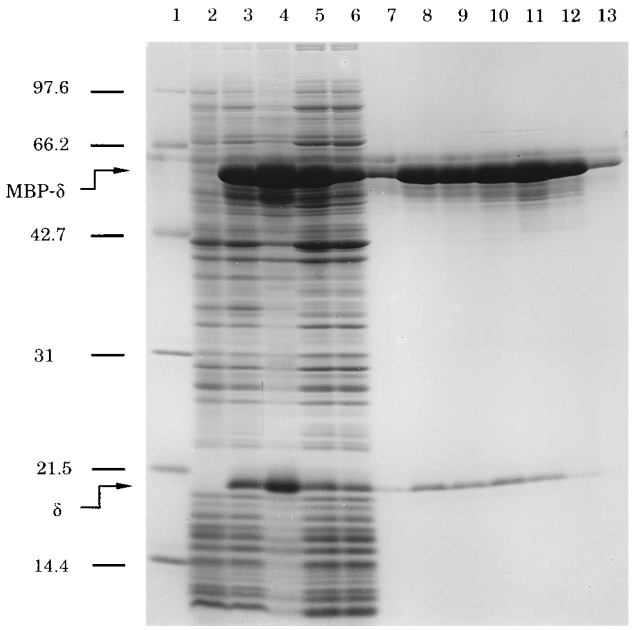

FIG. 2. Coomassie blue-stained sodium dodecyl sulfate (SDS)–12 to 19% polyacrylamide gel (16) of protein samples from the purification of δ. Lane 1 contains molecular mass markers (numbers on the left are in kilodaltons). Lanes 2 and 3 contain uninduced and induced cells, respectively, carrying pBJ60. Lane 4 contains proteins from the pellet dissolved in 8 M urea after cell lysis and centrifugation. Lane 5 contains proteins from the supernatant after lysis and centrifugation. Lane 6 contains proteins from the flowthrough. Lanes 7 to 13 are eluted fractions from the amylose column. The fusion is designated with an arrow labeled MBP-δ, and the unfused protein is designated with an arrow labeled δ.

plasmid, pBJ74, that has the initiating methionine of δ mutated to a glycine and, as a result, only produces the fusion was constructed. This plasmid does not support the growth of P2 *lg* Δ*ogr17*.

When cells containing pBJ60 are induced, they overproduce two proteins: a fusion of MalE and δ and an unfused form of δ (Fig. 2). When extracts of induced cells are applied to an amylose column, two proteins bind and are eluted with buffer containing maltose. The large protein is the MalE-δ fusion, and the small protein is the unfused form of δ. Since the unfused δ should not bind to the amylose resin, this indicates that, in solution, δ forms a multimer. A summary of the purification results is shown in Table 2. The total amount of recovered protein was 18%. The unfused δ constitutes approximately 15% of the molecules in the mixture, as measured by scanning protein gels with a densitometer. Because of the large amount of nonfunctional fusion, the specific activity did not increase significantly after use of the amylose column.

The purified δ was tested in the S30 extract for transcriptional activation, using a P4 P_{sid} or a P2 P_F *lacZ* fusion plasmid. Both plasmids were activated by purified δ (Fig. 3), and the

TABLE 2. Purification of δ

Fraction	Total protein (mg)	Total units[a]	Sp act (U/mg)	% Recovery
Crude extract	407	4.9×10^{10}	1.2×10^{8}	
Amylose column	75	1.0×10^{10}	1.4×10^{8}	20

[a] Units are defined as the amount of δ protein necessary to synthesize enough β-galactosidase in the S30 extract to convert 1 pmol of *o*-nitrophenyl-β-D-galactopyranoside to *o*-nitrophenyl per min.

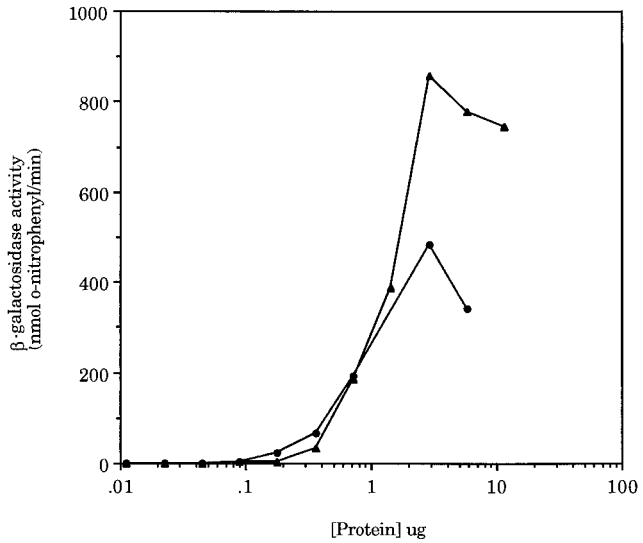

FIG. 3. Activation of the *sid* or *F* promoter by δ. Various concentrations of δ were assayed with either the P_{sid}-*lacZ* (●) or P_F-*lacZ* (▲) fusion plasmid with the S30 extract.

dose-response curves are similar. When protein is purified from cells expressing pBJ74, which produces only a fusion of MalE and δ, there is no transcriptional activation (data not shown). Therefore, it is concluded that the only transcriptionally active molecules in the mixture are the unfused forms of δ. However, we are unable to rule out the possibility that heteromultimers are able to activate transcription.

Since a cleavage site for factor Xa lies between MalE and δ of the fusion protein, attempts were made to cleave the fusion. Proteins containing only the fusion or containing a mixture of the fusion and unfused δ were incubated with factor Xa, and aliquots of the cleavage reaction mixture were tested for transcriptional activation at various times after incubation. Figure 4 shows that cleavage of the mixture of the fusion and unfused δ resulted in the loss of activity. However, incubating δ at room temperature did cause loss of some activity. When only the fusion is cleaved with factor Xa, no transcriptional activity is obtained (data not shown).

The reason for the loss of activity appears to be the insolubility of δ. During the cleavage reaction, the released δ molecules and the unfused δ probably form multimers. Because MalE is not present to keep the proteins in solution, δ precipitates. This explains why there is a loss of activity over time when factor Xa is present. The reason why there is a loss of activity when incubating δ at room temperature without factor Xa may be a higher affinity of δ to multimerize with other unfused δ molecules as opposed to the fusion. Over time, multimers of the unfused δ form, and because they are not linked to a molecule containing MalE, which helps keep δ soluble, they may now be unable to remain in solution and so may cause a loss of activity.

When the cleavage reaction is allowed to go to completion, approximately 15 h, the unfused δ is shown to be insoluble and the δ molecule released from the fusion appears to be degraded by factor Xa, even though there is no Ile-Glu-Gly-Arg sequence within δ. This is concluded from the results shown in Fig. 5. The protein gel shows that after complete digestion and a spin in a microcentrifuge, the MalE (MBP) portion of the cleaved fusion remains in solution while δ, both the unfused and the released molecules, is pelleted. The δ molecules that are released from the fusion do not accumulate. This is indicated by the lack of a protein band that corresponds to the size

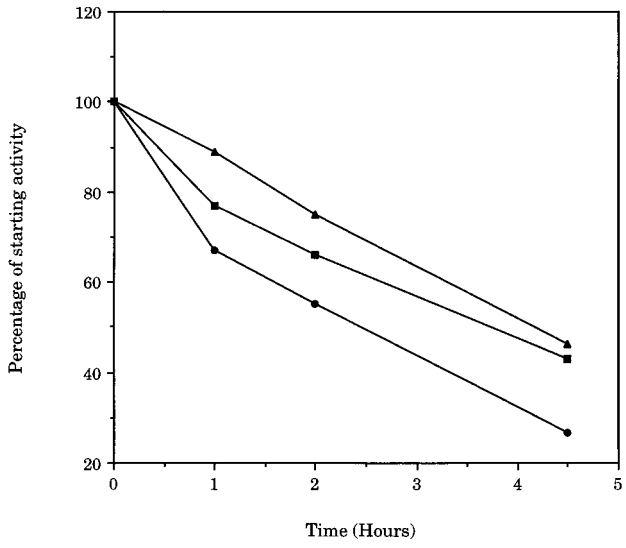

FIG. 4. Activity of δ after cleavage with factor Xa. At various times, aliquots of δ incubated with or without factor Xa were tested for activity in the S30 extract, using either the P_{sid}-*lacZ* (●) or P_F-*lacZ* (▲) fusion plasmid. δ incubated with no factor Xa (■) was assayed only with the P_{sid}-*lacZ* fusion.

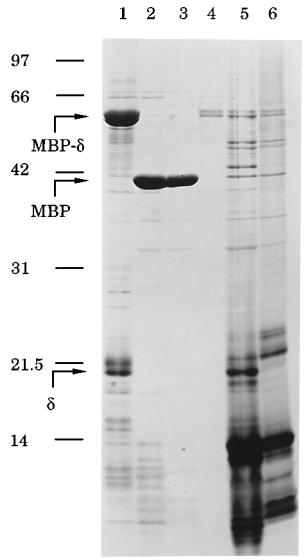

FIG. 5. Silver-stained (40) SDS–12 to 19% polyacrylamide gel of protein samples from the factor Xa cleavage reactions. The mixture of fused and unfused δ or the fused protein alone was incubated with factor Xa for 15 h. To remove any insoluble proteins, the samples were spun in a microcentrifuge for 5 min. The supernatant was removed, and the pellet was washed with 100 μl of cleavage buffer. The pellet was resuspended in protein loading dyes and loaded onto a polyacrylamide gel. Molecular mass markers are indicated on the left in kilodaltons. Lane 1 contains the supernatant of the mixture of fusion and unfused δ with no factor Xa. Lane 2 contains the supernatant of the mixture of δ proteins incubated with factor Xa. Lane 3 contains the supernatant of the fusion alone incubated with factor Xa. Lane 4 contains the pellet of the mixture of δ proteins incubated with no factor Xa. Lane 5 contains the pellet of the mixture of δ proteins incubated with factor Xa. Lane 6 contains the pellet of the fusion alone incubated with factor Xa. The fusion is indicated by an arrow labeled MBP-δ. The released MalE (MBP) portion of the fusion is indicated by an arrow labeled MBP. The unfused δ is indicated by an arrow labeled δ.

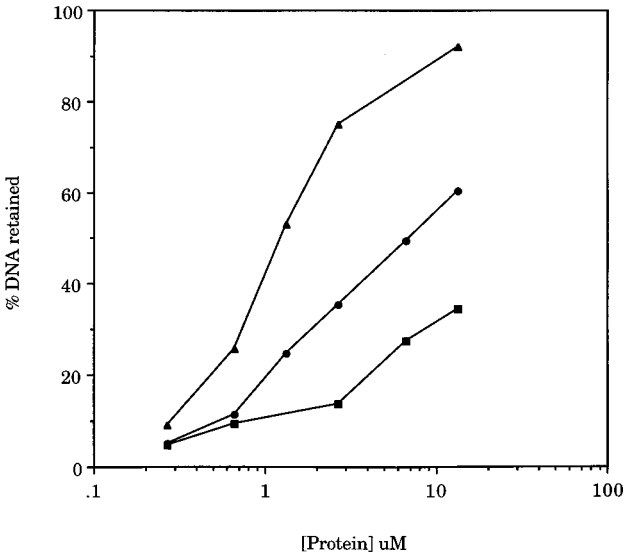

FIG. 6. Nitrocellulose filter binding experiment with either site from the *sid* promoter or the site from the *F* promoter. Various concentrations of δ were incubated with 100 fmol of DNA. ■, binding site from P_{sid} centered at −55; ●, binding site from P_{sid} centered at −18; ▲, binding site from P_F.

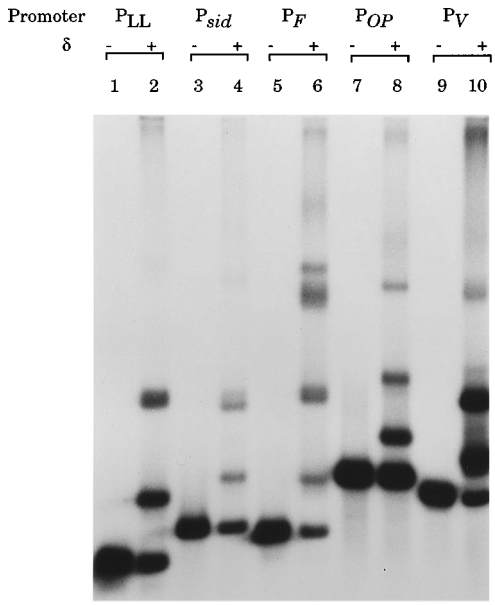

FIG. 7. Gel mobility shift experiment with δ and all of the late promoters of P2 and P4. Five micrograms of δ was incubated with 50 fmol of labeled DNA. The promoter fragment for each lane is shown at the top. The even-numbered lanes contain δ.

of the released δ molecule, 20.3 kDa. However, there is an accumulation of small proteins that are probably degradation products of the released δ protein. Interestingly, it appears that factor Xa does not cleave the unfused δ protein, as seen by the near equal amounts of δ protein in Fig. 5, lanes 1 and 5. This selective degradation of the released δ protein may be due to the 11 extra amino acids that remain after the cleavage of the fusion with factor Xa. These amino acids may destabilize the protein, making it possible for factor Xa to cleave at secondary recognition sites, which are characterized by the presence of an arginine residue (17, 34, 36). Because there is a loss of activity by cleavage with factor Xa, all subsequent experiments were done with the uncleaved fusion with or without the unfused δ form.

DNA binding studies with δ. Since it is proposed that δ is a DNA-binding protein, three methods were used to study its DNA binding property. The first method was nitrocellulose filtration. Using promoter fragments from the *F* promoter or either partial dyad sequence from the *sid* promoter, the percentage of DNA retained on the filter was measured. Figure 6 shows that δ binds to all three fragments. δ bound to the *F* promoter fragment with the highest affinity and to the partial dyad sequence centered at −55 from the *sid* promoter with the lowest affinity.

The second method used was a gel mobility shift assay. Using promoter fragments from each of the P2 and P4 late promoters, binding of δ was tested. In the presence of δ, all fragments show binding of δ (Fig. 7) and they show multiple shifted complexes. The *sid* promoter, which has two binding sites (shown below), does not show additional shifted complexes compared with the other promoters, which contain only one. At present, we do not know why there are not additional complexes. However, we do not believe that binding of δ at one site prevents binding at the other site, because we are able to see two sites by using DNase I footprinting.

We have not determined the composition of each complex, in regard to whether they contain a single or multiple δ molecules or if they contain unfused δ or the fusion. We do know that the fusion by itself is not able to bind to the P4 *sid*

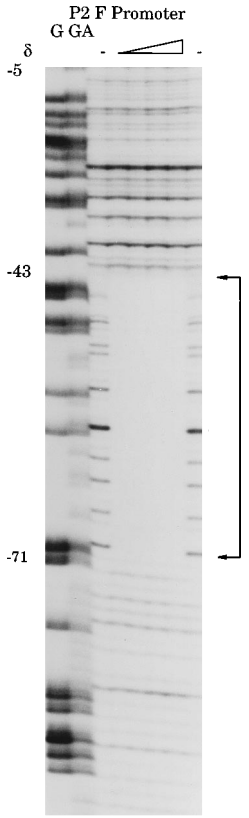

FIG. 8. Footprint of the P2 *F* promoter. The first two lanes contain the G and G+A Maxam-Gilbert sequencing reaction mixtures, respectively. The minus sign indicates that no δ protein was added to the reaction mixture. δ (2.8, 5.8, 11.5, and 23 μg) was used for the protection reactions. The protected region is designated with two connected arrows on the right, and the region where the protection occurs relative to the transcriptional start site is shown by the numbers on the left.

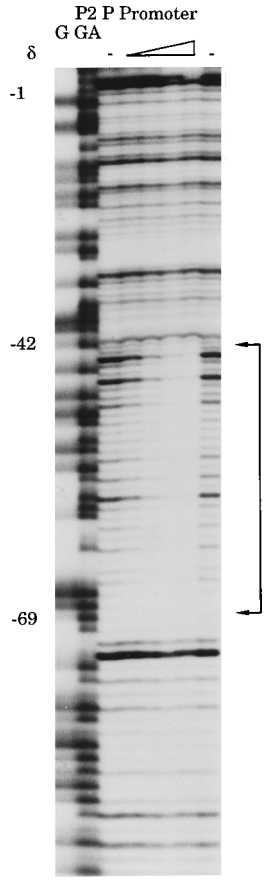

FIG. 9. Footprint of the P2 *P* promoter. The first two lanes contain the G and G+A Maxam-Gilbert sequencing reaction mixtures, respectively. The minus sign indicates that no δ protein was added to the reaction mixture. δ (2.8, 5.8, 11.5, and 23 μg) was used for the protection reactions. The protected region is designated with two connected arrows on the right, and the region where the protection occurs relative to the transcriptional start site is shown by the numbers on the left.

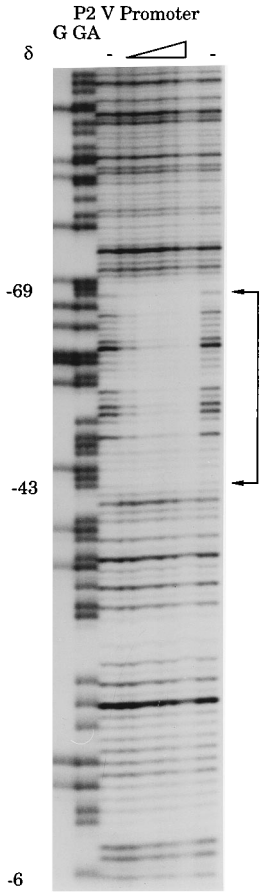

FIG. 10. Footprint of the P2 *V* promoter. The first two lanes contain the G and G+A Maxam-Gilbert sequencing reaction mixtures, respectively. The minus sign indicates that no δ protein was added to the reaction mixture. δ (2.8, 5.8, 11.5, and 23 μg) was used for the protection reactions. The protected region is designated with two connected arrows on the right, and the region where the protection occurs relative to the transcriptional start site is shown by the numbers on the left.

promoter or to the P2 *F* and *OP* promoters, as determined by DNase I footprinting (data not shown). However, we do not know if heteromultimers are able to bind. Binding of δ requires the presence of the partial dyad sequence, since a *cos*-containing piece of P4 DNA, without the dyad sequence, does not bind δ (data not shown).

Finally, DNase I footprinting experiments were performed to confirm the prediction that these dyad sequences serve as binding sites for δ. Figures 8 through 12 show that δ binds to the P2 and P4 late promoter fragments centered over the dyad sequences. In addition, δ binds to two sites within the P4 *sid* promoter but only to the upstream site within the P4 P_{LL} promoter. For convenience, the site centered at −55 in the *sid* promoter will be referred to as site I, and the one centered at −18 will be referred to as site II. It appears that δ has a higher affinity for site II in the *sid* promoter. This is concluded from the results of the nitrocellulose filtration and footprinting experiments. With the nitrocellulose filtration experiment, δ had a lower affinity for site I than for site II. Also in the footprinting experiments, site II appears to be fully occupied before site I. How this may affect transcription of the *sid* promoter is discussed below.

DISCUSSION

Our ability to study the transcriptional activation of the late promoters of P2 and P4 in vitro by δ has been hindered by the inability to obtain active δ protein. Here, we describe a new technique that allowed us to obtain large quantities of active δ protein and circumvented the need to denature and renature δ. This method used the commercially available *malE* fusion plasmid pMal-c2. By cloning the δ gene into this vector containing its own Shine-Dalgarno sequence, we were able to produce a fusion protein of MalE and δ, which does not bind DNA, as measured by DNase I footprint analysis of the P4 *sid* and P2 *F* and *OP* promoters, and which is unable to activate transcription by itself. Because δ forms multimers in solution, we were able to purify the unfused form indirectly by binding the fusion to an amylose column and then eluting both proteins. Along with simplifying the purification, the fusion is required to keep δ in solution, since removal of the MalE portion from the fusion results in precipitation of δ. This particular technique can be applied to any protein that forms dimers or higher-order multimers and others proteins that are difficult to keep in solution.

Transcription of the P4 *sid* promoter requires the host RNA polymerase with the σ^{70} subunit (24). Therefore, δ is not an

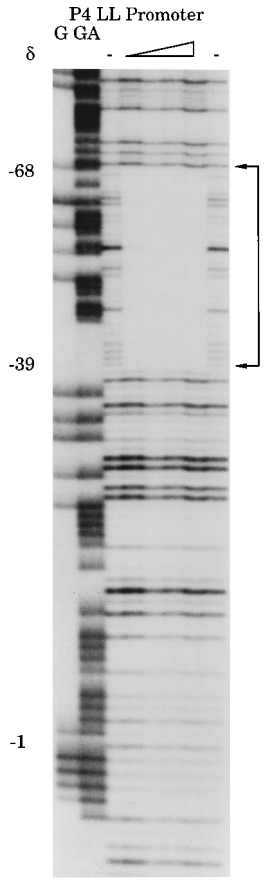

FIG. 11. Footprint of the P4 P_{LL} promoter. The first two lanes contain the G and G+A Maxam-Gilbert sequencing reaction mixtures, respectively. The minus sign indicates that no δ protein was added to the reaction mixture. δ (2.8, 5.8, 11.5, and 23 μg) was used for the protection reactions. The protected region is designated with two connected arrows on the right, and the region where the protection occurs relative to the transcriptional start site is shown by the numbers on the left.

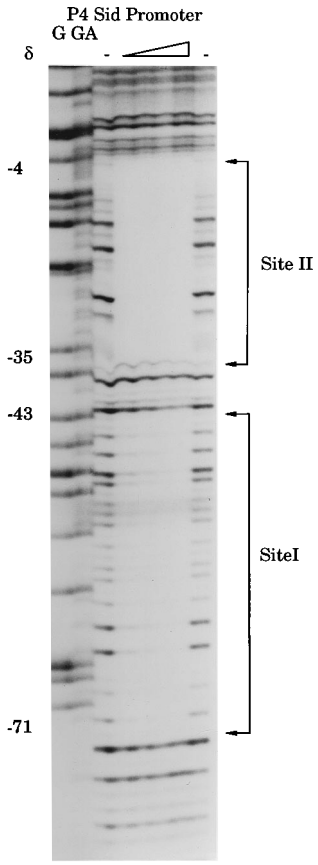

FIG. 12. Footprint of the P4 *sid* promoter. The first two lanes contain the G and G+A Maxam-Gilbert sequencing reaction mixtures, respectively. The minus sign indicates that no δ protein was added to the reaction mixture. δ (2.8, 5.8, 11.5, and 23 μg) was used for the protection reactions. The protected region is designated with two connected arrows on the right, and the region where the protection occurs relative to the transcriptional start site is shown by the numbers on the left.

alternate sigma factor. In addition, it has been shown with the P2 *F* promoter and the P4 *sid* promoter that transcriptional activation requires a region centered at approximately −55 (1, 19, 49). In all of the P2 and P4 late promoters, this region contains a partial dyad sequence which has been proposed to serve as a binding site for δ. Further sequence analysis has identified a second copy of the partial dyad sequence located between the −10 and −35 regions in the two late P4 promoters (15).

Using purified δ, we were able to show that δ binds to sequences centered at approximately −55 in the P2 and P4 late promoters. The footprinting data are summarized in Fig. 13, which shows the sequences of the P2 and P4 late promoters and the regions of protection. The divergent P2 *O* and *P* promoters contain a single binding site which serves to activate transcription in both directions. In addition to the dyad sequences centered at approximately −55, δ binds to a second region in the P4 *sid* promoter. However, it binds only to the upstream partial dyad sequence in the P4 P_{LL} promoter.

The binding of δ to the partial dyad sequences positioned at approximately −55 occurs within the region where most transcriptional activators, that also require RNA polymerase containing the σ^{70} subunit, bind (12). Many transcriptional activators that bind to regions upstream of the −35 region appear to contact the α subunit of the RNA polymerase. This has been demonstrated with purified RNA polymerase containing truncated α subunit and purified activators (reviewed in reference 22). Although binding to the α subunit has not been tested biochemically with δ, genetic evidence indicates that such binding occurs. The *rpoA109* mutation prevents the growth of P4, but suppressor mutations within δ that change the amino acid at position 127 from a threonine to an alanine have been isolated (21, 47). Therefore, it appears that δ interacts with the α subunit.

The importance of site II in the P4 *sid* promoter is unknown. When the initial mutational analysis of P_{sid} was performed, this second binding site had not been identified or thoroughly analyzed because of the fact that site I was absolutely required for promoter activity. In addition, multiple mutations in the left arm of the dyad sequence in site II did not affect promoter activity as much as mutations in site I (49). However, our results and those of others have revealed that the *sid* promoter is unique. When *lacZ* or *cat* fusions are made to each of the P2 and P4 late promoters and assayed in the presence of δ or Ogr, δ activates transcription better on the promoters with one binding site, whereas Ogr activates transcription better on the *sid* promoter, which has two binding sites (1, 22a).

The right arm of the partial dyad sequence of site II is part

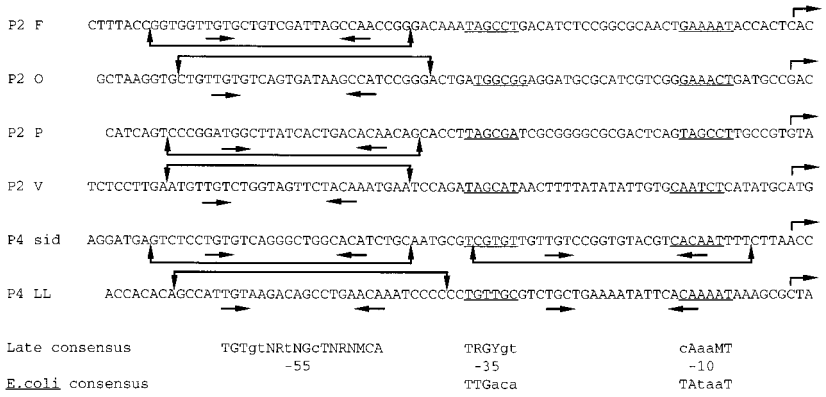

FIG. 13. Comparison of the P2 and P4 late promoters. The proposed −10 and −35 regions are underlined, with the consensus sequence for *E. coli* σ^{70} promoters shown at the bottom (32). A consensus sequence for the late promoters is also shown. Uppercase letters represent nucleotides conserved in five or more promoters. Lowercase letters represent nucleotides conserved in three or more promoters. The bent arrows represent the transcriptional start site for each promoter (8, 9, 13, 14). The partial dyad sequences are designated with inverted arrows. The half boxes with arrows show the region of DNase I protection; those with arrows pointing down designate that the top strand was footprinted, and those with arrows pointing up designate that the bottom strand was footprinted.

of the −10 region, which is where the σ^{70} subunit of the RNA polymerase holoenzyme contacts the DNA (43, 51). This explains how δ could decrease transcription of P_{sid}. However, if Ogr binds to site II, which it is presumed to do, then the same effect should be observed. It may be that Ogr binds site II to facilitate transcriptional activation, whereas δ does not. If this is the case, Ogr may be interacting with a different subunit of the RNA polymerase when bound at site II. When Ogr binds at site I, it is presumed to interact with the α subunit of the RNA polymerase (3). Recently, it has been shown that transcriptional activators that bind in the vicinity of the −35 region interact with the σ^{70} subunit of the RNA polymerase (25, 28, 33). At present, there is no evidence that Ogr interacts with the σ^{70} subunit.

The different activities observed for Ogr and δ on the *sid* promoter cannot be explained by the difference in the sizes of these two polypeptide chains. The bacteriophage φR73, which is similar to P4, also has a δ gene. However, δ from φR73 is 81 amino acids long, which is similar to the size of Ogr. Its activity on the *sid* promoter is lower than with P4 δ (22a). Therefore, the differences of activity on the *sid* promoter observed for δ and Ogr must be explained by differences in their three-dimensional structures and not their sizes. A mutational analysis of site II is being performed to determine its importance for Ogr and δ.

In summary, we have purified transcriptionally active P4 δ protein, using a modification of the MalE fusion system. With this pure δ, we have shown that it binds to sequences centered at approximately the −55 regions of the P2 and P4 late promoters and encompasses the partial dyad sequences found in these promoters. In addition, δ binds to a second site centered around −18 in the P4 *sid* promoter.

ACKNOWLEDGMENTS

We thank Barbara Bachmann, Hermann Bujard, Gail Christie, Elisabeth Haggård-Ljungquist, David Kennel, and Bob Simon for strains and plasmids.

This work was supported by NIH grant AI08722 to R.C.

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