Substitutions in Bacteriophage T4 AsiA and *Escherichia coli* σ^{70} That Suppress T4 *motA* Activation Mutations

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Bacteriophage T4 middle-mode transcription requires two phage-encoded proteins, the MotA transcription factor and AsiA coactivator, along with *Escherichia coli* RNA polymerase holoenzyme containing the σ^{70} subunit. A *motA* positive control (pc) mutant, *motA-pc1*, was used to select for suppressor mutations that alter other proteins in the transcription complex. Separate genetic selections isolated two AsiA mutants (S22F and Q51E) and five σ^{70} mutants (Y571C, Y571H, D570N, L595P, and S604P). All seven suppressor mutants gave partial suppressor phenotypes in vivo as judged by plaque morphology and burst size measurements. The S22F mutant AsiA protein and glutathione *S*-transferase fusions of the five mutant σ^{70} proteins were purified. All of these mutant proteins allowed normal levels of in vitro transcription when tested with wild-type MotA protein, but they failed to suppress the mutant MotA-pc1 protein in the same assay. The σ^{70} substitutions affected the 4.2 region, which binds the −35 sequence of *E. coli* promoters. In the presence of *E. coli* RNA polymerase without T4 proteins, the L595P and S604P substitutions greatly decreased transcription from standard *E. coli* promoters. This defect could not be explained solely by a disruption in −35 recognition since similar results were obtained with extended −10 promoters. The generalized transcriptional defect of these two mutants correlated with a defect in binding to core RNA polymerase, as judged by immunoprecipitation analysis. The L595P mutant, which was the most defective for in vitro transcription, failed to support *E. coli* growth.

Bacteriophage T4 uses three stages of transcription: early, middle, and late. This temporal progression is achieved by using three classes of promoters and by modifying the host RNA polymerase. Middle transcription requires two phage-encoded proteins, the MotA transcription factor and a coactivator, AsiA (for a review, see reference 37). AsiA binds to the σ^{70} subunit of host RNA polymerase and inhibits transcription from most *Escherichia coli* promoters. MotA binds to T4 middle promoters at the mot box, a consensus sequence centered at −30 (13, 30). The C- and N-terminal domains of MotA were solved by nuclear magnetic resonance and X-ray crystallography, respectively (9, 10). The C-terminal domain binds DNA, while the N-terminal domain is involved in transcriptional activation (10, 11). Based on the structural data, two residues (D30A and F31A) on an acidic, hydrophobic surface patch of the N-terminal domain were altered, resulting in a *motA* positive control mutant (*motA-pc1*). The mutant protein (full-length) binds normally to its DNA site but does not efficiently activate transcription (10).

MotA probably interacts directly with the σ^{70} subunit of host RNA polymerase during activation of middle-mode transcription. MotA and σ^{70} form a complex with unique mobility in native polyacrylamide gels, although this was tested in the absence of DNA and the other RNA polymerase subunits (11). Furthermore, MotA binds specifically to the −30 region of middle promoters, thus acting like a class II activator (see reference 16 for a review). Other class II activators generally interact with the 4.2 region of σ^{70}, which has a predicted helix-turn-helix motif and recognizes the −35 sequence of *E. coli* promoters (see reference 12 for a review). Various amino acid substitutions within the 4.2 region affect activation by transcription factors, including FNR, AraC, CRP, and PhoB (20, 21, 23, 24). The 4.2 region has also been shown to interact with phage λ cI repressor protein, which also binds at a site near the −30 region to activate transcription (22).

Additionally or alternatively, MotA may interact with the AsiA coactivator. AsiA has been shown to bind to σ^{70} in the 4.2 region (6, 32, 34, 35), implying that AsiA is in close proximity to DNA-bound MotA. Upon binding to σ^{70}, AsiA blocks transcription from host promoters requiring the −35 region (6, 33). Thus, AsiA blocks the 4.2 domain from recognizing the −35 region and possibly interacts directly with MotA to activate T4 middle promoters.

Using native polyacrylamide gel electrophoresis, Gerber and Hinton (11) detected a ternary MotA-AsiA-σ^{70} complex. This complex was detected in the absence of promoter DNA and other RNA polymerase subunits but it may well reflect interactions that occur during initiation of middle-mode transcription. Since MotA and AsiA can each bind separately to σ^{70}, the existence of this ternary complex does not clarify whether MotA and AsiA directly interact with each other.

To investigate these interactions, we conducted genetic selections to try to identify substitutions in AsiA and σ^{70} that suppress the activation defect caused by *motA-pc1*. In this report, we describe two *asiA* mutants and five *rpoD* (σ^{70}) mutants that gave moderate suppression in vivo. In vitro analysis

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TABLE 1. Genotypes of *E. coli* and phage T4 strains used[a]

Strain	Genotype	Reference
E. coli		
MCS1	*supD araD139 Δ(ara-leu7697) ΔlacX74 galU galK hsdR rpsL pro* (uncharacterized proline auxotrophy)	19
TabG	*E. coli* B, nonsuppressing, *hsdR gal met tabG*	29
B$_E$-BS	*E. coli* B, nonsuppressing	31
Phage T4		
T4 *denAB*	*denA (nd28) [denB-rII]^Δ (rIIPT8)*	2
T4 *denAB motA^Δ*	T4 *denAB motA^Δ*	2
T4 *denAB motA-pc1*	T4 *denAB motA-D30A/F31A*	This study
K10	T4 *denAB 38 (amB262) 51 (amS29)*	31
K10 *motA^Δ*	K10 *motA^Δ*	2
K10 *motA-pc1*	K10 *motA-D30A/F31A*	This study

[a] The *denA* and *denB* mutations inhibit host DNA degradation, and the *38^{am}* and *51^{am}* mutations block phage growth unless an amber suppressor is present.

of the mutant proteins failed to recapitulate the suppression but did reveal a novel class of $σ^{70}$ mutants.

MATERIALS AND METHODS

Media and chemicals. L broth consisted of NaCl (10 g/liter), Bacto Tryptone (Difco; 10 g/liter), and yeast extract (Difco; 5 g/liter). Hershey plates contained NaCl (8 g/liter), Bacto agar (10 g/liter), sodium citrate (2 g/liter), glucose (1.3 g/liter) and Bacto Tryptone (13 g/liter). Hershey top agar had the same composition except that glucose was at 3 g/liter and Bacto agar at 6.5 g/liter. Oligonucleotides were prepared by the Duke University DNA Core Facility.

Bacterial and phage strains. *E. coli* and phage strains are listed in Table 1. T4 *denAB motA-pc1* was created by homologous recombination (marker rescue) with plasmid pMotA-pc1. Progeny phage with *motA-pc1* were identified by their inability to grow on *E. coli* TabG, and the presence of the two mutations was confirmed by sequencing. K10 *motA-pc1* was created by a genetic cross between T4 *denAB motA-pc1* and K10 *motA^Δ*.

Construction of plasmids. Plasmids are listed in Table 2. Plasmid pMPC43, which expresses MotA-pc1, was used in the AsiA suppressor selection. This plasmid was created by inserting the 1.1-kb *Sal*I/*Bam*HI fragment from pMotA-pc1 into *Sal*I/*Bam*HI-cleaved pSU18 (25). Plasmid pMPC34 was created as follows. The small *ori(34)*-containing *Hin*dIII/*Sal*I fragment from pKK061-1 (26) was inserted into *Hin*dIII/*Sal*I-cleaved pSU18, and then the resulting plasmid was cleaved with *Hin*dIII and ligated to the 4.9-kb *rpoD*-containing *Hin*dIII fragment of pJH62 (kindly provided by D. Siegele, Texas A & M University). After isolation of a clone with the insert in the proper orientation, most of the *dnaG* gene (originally from pJH62) was removed by cleaving with *Hpa*I and religating, which removes a 1.7-kb fragment. The resulting plasmid, pMPC34, contains T4 *ori(34)* and the plasmid vector origin of replication, along with *rpoD*, which is apparently transcribed from the upstream *cam* gene promoter.

The wild-type glutathione *S*-transferase (GST)-$σ^{70}$ fusion plasmid, pGEX-$σ^{70}$, contains residues 8 to 613 of *rpoD*, in frame and downstream of the GST reading frame (7, 23). Each GST-$σ^{70}$ mutant was cloned by purifying a 980-bp *Nco*I fragment from the appropriate pMPC34-derived plasmid and ligating it into *Nco*I-cleaved pGEX-$σ^{70}$. The proper insert orientation was determined by restriction mapping, and the 4.2 region was sequenced to confirm the appropriate mutation.

The pBSPLO+-AsiA plasmid used in the *asiA* suppressor selection was created by ligating the 300-bp *Nde*I/*Bam*HI fragment from pAsiA (14) (kindly provided by D. Hinton, National Institutes of Health) to *Nde*I/*Bam*HI-cleaved pBSPLO+ (31). The AsiA overexpression plasmid, pAsiA, contains the *asiA* gene cloned into pET-21 vector between the *Nde*I and *Bam*HI sites (14). A comparable AsiA-S22F overexpression plasmid was created by amplifying the *asiA-S22F* gene from the T4 genome using PCR primers containing flanking *Nde*I and *Bam*HI sites. After digestion with *Nde*I and *Bam*HI, the fragment was ligated into *Nde*I/*Bam*HI-cut pET-21A vector. The insert was verified by sequencing.

Proteins. MotA and MotA-pc1 were overexpressed and purified as described earlier (10). RNA polymerase holoenzyme was purchased from Boehringer Mannheim. RNA polymerase core was purchased from Epicentre Technologies, except for the experiment in Fig. 7, in which core was purified as previously described (36). The GST-$σ^{70}$ proteins were purified as described before (23).

Overexpression and purification of AsiA proteins followed the procedures previously described (14). Briefly, cells were harvested, frozen at −80°C, resuspended in AsiA sonication buffer (20 mM Tris-Cl [pH 8], 1 mM EDTA, 10% glycerol, 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaCl), and sonicated until lysed. After centrifugation at $100,000 × g$ for 90 min, ammonium sulfate (60% saturation) was added to the supernatant and the protein was collected by centrifugation. The protein was resuspended and dialyzed against sonication buffer and then purified sequentially by column chromatography on Q-Sepharose, Affi-Gel Blue, and Sephadex G-50.

Isolation of AsiA suppressor mutants. The first method involved mutagenesis of T4 *denAB motA^Δ* with 2-hydroxylamine following the protocol of Drake and Ripley (8). Mutagenized phage (roughly 10^7 PFU) were plated on TabG cells harboring pMotA-pc1, and plaques were found at a frequency of about 10^{-5}. Phage from plaques were recovered and streaked on lawns of TabG and TabG pMotA-pc1 to verify MotA-dependent growth (no growth on TabG) and to isolate individual plaques (on TabG pMotA-pc1). The *asiA* gene from candidate mutants was amplified by PCR and sequenced.

In the second method, the *asiA* gene was mutagenized by PCR, cloned into a plasmid, and then rescued into the phage genome by homologous recombination. PCR mixtures contained 20 mM Tris-Cl (pH 8.4), 50 mM KCl, 5 mM MgCl$_2$, 150 μM (each) dATP, dGTP, TTP, and dCTP, 50 pmol each of primer A (5′-CCC GACGCATATGAATAAAAACATTGATACAGTTCG-3′) and primer B (5′-GCCGGATCCAGAATATTAGGAAGGGCTA-3′), 10 U of *Taq* polymerase (Gibco BRL), and 50 ng of pBSPLO+-AsiA DNA. Roughly 15 to 20% of the PCR products should contain at least one point mutation after 30 amplification cycles (39). The PCR primers introduced *Nde*I and *Bam*HI restriction sites, which were cleaved prior to ligation to *Nde*I/*Bam*HI-cleaved pBSPLO+. The DNA was electroporated into MCS1 cells harboring the integrated plasmid pMPC43, resulting in >50,000 viable transformants (determined by plating a small aliquot on selective plates). The pool of transformants was grown in L broth containing chloramphenicol (10 μg/ml) and ampicillin (40 μg/ml) and then infected with K10 *motA^Δ* at a multiplicity of infection (MOI) of 3. The pBSPLO+-AsiA plasmid inserted into the phage genome by homologous recombination at a frequency of about $3.5 × 10^{-4}$ during this infection. About 5,000 to 10,000 integrant plaques, selected by growth on a nonsuppressing host strain (B$_E$-BS), were pooled. The integrated plasmid in the phage genome is lost at a high frequency by homologous recombination, frequently leaving behind any mutations in the cloned segment (*asiA* in this case). Phage with suppressors of the *motA-pc1* defect were recovered by plating on TabG pBSPLO+ cells at a frequency of $5.5 × 10^{-4}$, about twice that measured in a parallel procedure using unmutagenized pBSPLO+-AsiA. After verifying that each candidate mutant could grow on TabG pMPC43 pBSPLO+ (to test *motA-pc1* suppression) but not on B$_E$-BS pMPC43 (to test for loss of the integrated plasmid), the *asiA* gene from the mutant was PCR amplified and sequenced.

Isolation of $σ^{70}$ suppressor mutants. Plasmid pMPC34, which contains *rpoD*, was mutagenized by growing MCS1 cells harboring the plasmid in L broth containing chloramphenicol (10 μg/ml) and N^4-aminocytidine (50 μg/ml; causes both transitions and transversions [38]). Plasmid DNA was isolated from the mutagenized cells and transformed into TabG pMotA-pc1. A pool consisting of about 10^6 transformants (determined by plating a small aliquot) was incubated for a 2-h outgrowth without selection and then grown in L broth containing chloramphenicol (10 μg/ml) and ampicillin (40 μg/ml) to an optical density at 560 mm of 0.4. The cells were infected with T4 *denAB motA^Δ* (MOI = 3), and the resulting lysate was used to transduce MCS1 cells to chloramphenicol resistance. Plasmid DNA from the transductant pool was isolated and used to repeat the selection scheme (i.e., transformation into TabG pMotA-pc1, infection with T4 *denAB motA^Δ*, and transduction into MCS1 cells). After the second round of selection, the pool of plasmid DNA was used to transform TabG pMotA-pc1.

TABLE 2. Plasmids used

Plasmid	Vector backbone	Cloned gene	Reference(s)
pAsiA	pET21a	*asiA*	14
pAsiA-S22F	pET21a	*asiA-S22F*	This study
pBSPLO+	pBR322	*supF*	31
pBSPLO+-AsiA	pBR322	*supF asiA*	This study
pMotA-pc1	pET3b	*motA-pc1*	10
pMPC34	pSU18	*rpoD ori(34)*	This study
pMPC43	pSU18	*motA-pc1*	This study
pJH62	ColE1	*rpoD* operon	15
pGEX-$σ^{70}$	pGEX-2T	*rpoD*	7, 23

Individual transformants were tested for the ability to support plaque formation by T4 *denAB motA*$^\Delta$. Transformants harboring a suppressor mutation in *rpoD* allowed small plaques to form, while comparable cells harboring the wild-type plasmid did not allow plaque formation. The *rpoD* gene from each suppressor plasmid was subcloned to map the causative mutation(s), which in each case was within the *Bgl*I-*Xho*I fragment (contains the C-terminal 85 codons of *rpoD*).

Plaque spot tests. To test suppression by the *asiA* mutants, TabG cells harboring pBSPLO+ were mixed with Hershey top agar and overlaid on a square Hershey plate. Appropriate dilutions of the indicated phage were then spotted on the plate in 3-μl aliquots, and the plate was incubated at 37°C overnight. To test suppression by the *rpoD* mutants, TabG cells harboring pMotA-pc1 and the indicated σ^{70}-expression plasmid were mixed with Hershey top agar, overlaid on a square Hershey plate, and spotted with the indicated phage as described above.

Burst size experiments. Burst experiments were conducted as described previously (10) with the following exceptions. For the suppressor mutants in *rpoD*, TabG cells harboring pMPC34 expressing either wild-type or mutant σ^{70} were infected with T4 *denAB motA-pc1* at an MOI of 0.1, and samples were taken at 90 min postinfection. To measure burst size of the suppressor mutations in *asiA*, TabG cells harboring pBSPLO+ were infected at an MOI of 0.1 with K10 *motA-pc1* strains containing either wild-type or mutant *asiA*, and samples were taken at 90 min postinfection. Progeny phage titers were determined on MCS1 cells, and the burst size was corrected for free (unattached) phage as previously described (10).

In vitro transcription. The transcription buffer contained 175 mM KCl, 10 mM Tris-Cl [pH 7.9], 10 mM MgCl₂, 1 mM dithiothreitol, and bovine serum albumin at 50 μg/ml. Transcription assays (10 μl) also contained 400 μM (each) rATP, rGTP, and rCTP, 40 μM UTP, 4 U of RNasin (Promega), 25 to 50 fmol of DNA template, and 0.7 μM [α-³²P]UTP (3,000 Ci/mmol), along with the indicated proteins. For the T4 middle-mode transcription assays, MotA⁺ or MotA-pc1 (2 pmol) was preincubated with the DNA and ribonucleoside triphosphates in 5 μl of transcription buffer for 5 min at 37°C. Similarly, RNA polymerase (core or holoenzyme; 0.05 pmol), AsiA (2 pmol), and GST-σ^{70} (0.5 pmol) were preincubated in another 5-μl aliquot of transcription buffer for 5 min at 37°C. The two preincubations were then mixed to initiate the reaction. For *E. coli* promoter transcription assays, RNA polymerase core enzyme (0.05 pmol) was preincubated with the indicated GST-σ^{70} protein (0.5 pmol) in 5 μl of transcription buffer for 5 min at 37°C before addition of the DNA substrate (also in 5 μl of transcription buffer). After the preincubations, the mixed reactions (10 μl) were incubated at 37°C for 30 min and then placed on ice. The reactions were terminated by adding 10 μl of stop solution (95% [vol/vol] formamide, 2 mM Na₂-EDTA, 0.05% bromphenol blue). Samples were heated at 95°C for 4 min and subjected to electrophoresis through a denaturing 8% polyacrylamide gel. Electrophoretic bands were quantitated with an AMBIS direct radioisotope counting system.

T4-modified DNA templates containing middle promoters P$_{uvsY}$ and P$_{ori(34)}$ were prepared from plasmids pGJB1 and pGJB4, respectively (26), as previously described (30). The P$_{uvsY}$ template was cleaved with *Ssp*I and *Eco*RV to yield a 221-base runoff transcript, while the P$_{ori(34)}$ template was cleaved with *Ase*I and *Eco*RV to yield a 320-base runoff. For the *E. coli* transcription assays, the *tac* promoter template was prepared by purifying the 370-bp *Ssp*I/*Bam*HI fragment of pPH310 (4), resulting in a 320-base runoff transcript. The unmodified P$_{uvsY}$ DNA template was a gel-purified 467-bp *Ssp*I/*Eco*RV fragment of pGJB1, which yields a 221-base runoff. The KAB promoter templates were obtained from plasmid pRW50 derivatives (kindly provided by S. Minchin, University of Birmingham, United Kingdom) for promoters KAB-TG and KAB-TT (1). The gel-purified 1.5-kb *Hpa*I/*Sac*II fragment yields a runoff transcript of about 900 bases.

σ-core binding assay. Binding of the GST-σ^{70} mutants to core RNA polymerase was analyzed essentially as described by Sharp et al. (36). Wild-type or mutant GST-σ^{70} was added in increasing concentrations in a competition against wild-type (His-tagged) σ^{70}, labeled by a kinase reaction as previously described (36). The buffer for this experiment contained 10 mM Tris-Cl [pH 8], 5% glycerol, 1 mM β-mercaptoethanol, 0.3 M NaCl, 10 mM MgCl₂, and bovine serum albumin at 200 μg/ml. Siliconized Eppendorf tubes were used to decrease nonspecific binding. Each reaction contained 100 nM labeled His-σ^{70} and 30 nM core, along with 0, 0.25, 0.5, 1, or 2 μM cold σ^{70} competitor. After a 1-h incubation at 37°C, the reaction mix was added to 100 μl of preequilibrated protein A-Sepharose beads (10% volume) coupled to polyclonal anti-core antibody (Animal Pharm Services Inc., Healdsburg, Calif.). The beads were rocked at 4°C for 1 h, washed with 1 ml of buffer, collected by centrifugation, aspirated to remove residual liquid, resuspended in water, and finally added to scintillation fluid for counting. Coimmunoprecipitation of the labeled σ^{70} is dependent on the presence of core RNA polymerase (data not shown).

Test of GST-σ^{70} mutants for in vivo function in *E. coli*. The p*trp-rpoD* gene (23) was introduced into the chromosome of *E. coli* strain MCS1 by P1 trans-

duction, using the linked chloramphenicol-resistance marker for selection. Wild-type and mutant GST-σ^{70} fusion plasmids (pGEX-σ^{70} and derivatives) were transformed into this strain and incubated for 1 h at 37°C in L broth for phenotypic expression. The cells were then serially diluted and spotted onto chloramphenicol-ampicillin L broth plates either with or without 0.2 mM indole-3-acrylic acid (Aldrich). The plates did not contain isopropyl-β-D-thiogalactopyranoside (IPTG), because the *tac* promoter on the GST-σ^{70} plasmids is leaky enough to allow sufficient σ^{70} expression for cell growth without induction (23). Plates were incubated at 37°C for 18 h.

RESULTS AND DISCUSSION

Selection of *asiA* and *rpoD* mutants that suppress *motA-pc1*. The *pc1* mutation in *motA* prevents T4 growth in *E. coli* TabG but not in wild-type *E. coli* (10). All *motA* point mutants, including amber and temperature-sensitive mutants, have this same characteristic, but a *motA* deletion mutant is lethal even in wild-type *E. coli* strains (2). Apparently, a very small amount of MotA activity is sufficient for growth in wild-type *E. coli*, but the mutation in TabG somehow increases this requirement (2, 10, 29, 37). Although the *motA-pc1* mutant grows in wild-type *E. coli*, the MotA-pc1 protein is defective for in vitro transcription with RNA polymerase purified from wild-type (non-TabG) *E. coli* (10). Furthermore, the *pc1* mutation decreases T4 origin-dependent plasmid replication in wild-type *E. coli* (5). Thus, while *motA-pc1* defects can be observed without the mutation in the TabG strain, we used this host strain for genetic selections of suppressor mutants.

Two methods were used to isolate suppressor mutations in the T4 *asiA* gene (also see Materials and Methods). The first was to mutagenize a T4 *denAB motA*$^\Delta$ strain with hydroxylamine and select for growth in the presence of plasmid-produced MotA-pc1 protein. The second method involved PCR mutagenesis of the *asiA* gene on a plasmid, substituting the mutagenized gene into a T4 *denAB motA*$^\Delta$ phage genome by homologous recombination and selecting for phage that grow in the presence of plasmid-produced MotA-pc1. Many of the isolated suppressor mutants from both procedures did not contain a mutation in *asiA*; these were not pursued. We did identify two *asiA* substitution mutations (S22F and Q51E) that each allowed growth with MotA-pc1. Each mutation was substituted into the *asiA* gene of an unmutagenized K10 *motA-pc1* genome using the T4 insertion/substitution system (31), and this recapitulated the suppressor phenotype.

The selection to isolate suppressors in *rpoD* modified a scheme originally used by Kreuzer and Alberts (18, 19) to isolate T4 replication origins. Plasmid pMPC34 was constructed with *rpoD* and the MotA-dependent T4 origin, *ori(34)* (Fig. 1). This plasmid was randomly mutagenized and then introduced into TabG cells that also contained a plasmid expressing MotA-pc1. The TabG cells harboring both plasmids were infected with T4 *denAB motA*$^\Delta$, and the lysate was collected. Since only MotA-pc1 is present, almost all infected cells undergo an abortive infection. However, a productive infection could occur in any cell that contains a plasmid with an *rpoD* mutation that suppresses the MotA defect. The mutant pMPC34 plasmid would replicate due to the T4 origin of replication, and the resulting concatemers of plasmid DNA would get packaged into phage heads and thereby produce plasmid-transducing particles in the lysate. The lysate was used to transduce cells that were restrictive for growth of T4 *denAB motA*$^\Delta$ (so that the transductants would not be killed by phage
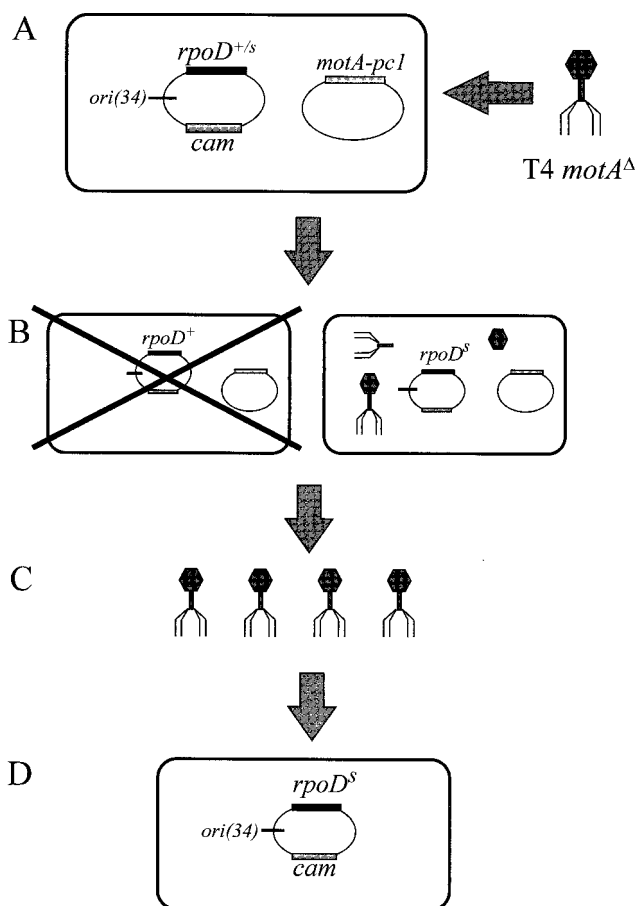
FIG. 1. Schematic for σ^{70} suppressor selection. (A) TabG cells contain a MotA-pc1 expression plasmid and mutagenized plasmid pMPC34, which contains the σ^{70} gene (*rpoD*) and the T4 origin, *ori(34)*. The cells are infected with T4 *denAB motA*$^\Delta$. (B) Most infected cells contain a wild-type *rpoD* gene (or *rpoD* mutations that do not suppress) and therefore lead to an abortive infection (left cell). A viable infection should occur only if a mutant σ^{70} is expressed from the plasmid and suppresses the MotA-pc1 defect (right cell). The suppressing mutation in *rpoD* is designated *rpoD*^{S} in this diagram. The viable infection allows T4 growth and replication of the pMPC34, which then forms long concatemers of plasmid DNA. (C) The concatemeric plasmid DNA is packaged into some of the phage heads to produce transducing phage in the lysate. The lysate is then used to transduce *E. coli* cells, and transductants are selected by the *cam* marker on pMPC34 (D).

growth), and transductants were selected by growth in the presence of chloramphenicol. This procedure enriches for plasmids that contain a mutant *rpoD* gene that suppresses the MotA-pc1 activation defect, and individual candidate plasmids were analyzed after two rounds of enrichment (see Materials and Methods). Selected plasmids were subcloned to localize the *rpoD* mutation and were sequenced to identify the mutation. Five point mutations were identified, and each affects the 4.2 region of σ^{70}: Tyr-571 to Cys (Y571C), Tyr-571 to His (Y571H), Leu-595 to Pro (L595P), Ser-604 to Pro (S604P), and Asp-570 to Asn (D570N).

The 4.2 region has also been shown to be involved in activation by a number of other transcription factors, including λ cI protein, FNR, PhoB, CRP, and AraC (16). Each of these activators has a binding site very near -35, similar to the lo-

cation of the T4 mot box. Interestingly, different substitutions in residues D570 and Y571 affect activation by PhoB (17, 24) and by MotA (this work). The 4.2 region of σ^{70} is also important in the interaction with AsiA. Fragments of σ^{70} containing either residues 568 to 600 or residues 547 to 603 bind AsiA, and furthermore, AsiA protein creates a hydroxyl radical footprint at residues 572 to 588 of σ^{70} (6, 32, 34, 35). With one exception (S604P), the suppressor mutations that we isolated (D570N, Y571C, Y571H, and L595P) are within the σ^{70} fragments that bind AsiA and are from one to seven residues from the footprinted region.

Particularly given their locations, there are several possible explanations for suppression by the σ^{70} suppressor mutations. For example, MotA and σ^{70} may interact directly, with the MotA-pc1 substitutions weakening this interaction and the suppressor substitutions restoring it. Alternatively, MotA may interact predominantly with AsiA, with the σ^{70} suppressor mutations acting indirectly (e.g., inducing a conformational change in AsiA that allows better interaction with MotA-pc1).

In vivo tests for suppression by *asiA* and *rpoD* mutants. We tested the ability of each isolated mutant to restore growth in the presence of MotA-pc1 by observing plaque formation on *E. coli* strain TabG. To test suppression by the *asiA* mutants, spot tests were performed with T4 strains containing *motA-pc1* and either the wild-type or mutant *asiA* gene. The presence of each *asiA* mutation led to plaque formation, but the plaque size was still smaller than that of the *motA*$^+$ control phage (Fig. 2A). Therefore, the *asiA* mutations partially suppress the growth defect caused by *motA-pc1*.

Spot tests were also performed with cells containing a MotA-pc1 expression plasmid along with each mutant σ^{70} expression plasmid (Fig. 2B). Each mutant σ^{70} allowed small to moderate-sized plaques, with the L595P and S604P mutants giving the strongest suppression, Y571C and Y571H intermediate suppression, and D570N weak suppression (Fig. 2B and data not shown). However, none of the mutant σ^{70} proteins returned growth to the level achieved by the control *motA*$^+$ strain, again indicating partial suppression.

We next quantitated in vivo suppression by each *asiA* mutation by measuring burst size (average number of phage progeny produced from each infected cell). Each of the *asiA* mutations caused an increase in burst size compared to the wild-type *asiA* strain, but the burst sizes were still quite low compared to the *motA*$^+$ infection (Table 3). To measure changes in burst size with the *rpoD* mutants, cells harboring either wild-type or mutant σ^{70} expression plasmid were infected with T4 *motA-pc1*. As with the *asiA* suppressors, each mutant σ^{70} caused a several-fold increase in burst size, but it was still lower than that of a *motA*$^+$ infection (Table 3). The σ^{70} in vivo assays may have been affected by the presence of wild-type σ^{70} protein expressed from the genomic copy of *rpoD*. (For unknown reasons, we were unable to construct a TabG strain in which the *trp* promoter controlled expression of the genomic copy of *rpoD*.) In summary, plaque and burst size measurements demonstrate that the two *asiA* mutations and the five *rpoD* mutations allow partial suppression of the *motA-pc1* defect in vivo.

Transcription from T4 middle-mode promoters. We next attempted to measure suppression of the MotA-pc1 defect with in vitro transcription assays using purified proteins (see
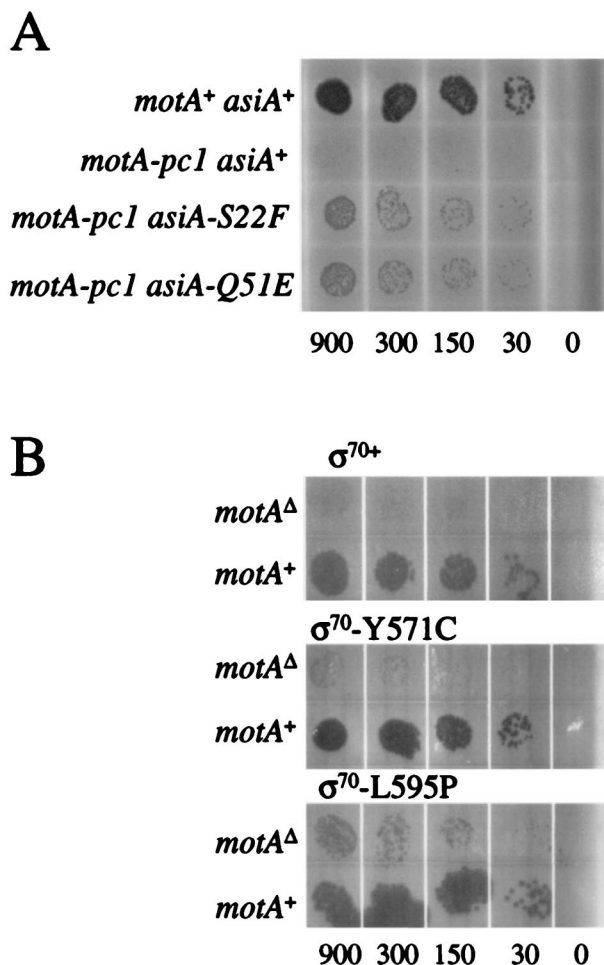
A

B

FIG. 2. Plaque spot tests to visualize suppression. (A) The plate contained a lawn of TabG pBSPLO+ cells, and the indicated T4 K10 strain was spotted on the lawn in 3-μl aliquots. The aliquots contained approximately the number of phage indicated below the plate. The positive control was T4 strain K10 (*motA*+ *asiA*+), which resulted in full growth (top row), while the negative control K10 *motA-pc1* (*asiA*+) showed very little growth (second row). (B) The plates contained lawns of TabG cells with the indicated σ70 expression plasmid and a MotA-pc1 expression plasmid. Phage T4 *denAB motA*Δ (top row) or T4 *denAB* (*motA*+; bottom row) were spotted on the lawn in 3-μl aliquots that contained approximately the number of phage indicated below the plate. All plates were incubated at 37°C overnight.

Materials and Methods). Activation was tested using T4-modified (glucosylated hydroxymethyl-cytosine-containing) templates with either of two T4 middle-mode promoters (P_{uvsY} and $P_{ori(34)}$) in the presence of RNA polymerase holoenzyme, wild-type or mutant MotA, and wild-type or S22F mutant AsiA. As expected from previous work (28), transcription did not occur in the absence of AsiA protein (Fig. 3, lanes 1, 2, 7, and 8). Wild-type MotA induced a large amount of transcription with either AsiA protein, indicating that the S22F substitution does not affect activation with wild-type MotA (lanes 3, 4, 9, and 10). Transcription was dramatically decreased in the presence of MotA-pc1 and wild-type AsiA (lanes 5 and 11), as previously reported (10). However, we did not detect an increase in transcription when the AsiA-S22F was present with MotA-pc1 (lanes 6 and 12).

We also analyzed transcription with the mutant σ70 proteins. Wild-type and each mutant σ70 were overexpressed and purified as GST fusions (see Materials and Methods). GST-σ70 fusions have been analyzed in previous studies and found to behave much like native σ70 (7). Transcription was tested using the $P_{ori(34)}$ substrate with wild-type or mutant GST-σ70, core RNA polymerase, MotA (wild type or pc1) and wild-type AsiA (Fig. 4). Comparable amounts of transcription were induced with each σ70 protein and wild-type MotA protein, indicating that the mutations in σ70 do not affect activation with wild-type MotA (compare lane 4 with lanes 7, 10, 13, 16, and 19). Also, transcription with each GST-σ70 still required MotA (lanes 3, 6, 9, 12, 15, and 18). As above, transcription was reduced with MotA-pc1 and wild-type σ70 (lane 5). Once again we did not observe a noticeable suppression of the transcription defect with any mutant σ70 compared to wild type (compare lane 5 with lanes 8, 11, 14, 17, and 20). We also failed to observe suppression with a template containing the P_{uvsY} middle promoter and in assays that contained AsiA-S22F and either σ70-L595P or σ70-D570N (data not shown).

The transcription assay may not be able to measure suppression for any number of reasons. First, the composition of the transcription buffer may prevent the mutations from suppressing the activation defect, especially if suppression occurs by a weak protein-protein interaction (although we failed to detect suppression with a variety of salt concentrations; data not shown). Second, the unmodified RNA polymerase may be lacking a T4-directed modification required to obtain suppression, even though unmodified polymerase is adequate for observing MotA-dependent activation from middle promoters. RNA polymerase undergoes several modifications during a T4 infection, including ADP ribosylation of the α subunits and binding of T4-encoded RpbA and Alc proteins (for review, see reference 37). Third, suppression may require RNA polymerase from strain TabG, since that strain was used for in vivo selections and suppression tests (TabG apparently contains a mutation within or closely linked to the gene for the β subunit of RNA polymerase [29]). Fourth, suppression may occur only at a subset of middle promoters, and we tested only two. Middle promoters are used to express a number of essential replication proteins, and a large suppression effect at one or two promoters may be adequate to cause the in vivo pheno-

TABLE 3. Burst size analysis of suppressor mutants[a]

T4 strain	Plasmid gene	Burst size
K10 (*motA*+ *asiA*+)	*supF*	45
K10 *motA-pc1* (*asiA*+)	*supF*	0.4
K10 *motA-pc1 asiA-S22F*	*supF*	2.0
K10 *motA-pc1 asiA-Q51E*	*supF*	1.3
T4 *denAB* (*motA*+ *asiA*+)	*rpoD*+	73
T4 *denAB motA-pc1*	*rpoD*+	0.5
T4 *denAB motA-pc1*	*rpoD-D570N*	2.6
T4 *denAB motA-pc1*	*rpoD-Y571C*	4.6
T4 *denAB motA-pc1*	*rpoD-Y571H*	5.5
T4 *denAB motA-pc1*	*rpoD-L595P*	2.8
T4 *denAB motA-pc1*	*rpoD-S604P*	2.3

[a] The host cells for the infections with K10 and its derivatives contained plasmid pBSPLO+, which carries *supF* and thereby allows suppression of the amber mutations in this phage background. The host cells for the infections with T4 *denAB* and its derivatives contained a plasmid encoding either wild-type or mutant σ70 (*rpoD* gene), along with the normal (wild-type) chromosomal *rpoD* gene.
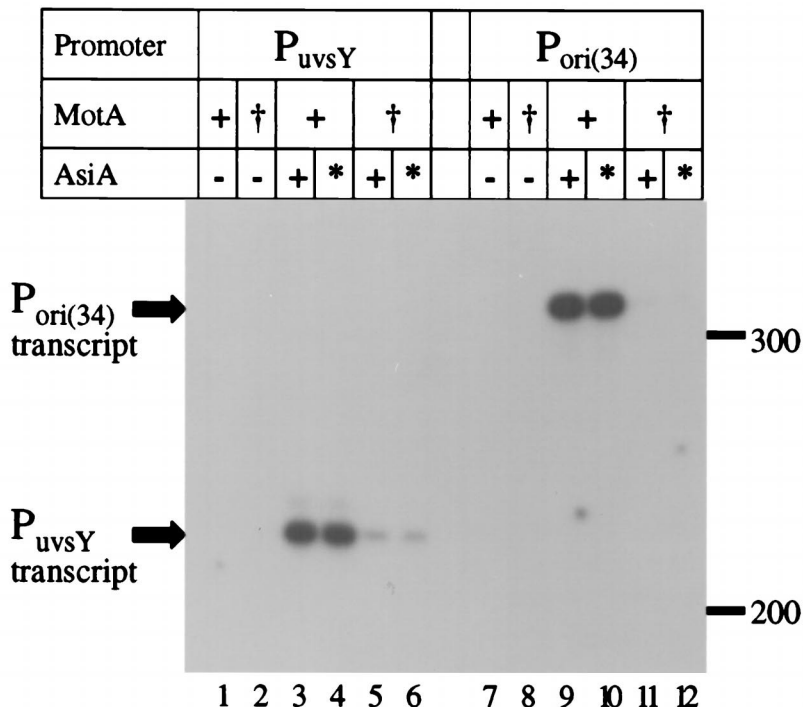
Promoter	P$_{uvsY}$							P$_{ori(34)}$				
MotA	+	†	+		†			+	†	+		†	
AsiA	-	-	+	*	+	*		-	-	+	*	+	*

P$_{ori(34)}$ transcript →

P$_{uvsY}$ transcript →

— 300

— 200

1 2 3 4 5 6   7 8 9 10 11 12

FIG. 3. In vitro transcription from T4 middle promoters with AsiA or AsiA-S22F. Transcription assays contained RNA polymerase holoenzyme, wild-type MotA (+) or MotA-pc1 (†) (2 pmol each), and wild-type AsiA (+) or AsiA-S22F (∗) (2 pmol each) as indicated. Each T4-modified DNA template contained the middle promoter indicated at the top. Size standards (in bases) are indicated to the right of the gel.

type. Fifth, suppression may not affect transcription per se, but rather activation of DNA replication at T4 origins (which contain middle-mode promoters). Indeed, restoration of origin function was critical for the σ^{70} suppressor selection.

Transcription of *E. coli* promoters with mutant σ^{70} proteins. To better understand the nature of the σ^{70} mutants, transcription assays were performed using *E. coli* promoters in the absence of T4 proteins. We first analyzed transcription from

GST-σ^{70}	-	wt				Y571C			L595P			S604P			Y571H			D570N		
MotA	-	-	-	+	†	-	+	†	-	+	†	-	+	†	-	+	†	-	+	†
AsiA	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

500 —

400 —

300 —

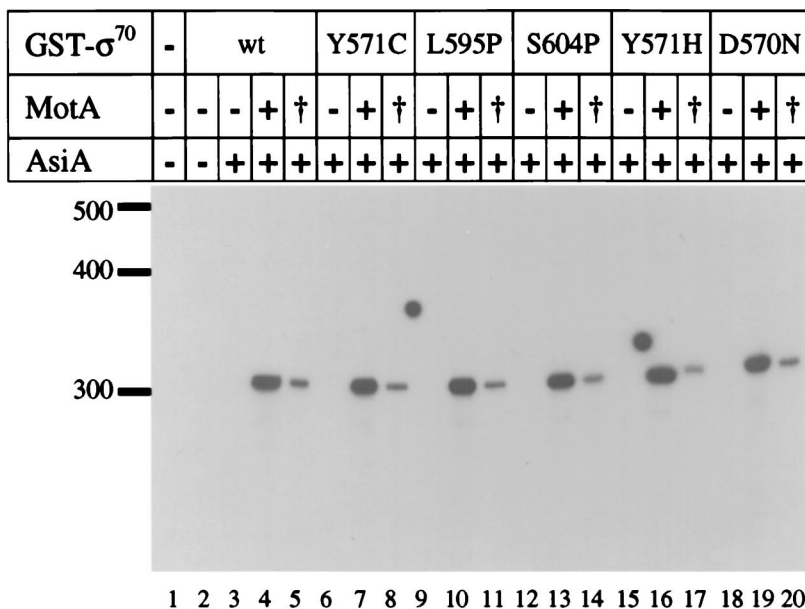1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

FIG. 4. In vitro transcription with σ^{70} suppressor mutants from the T4 *ori(34)* promoter. Reactions contained RNA polymerase core enzyme, AsiA (2 pmol, where indicated), wild-type MotA (+) or MotA-pc1 (†) (2 pmol each), and the indicated GST-σ^{70} fusion protein (0.5 pmol). Runoff transcripts (320 bases) were synthesized from a linear T4-modified DNA template containing the *ori(34)* middle promoter. Size standards (in bases) are indicated to the left of the gel.
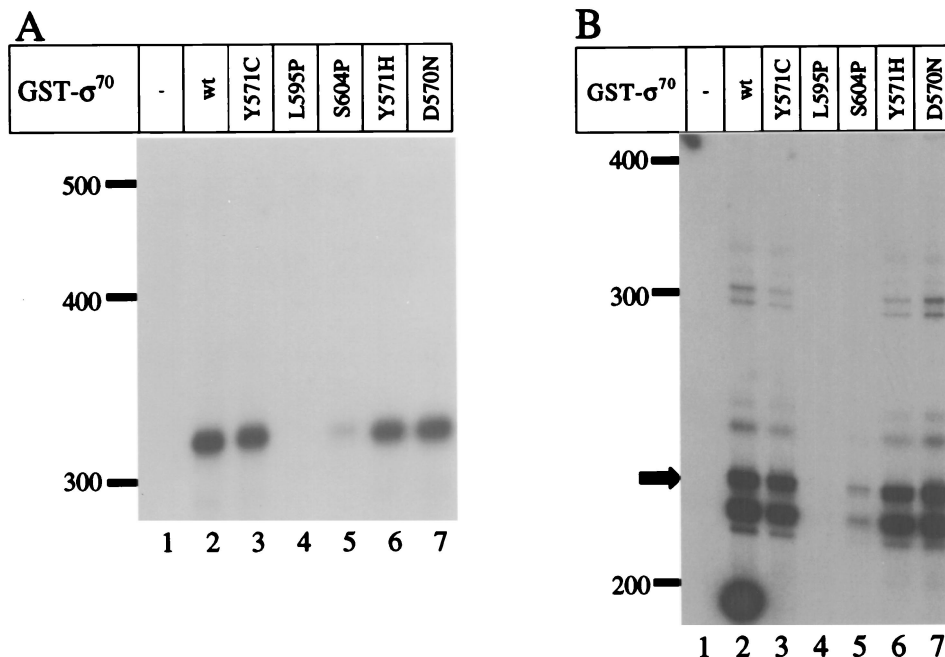
FIG. 5. In vitro transcription with σ^{70} mutants from the *tac* and T4 *uvsY* promoters. Reactions contained RNA polymerase core enzyme and the indicated GST-σ^{70} protein (0.5 pmol). Runoff transcripts were synthesized from a linear template containing either the *tac* promoter (A) (320-base transcript) or the T4 *uvsY* promoter (B) (221-base transcript, indicated by the arrow; the template was not T4 modified). Size standards (in bases) are indicated to the left of the gel. The heavy spot at the bottom left corner (just above the numeral 2) is a film artifact.

the *tac* promoter, which has strong −10 and −35 consensus elements (but no extended −10 element). Transcription by core RNA polymerase was strictly dependent on σ^{70} (Fig. 5A, lanes 1 and 2), and nearly normal transcription occurred with the Y571C, Y571H, and D570N mutants (lanes 3, 6, and 7; see figure legend). However, the L595P and S604P substitutions caused dramatic defects in transcription from P_{tac} (<1 and 15%, respectively, compared to wild-type σ^{70}; lanes 4 and 5). The defects caused by the proline substitutions were surprising since these substitutions did not reduce transcription from T4 middle promoters.

The two proline substitutions are at the C-terminal end of the 4.2 region, which recognizes the −35 sequence of promoters and is predicted to form a helix-turn-helix structure. The proline substitutions may simply change the structural conformation of the domain and block the ability of σ^{70} to recognize the −35 sequence, thus preventing transcription from P_{tac}. We analyzed this possibility by measuring transcription from extended −10 promoters. These promoters contain a 5′-TG-3′ positioned at −15 and −14 and do not require a −35 sequence for activity (3). If the proline substitutions block recognition solely of the −35 sequence, normal transcription should occur with an extended −10 promoter. The T4 *uvsY* promoter contains an extended −10 sequence and no consensus −35 sequence (37). As predicted from previous experiments (30), transcription from P_{uvsY} occurred without MotA when AsiA was not included in the reaction and the template was not T4 modified (Fig. 5B, lane 2). Results with the σ^{70} substitution mutants were nearly identical to those with the *tac* promoter (compare Fig. 5A and B). Most importantly, the L595P and S604P substitutions greatly decreased transcription (<1 and 10%, respectively, compared to wild type [lanes 2, 4, and 5]).

This result was confirmed using a second substrate, KAB-TG, which contains a semisynthetic promoter that is dependent on its extended −10 sequence (1). The proline substitutions greatly decreased transcription from this extended −10 promoter (Fig. 6, left lanes). As a control, we also tested a second substrate, KAB-TT, which has a T nucleotide substituted at position −14, thus eliminating the extended −10 element. As expected (1), transcription did not occur with the KAB-TT substrate with any σ^{70} protein, demonstrating that efficient transcription of KAB-TG depends on the extended −10 sequence (Fig. 6, right lanes). The main conclusion is that the two proline substitutions, at least in the context of these GST fusions, cause a major defect in transcription from both standard *E. coli* promoters and those with an extended −10 and yet do not block MotA-activated T4 middle-mode transcription.

The proline substitutions affect the interaction of sigma with core. Previous studies have implicated 4.2 as one of several σ^{70} regions that are involved in the interaction of the protein with core RNA polymerase (27, 36). At present, it is not clear which subunit(s) of core polymerase interacts with the 4.2 region. One possible model to explain the transcriptional defect of the proline substitution mutants is that the altered σ^{70} is unable to interact with core RNA polymerase. We used a competitive binding assay to determine the relative binding affinity of each mutant σ^{70} compared to the wild-type protein. His-tagged σ^{70}* labeled with ^{32}P (by a kinase reaction) was competed against increasing concentrations (250 nM to 2 μM) of nonradioactive wild-type or mutant σ^{70} for binding to a limiting amount of core. The complexes were separated by immunoprecipitation with an antibody directed against core and then analyzed for the amount of radioactive σ^{70} bound. As
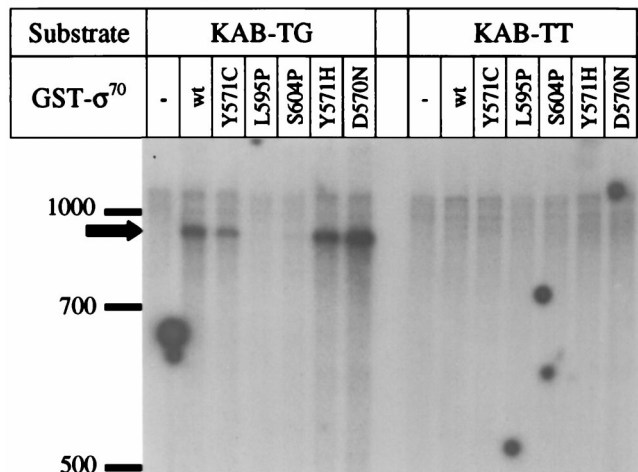
FIG. 6. In vitro transcription with σ^{70} mutants from KAB substrates. Reactions contained RNA polymerase core enzyme and the indicated GST-σ^{70} fusion protein (0.5 pmol). The substrates contained either the extended −10 promoter (KAB-TG, left side) or a variant without the extended −10 element (KAB-TT, right side). The arrow indicates the runoff transcripts, roughly 900 bases in length. With the extended −10 substrate, GST-σ^{70} Y571C produced about half as many transcripts as wild-type σ^{70} and D570N produced about twice as many as wild type. Transcripts were not detected with any of the σ^{70} proteins from the KAB-TT substrate. Size standards (in bases) are indicated to the left of the gel, and the round spots are film artifacts.

FIG. 7. Competition assay for binding of σ^{70} mutants to core RNA polymerase. Wild-type His-σ^{70} labeled with [γ-^{32}P]ATP and kinase was added to nonradioactive core RNA polymerase and Sepharose beads coated with polyclonal antibodies against the core. The amount of radioactivity bound to the beads was measured as a function of increasing concentrations of cold competitor proteins (0, 0.25, 0.5, 1, and 2 μM). Each point in this figure shows the average of three determinations.

expected (36), nonradioactive His-WT competed strongly, with nearly complete competition at a 20-fold molar excess (Fig. 7). The GST-tagged wild-type σ^{70} also competed well, though not as strongly as the His-tagged protein, consistent with results from previous competition experiments (M. M. Sharp and C. A. Gross, unpublished data). The D570N, Y571C, and Y571H mutant proteins all competed nearly as well as the wild-type GST fusion protein, consistent with their ability to transcribe *E. coli* promoters. Interestingly, however, the L595P and S604P proteins both showed strong core binding defects (Fig. 7). These two proteins were essentially unable to compete under the conditions of this assay. The lack of competition of these two mutant proteins cannot be attributed to inactive protein because each induces MotA-dependent transcription from T4 middle-mode promoters normally (Fig. 4).

The binding assay can be quantified by comparing the concentrations of wild-type and mutant σ^{70} required for equivalent competition. We can reliably quantify our results in the range of 25 to 90% of σ^{70*} competed, thus setting upper and lower limits for the assay (36). Since 2 μM concentrations of the GST-S604P and GST-L595P proteins were unable to compete but 250 nM GST-WT showed significant competition, the mutants exhibit a specific core-binding defect of greater than 10-fold.

The transcriptional defects of the S604P and L595P proteins may therefore be caused by an inability to bind or alteration in binding to core polymerase. Unlike the proline substitution, the alanine substitution of S604 did not suppress the *motA-pc1* mutation, nor did it exhibit a core-binding defect (data not shown). This suggests that the proline substitutions may be disrupting the region 4.2 helix, causing localized misfolding of a region required to interact with the core. Nonetheless, the mutant 4.2 region must still be able to interact with MotA and/or AsiA,
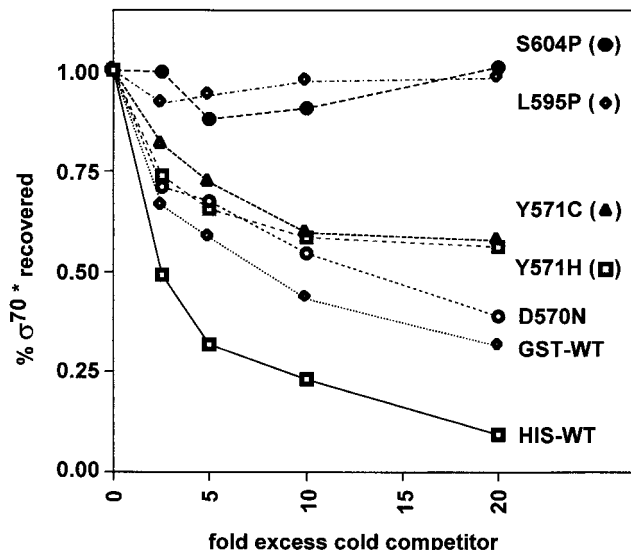
because the proline substitutions do not block MotA-dependent transcription from T4 middle-mode promoters (Fig. 4). The proficiency in T4 middle-mode transcription also demonstrates that the S604P and L595P proteins can interact productively with the rest of the transcription complex under some conditions and that the proline substitutions do not result in gross misfolding of σ^{70}. The presence of the T4 activator proteins apparently overcomes the generalized defect in the proline-substitution mutants. Per-
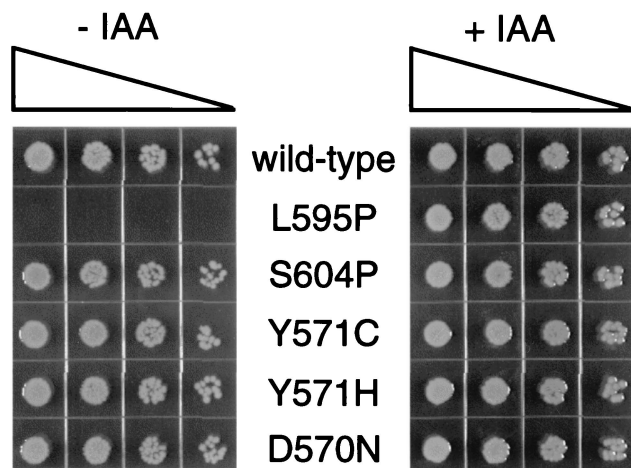

FIG. 8. *E. coli* viability assay for GST-σ^{70} mutants. Wild-type and mutant GST-σ^{70} expression plasmids were transformed into MCS1 p*trp-rpoD* cells (see Materials and Methods). After outgrowth, 4-μl aliquots of fourfold serial dilutions of each transformation were spotted onto L broth plates that contained ampicillin to select for the plasmid, either with or without 0.2 mM indole-3-acrylic acid (IAA) to induce σ^{70} expression from the chromosomal p*trp-rpoD* (provides wild-type σ^{70}).

haps AsiA and or MotA also contacts the core, replacing the defective interaction, or alters the structure of σ^{70} so that it can interact productively with the core.

σ^{70} **L595P does not support *E. coli* growth.** Finally, we also tested whether the mutant σ^{70} proteins are sufficient for *E. coli* viability. An *E. coli* strain with the chromosomal *rpoD* under the control of a *trp* promoter (and no other source of σ^{70}) is inviable on rich media unless the *trp* repressor is inactivated by the antagonist indole-3-acrylic acid (23). As expected from the past study, a plasmid expressing the wild-type GST-σ^{70} rescued survival in the absence of indole-acrylic acid (Fig. 8, top row). We found that the L595P fusion protein was not able to rescue survival, correlating to its gross defect in transcription. However, expression of any of the other four mutants, including S604P, allowed apparently normal growth (Fig. 8). The small amount of residual transcription by the S604P mutant (Fig. 5) is apparently sufficient for cell viability.

Summary. We isolated two *asiA* and five *rpoD* mutations that suppress the *motA-pc1* activation defect. Each of the seven mutations caused partial suppression in vivo, but suppression was not detected with in vitro transcription assays. Nonetheless, two of the σ^{70} mutants, L595P and S604P, had novel biochemical properties. Both proteins showed greatly reduced transcription from *E. coli* promoters (both normal and extended −10) and defective binding to core polymerase, yet were fully functional for MotA-dependent transcription from T4 middle-mode promoters.

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