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Role of Operator Subsites in Arc Repression

Tracy L. Smith and Robert T. Sauer*

Department of Biology Massachusetts Institute of Technology, Cambridge MA 02139, USA

**Corresponding author*

By binding to adjacent subsites in its 21 base-pair operator, Arc represses transcription from two divergent promoters, P_{ant} and P_{mnt}, in the *immunity I* operon of bacteriophage P22. Arc dimers bind to each subsite with nanomolar affinities and interact through protein–protein interactions to stabilize binding further. Here, we show that an Arc dimer bound to a single subsite reduces the rate of RNA polymerase open-complex formation and represses transcription from P_{ant} and P_{mnt} promoter variants to varying degrees. Occupancy of the subsite proximal to the P_{ant} – 35 region results in significantly greater repression than occupancy of the –10 proximal subsite. For repression of P_{mnt}, Arc bound at the –10 proximal subsite is more effective than Arc bound at the –35 proximal subsite. Because of the divergent orientations of the two promoters, the –35 proximal site in P_{ant} is the same as the –10 proximal site in P_{mnt}. Thus, in both cases, the same operator subsite is primarily responsible for repression of transcription initiation.

Keywords: transcriptional regulation; multimerization; cooperative DNA binding; bacteriophage P22

Introduction

It is relatively common for transcription factors to bind to adjacent DNA regulatory sites near the promoters they control (Carlson & Little, 1993; Collado-Vides *et al*., 1991; Kim & Little, 1992; Phillips *et al*., 1989; Ptashne, 1986). Proteins bound at tandem sites of this type have the potential to interact through cooperative protein–protein contacts, which would serve to increase both the affinity and the specificity of DNA binding. In principle, binding to adjacent sites could also permit regulatory proteins to serve distinct functional roles. In this paper, we analyze the functional importance of the binding of Arc dimers to individual subsites in the arc operator. During lytic growth of bacteriophage P22, Arc dimers bind to adjacent DNA subsites in the *immunity I* operon (Figure 1), thereby repressing transcription from the divergent P_{ant} and P_{mnt} promoters (Susskind & Youderian, 1983). The DNA sequences of the two operator subsites and their affinities for Arc are similar but not identical (Brown & Sauer, 1993). Protein–protein interactions between adjacently bound Arc dimers stabilize DNA binding (Brown & Sauer, 1993; Raumann *et al*., 1994b), and Arc variants with normal subsite binding but diminished cooperativity are poorer repressors *in vivo* than wild-type Arc (Smith & Sauer, 1995). However, cooperativity defective mutants are fully active at high protein concentrations *in vitro*, suggesting that cooperative binding to adjacent subsites is required for optimal function *in vivo* principally to allow saturation of the operator at low Arc concentrations (Smith & Sauer, 1995).

The studies presented here show that Arc dimers bound to single operator subsites can repress transcription of variants of both P_{ant} and P_{mnt}. The efficiency of this repression, however, is subsite and promoter dependent. Occupancy of the subsite proximal to the P_{ant} –35 region and the P_{mnt} –10 region (the left subsite in Figure 1), results in stronger repression of both promoters than occupancy of the other subsite (the right subsite in Figure 1). Thus, the left operator subsite appears to play the dominant role in mediating repression, and the right subsite may be needed primarily to allow cooperative stabilization of the dimer bound to the left subsite.

Abbreviations used: CRP, cAMP receptor protein; PCR, polymerase chain reaction.

Results

Construction of promoters containing single subsites for Arc binding

The positions of the two subsites of the arc operator in relation to the overlapping P_{ant} and P_{mnt}

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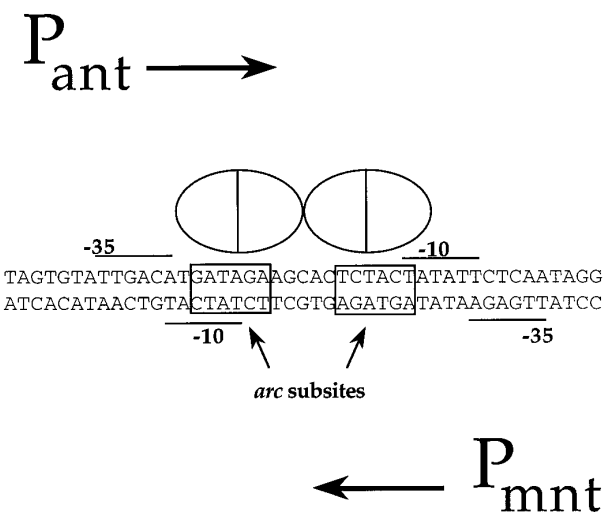

Figure 1. Wild-type *immunity I* region of bacteriophage P22. The –35 and –10 regions of the P_{ant} and P_{mnt} promoters are indicated. The 6-bp sequences to which Arc dimers make base-specific contacts in the Arc-operator cocrystal structure are boxed (Raumann *et al*., 1994b). Arc dimers, shown as ovals, interact to form a cooperatively stabilized tetramer on the DNA.

promoters are shown in Figure 1. Promoter variants with only a single active subsite were constructed by changing the sequence of the other subsite to eliminate Arc binding (Vershon *et al*., 1989). In the wild-type context, transcription from the P_{ant} promoter interferes with transcription from the weaker P_{mnt} promoter (Vershon *et al*., 1987a,b). To avoid complications caused by this interaction, we also inactivated one promoter or the other with mutations, allowing the effects of Arc binding to single subsites to be independently assayed for the variants of P_{ant} and P_{mnt}. The promoter variants used for these studies are shown diagrammatically in Figure 2 and the associated sequence changes are listed in Table 1. We refer to the single-subsite variants using the promoter name and a number indicating whether the active subsite is proximal to the –35 region or the –10 region (e.g. P_{mnt} / 35). For the P_{ant} / 35 variants, the subsites are further designated A (wild-type sequence) or B (–35 proximal subsite has the sequence of the wild-type –10 proximal site). The latter construct controls for the minor sequence differences between subsites. There are also A and B variants of the P_{ant} / 10 constructs, but in this case the promoters differ at a position (–30) that is not important for Arc binding but has been shown to affect recognition by RNA polymerase (McClure, 1985). Promoters with two active subsites are designated P_{ant} / both and P_{mnt} / both, whereas those with no active subsites are designated P_{ant} / none and P_{mnt} / none.

On each promoter construct, only one RNA polymerase open-complex footprint was seen at the expected position (not shown) and only one major transcript of the expected size was observed in run-off transcription assays (see below). In addition, footprinting with saturating concentrations of the cooperativity defective mutant, Arc-SL35, showed that each construct bound Arc only at the expected subsites (Figure 3). Arc-SL35 was used throughout these studies instead of wild-type Arc because this mutant does not stabilize non-specific binding of additional dimers through cooperative protein–protein interactions (Smith & Sauer, 1995).

Run-off transcription experiments

Run-off transcription experiments with RNA polymerase in excess of template DNA were performed in the absence of repressor or in the presence of a concentration of Arc-SL35 sufficient to saturate the subsite in the footprinting assay. Figure 4(a) shows an example of a run-off transcription experiment with the P_{ant} / both promoter variant. In the absence of Arc, the major bands are the run-off transcript of approximately 85 nucleotides and the ^{32}P-labeled DNA template. In addition, an end-to-end transcript was seen when no promoter was present (not shown) or when the promoter was strongly repressed (+Arc-SL35 lane in Figure 4(a)).

The results of run-off transcription experiments with each promoter variant are shown in Figure 4(b). Some promoter variants containing only a single subsite can be significantly repressed by the binding of an Arc-SL35 dimer. For the P_{ant} promoter variants, Arc-SL35-mediated repression was about 80 to 90% efficient under the conditions of this assay when both operator subsites were present, about 30 to 50% efficient with an active subsite proximal to the –35 region, and only 0 to 20% efficient when the active subsite was proximal to the –10 region (Figure 4(b)). Differences in the overall promoter strengths of these variants were less than twofold (Table 1). Thus, the main variables affecting repression seem to be the number and positions of active subsites. Changing the sequence of the –35 proximal active subsite from wild-type (P_{ant} / 35A) to that of the normal –10 proximal subsite (P_{ant} / 35B) did not significantly change the ability of that position to mediate repression, indicating that the minor sequence and affinity differences between subsites are not important determinants of repression efficiency. Altering the promoter context by changing position –30 from a consensus (P_{ant} / 10A) to a non-consensus bp (P_{ant} / 10B) resulted in slightly better repression, indicating that promoter context may affect regulation by Arc (see Discussion).

Figure 4(c) shows run-off transcription as a function of Arc-SL35 concentration for the P_{ant} / both, P_{ant} / 35A, P_{ant} / 10A, and P_{ant} / none promoters. At concentrations of Arc-SL35 above 100 nM, repression of the promoter with the active –35 proximal subsite approaches that of the promoter with both active subsites. However, at these high concentrations, non-specific binding of Arc-SL35 is observed in footprinting experiments (not shown) and thus some of the repression of P_{ant} / 35A may be mediated by non-specifically bound dimers under

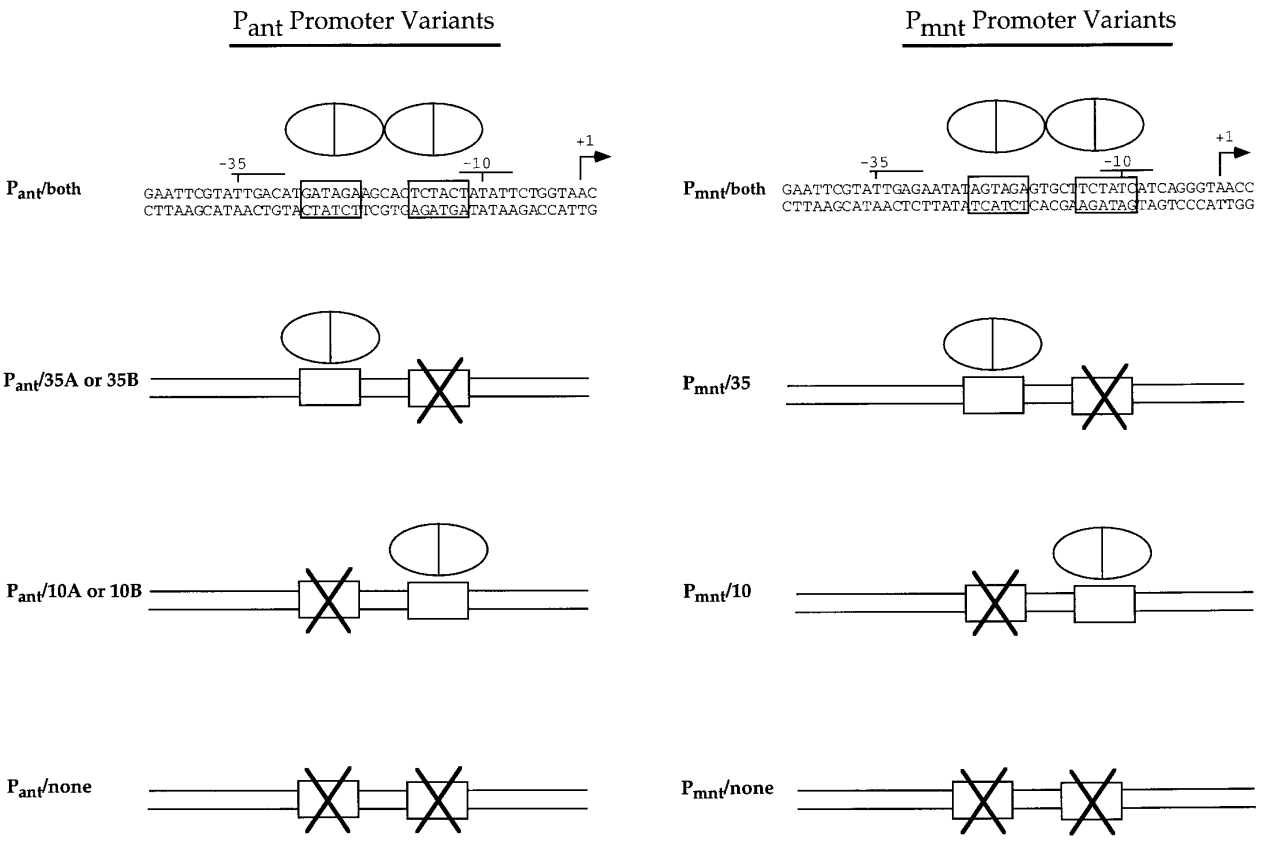

Figure 2. Promoter variants. The *arc* operator subsites are shown as boxes and the Arc dimers as ovals. Subsites inactivated by mutations are marked by crosses. The sequences of the reference P_{ant}/both and P_{mnt}/both promoters are shown. Sequence changes in the remaining variants are listed in Table 1.

these conditions. Nevertheless, more than 50% repression of P_{ant}/35A occurs at 75 nM Arc-SL35, a concentration where the active operator subsite is saturated in footprinting but non-specific binding is not observed (Figure 3). Moreover, at the highest repressor concentrations tested, the promoter variant with the –10 proximal subsite shows almost no repression. Thus, the subsite next to the –35 promoter element of P_{ant} appears to play the major role in repression.

Repression efficiency of the P_{mnt} variants by Arc also varied depending on the position of the active subsite (Figure 4(b)). In this case, however, the variant with the active subsite proximal to the –10 region (P_{mnt}/10) was repressed to the same extent ($\approx$75%) as the variant with two active subsites

Table 1. Mutations in promoter variants relative to P_{ant}/both or P_{mnt}/both

Promoter variant	Mutations	Promoter strength
P_{ant}/both	—	1.00
P_{ant}/35A	GTGC at –17 to –14	0.70 ± 0.04
P_{ant}/35B[a]	AG at –28 to –27, GT at –22 to –21, GTGC at –17 to –14	0.85 ± 0.14
P_{ant}/10A	AGGCACGT at –28 to –21	1.50 ± 0.05
P_{ant}/10B[b]	G at –30, AGGCACGT at –28 to –21	1.53 ± 0.07
P_{ant}/none	AGGCACGT at –28 to –21, GTGC at –17 to –14	1.29 ± 0.01
P_{mnt}/both	—	0.26 ± 0.09
P_{mnt}/35	GT at –14 to –13	1.13 ± 0.26
P_{mnt}/10	AC at –21 to –20	0.43 ± 0.04
P_{mnt}/none	AC at –21 to –20, GT at –14 to –13	1.74 ± 0.40

Promoter strengths (averages ± standard deviation, $n \geqslant 3$) *in vitro* relative to P_{ant}/both in the absence of Arc. The sequences of the reference promoter variants, P_{ant}/both and P_{mnt}/both, are shown in Figure 2; sequence substitutions refer to the top strands shown in Figure 2.

[a] In the P_{ant}/35B variant, the sequence of the wild-type P_{ant} –35 proximal arc subsite is replaced by the sequence of the –10 proximal subsite.

[b] In the P_{ant}/10B variant, there is a change from a consensus to a non-consensus bp in the –35 promoter region relative to the P_{ant}/10A variant.

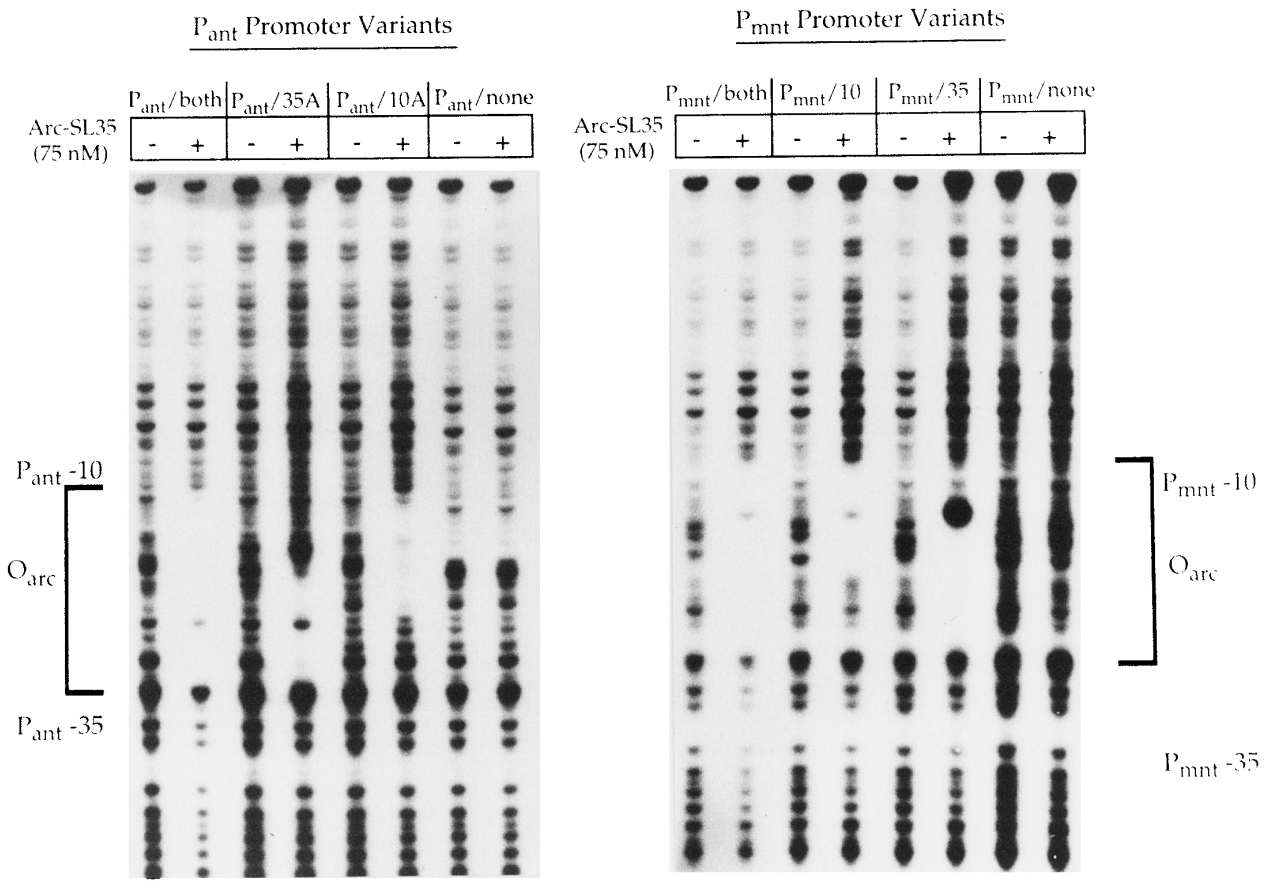

Figure 3. DNase I footprinting assays of the binding of Arc-SL35 to promoter variants. Protection of only the active subsites is observed. Arc-SL35 footprints on the P_{ant}/35B and the P_{ant}/10B variants (not shown) were similar to those on P_{ant}/35A and P_{ant}/10A, respectively.

(P_{mnt}/both). The promoter with the single active subsite proximal to the –35 region (P_{mnt}/35) was repressed at only about 25% efficiency. The P_{mnt} variants have significantly different promoter strengths, with the strongest promoters (P_{mnt}/35 and P_{mnt}/none) containing the consensus base for σ^{70} promoters at position –14 (McClure, 1985). Because the weaker P_{mnt}/both and P_{mnt}/10 promoter variants are repressed most efficiently, we cannot exclude a causal relationship between promoter strength and poor repressibility for the P_{mnt} variants. Nevertheless, it is still clear that Arc-SL35 bound only to the –10 proximal subsite is as efficient as Arc-SL35 bound to both subsites because P_{mnt}/10 and P_{mnt}/both, which have reasonably comparable promoter strengths, are repressed equally well.

DNA mobility shift assays for open-complex formation

As an independent test of the effects of Arc-SL35 binding to different subsites, DNA mobility shift assays were used to monitor the extent of open-complex formation by RNA polymerase in the absence and the presence of Arc-SL35. Figure 5(a) shows an example of an assay for open-complex formation with the P_{ant}/both variant at a single time point (two minutes) in which Arc-SL35 effectively prevented RNA polymerase open-complex formation. A summary of similar assays for the extent of open-complex formation with each promoter construct in the presence and absence of Arc-SL35 (75 nM) is shown in Figure 5(b). For the P_{ant} variants, Arc-SL35 repressed open-complex formation by RNA polymerase most efficiently when both subsites were filled (≈90 to 95%), next most efficiently when the subsite proximal to the –35 region was occupied (≈60 to 75%), and least efficiently when the subsite proximal to the –10 region was bound (≈0 to 30%). Figure 5(c) shows an assay of open-complex formation at two minutes in the presence of increasing concentrations of Arc-SL35 for the P_{ant}/both, P_{ant}/35A, P_{ant}/10A, and P_{ant}/none promoters. Again, it is clear that an Arc-SL35 dimer bound just to the –35 proximal subsite is an efficient repressor whereas a dimer bound just to the –10 proximal subsite is a very poor repressor.

For the P_{mnt} variants (Figure 5(b)), repression of open-complex formation by Arc was most efficient when both subsites were present (≈95%), slightly less efficient when only the –10 proximal subsite was functional (≈80%), and only marginally

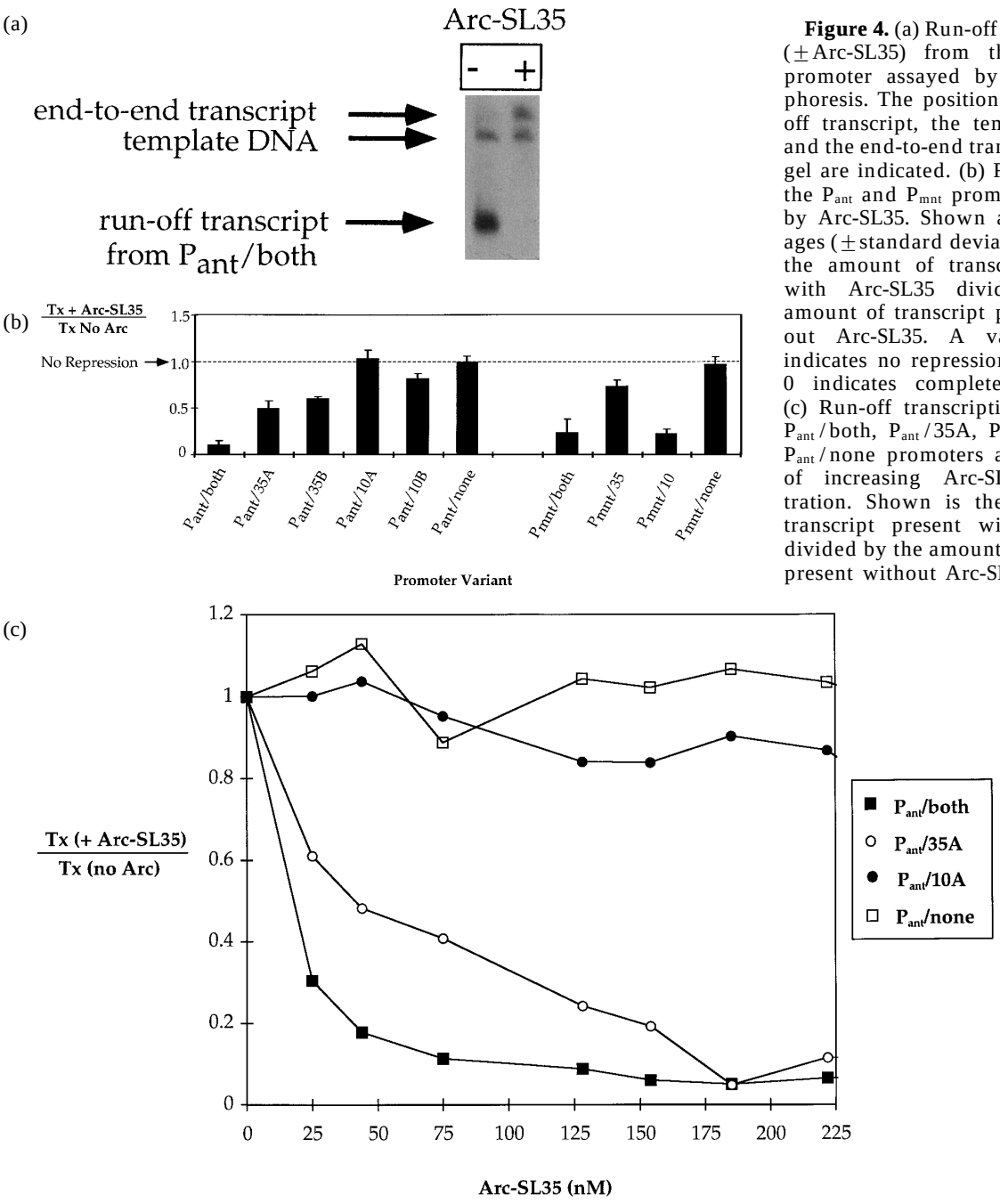

Figure 4. (a) Run-off transcription (±Arc-SL35) from the P_{ant}/both promoter assayed by gel electrophoresis. The positions of the run-off transcript, the template DNA, and the end-to-end transcript on the gel are indicated. (b) Repression of the P_{ant} and P_{mnt} promoter variants by Arc-SL35. Shown are the averages (±standard deviation, $n \geqslant 4$) of the amount of transcript present with Arc-SL35 divided by the amount of transcript present without Arc-SL35. A value of 1.0 indicates no repression; a value of 0 indicates complete repression. (c) Run-off transcription from the P_{ant}/both, P_{ant}/35A, P_{ant}/10A, and P_{ant}/none promoters as a function of increasing Arc-SL35 concentration. Shown is the amount of transcript present with Arc-SL35 divided by the amount of transcript present without Arc-SL35.

efficient when just the –35 proximal subsite was active (≈25%). Similar results for the P_{ant} and P_{mnt} variants were obtained when open-complex formation was assayed at an earlier time point (20 seconds) at which open-complex formation was less complete (not shown). Repression ratios calculated from the open-complex formation assays correlate well ($r = 0.967$) with those calculated from the run-off transcription assays, suggesting that repression by Arc-SL35 dimers bound to one or two subsites largely reflects its activity in slowing the rate of open-complex formation by RNA polymerase.

Assays for dimer repression *in vivo*

Plasmids containing the P_{ant}/both, P_{ant}/35A, P_{ant}/10A, and P_{ant}/none promoters fused to the *lacZ* gene were constructed to allow assays of repression *in vivo*. As shown in Table 2, repression of the P_{ant}/both-*lacZ* fusion by Arc-SL35 was about 75% complete and repression of the P_{ant}/35A-*lacZ* fusion was approximately 50% complete. No significant repression was observed for the P_{ant}/10A-*lacZ* and P_{ant}/none-*lacZ* constructs. Each of these promoters had similar promoter strengths *in vivo* (Table 2). Controls also showed that Arc-RA13, a mutant

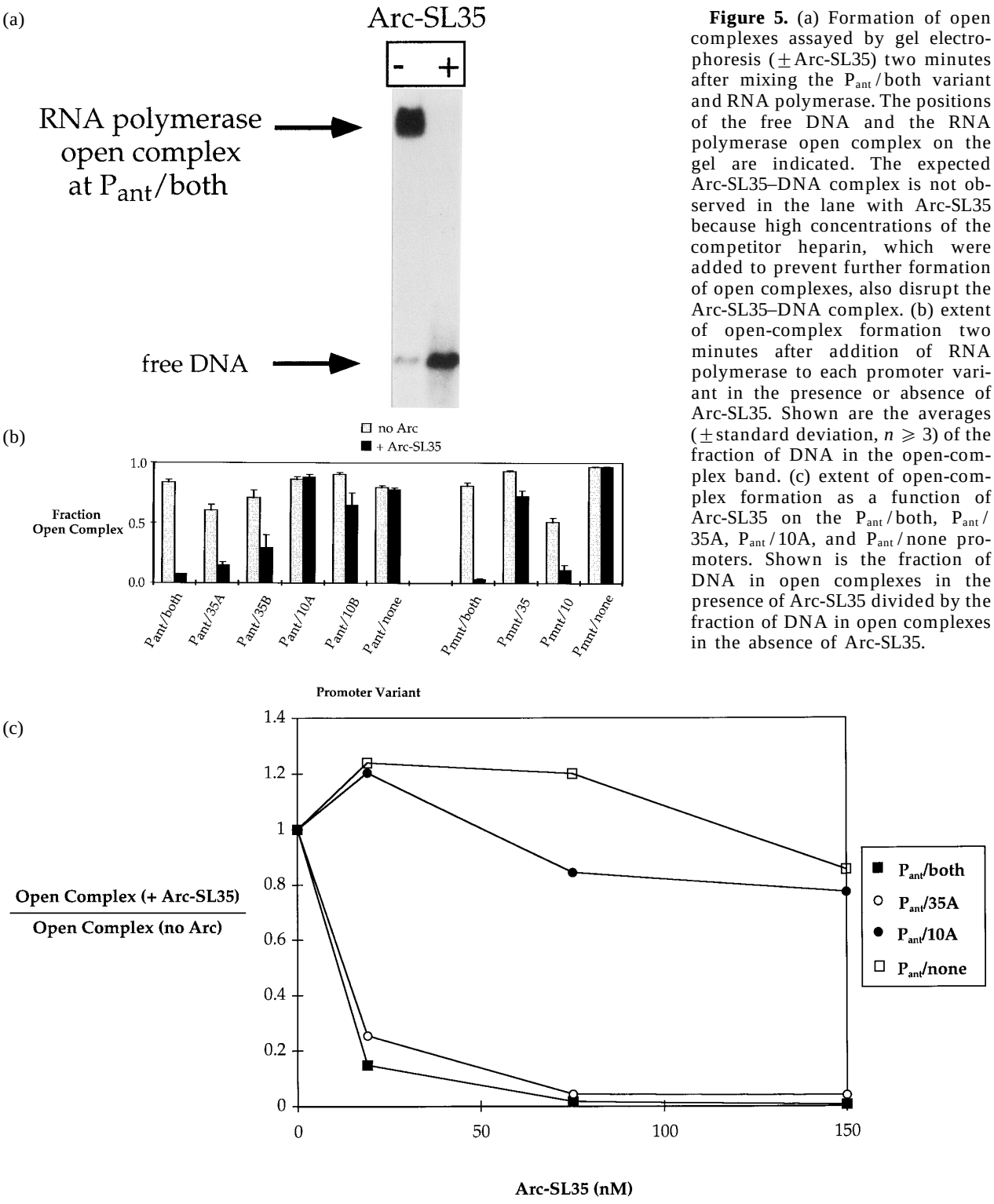

Figure 5. (a) Formation of open complexes assayed by gel electrophoresis (±Arc-SL35) two minutes after mixing the P_{ant}/both variant and RNA polymerase. The positions of the free DNA and the RNA polymerase open complex on the gel are indicated. The expected Arc-SL35–DNA complex is not observed in the lane with Arc-SL35 because high concentrations of the competitor heparin, which were added to prevent further formation of open complexes, also disrupt the Arc-SL35–DNA complex. (b) extent of open-complex formation two minutes after addition of RNA polymerase to each promoter variant in the presence or absence of Arc-SL35. Shown are the averages (±standard deviation, $n \geqslant 3$) of the fraction of DNA in the open-complex band. (c) extent of open-complex formation as a function of Arc-SL35 on the P_{ant}/both, P_{ant}/35A, P_{ant}/10A, and P_{ant}/none promoters. Shown is the fraction of DNA in open complexes in the presence of Arc-SL35 divided by the fraction of DNA in open complexes in the absence of Arc-SL35.

defective in operator binding (Brown *et al*., 1994), failed to repress any of the constructs. These repression assays *in vivo* are generally consistent with the results obtained *in vitro* with the same promoter variants. In both circumstances, an Arc dimer bound to the P_{ant} –35 proximal subsite is more efficient at repressing transcription than a dimer bound to the –10 proximal subsite.

Discussion

Arc is a member of the ribbon-helix-helix family of DNA binding proteins (Raumann *et al*., 1994a). Arc and the other well-characterized members of this family, the Mnt and MetJ repressors, use protein–protein interactions between DNA-bound dimers to stabilize binding to tandemly arrayed

Table 2. β-Galactosidase expression *in vivo* from fusions of P_{ant} promoter variants to *lacZ* assayed in the absence or presence of Arc-SL35 or the inactive Arc-RA13 mutant

Promoter variant	Protein	β-Galactosidase (Miller units)
P_{ant} / both	No Arc	$3.0(\pm 0.5) \times 10^4$
	Arc-RA13	$3.0(\pm 0.8) \times 10^4$
	Arc-SL35	$0.7(\pm 0.1) \times 10^4$
P_{ant} / 35A	No Arc	$2.6(\pm 0.5) \times 10^4$
	Arc-RA13	$2.7(\pm 0.6) \times 10^4$
	Arc-SL35	$1.2(\pm 0.2) \times 10^4$
P_{ant} / 10A	No Arc	$2.6(\pm 0.4) \times 10^4$
	Arc-RA13	$3.1(\pm 0.8) \times 10^4$
	Arc-SL35	$2.1(\pm 0.3) \times 10^4$
P_{ant} / none	No Arc	$2.6(\pm 0.5) \times 10^4$
	Arc-RA13	$2.6(\pm 0.2) \times 10^4$
	Arc-SL35	$2.4(\pm 0.3) \times 10^4$

Values shown are averages (±standard deviations, $n \geq 3$).

operator subsites (Brown & Sauer, 1993; He *et al*., 1992; Waldburger & Sauer, 1995). Are tandem binding sites required for regulation by proteins in this family? Here, we addressed this question by using a set of designed promoter variants to test whether the reiterated subsites in the Arc system are required for repression. We found that an Arc dimer bound to a single subsite could repress transcription of certain P_{ant} and P_{mnt} variants *in vitro* and *in vivo*. Thus, tandem binding of Arc to both subsites is not essential for regulation although it may be required to achieve maximal levels of repression in some circumstances.

Arc dimers bound to the left or right operator subsites have significantly different repressor activities. In particular, for the P_{ant} variants, a dimer bound at the –35 proximal position slowed open-complex formation and repressed transcription reasonably efficiently, whereas a dimer bound at the –10 proximal position was much less effective. In the case of the P_{mnt} variants, a dimer bound proximal to the –10 region was more effective than a dimer bound near the –35 region. In the wild-type context, the subsite proximal to the –10 region of P_{mnt} is the same as the subsite proximal to the –35 region of P_{ant} (Figure 1). Hence, the left arc subsite shown in Figure 1 appears to be the primary mediator of Arc repression. Since cooperative DNA binding of two Arc dimers is important for normal repression efficiency *in vivo* (Smith & Sauer, 1995), occupancy of the right subsite may be required mainly to facilitate cooperative stabilization of the dimer bound to the left subsite. However, since transcriptional repression of P_{ant} is slightly more efficient *in vitro* and *in vivo* when both subsites are filled than when just the left subsite is filled (Figure 4(c) and Table 2), non-cooperative occupancy of the right subsite may also have a small additive or synergistic effect on repression.

The sequence changes used to inactivate the –35 and/or –10 proximal operator subsites result, in some cases, in changes in promoter strength (Table 1). This makes it difficult to know whether the observed changes in repression arise solely from changes in the number or positions of active subsites or if repression is also influenced by alterations in the kinetics of transcription initiation for the variant promoters. For the P_{ant} variants, the largest changes in promoter strength are only about 50% *in vitro* (Table 1) and less than 15% *in vivo* (Table 2). Given the large differences in repression of these variants, it seems likely that the position of the active subsite(s) is the major factor in determining repression by Arc. For the P_{mnt} variants, however, larger changes in promoter strength are observed *in vitro* (Table 1), which correlate with the repressibility of the different promoters. In these cases, both the position of the active subsite and the intrinsic promoter strength may contribute to repression efficiencies.

Transcription initiation by *Escherichia coli* RNA polymerase is a multistep process involving binding of the enzyme to the promoter in an unstable closed complex, isomerization to a stably bound open complex, initiation of transcript formation, and clearance from the promoter (McClure, 1985). The experiments presented in Figure 4 and Figure 5 show that repression of a variety of promoter constructs by Arc-SL35 is strongly correlated with its ability to decrease the rate of RNA polymerase open-complex formation on these promoters. Experiments performed by Liao and McClure suggest that wild-type Arc represses the wild-type P_{ant} promoter by slowing the rate at which closed-promoter complexes isomerize to form open complexes (Liao, 1988). We have recently found that Arc can also increase the rate of RNA polymerase clearance from certain P_{ant} promoter variants with an active –35 proximal subsite but not from a variant with only an active –10 proximal subsite (Smith & Sauer, 1996). For Arc to slow isomerization or to accelerate promoter clearance likely requires that it bind to the promoter simultaneously with RNA polymerase. The multiple activities of Arc and potential concomitant binding of Arc and RNA polymerase suggest the possibility of interactions between Arc and RNA polymerase, at least at the P_{ant} promoter. Arc dimers bound to the left and right operator subsites might have unequal repression efficiencies because only one Arc subunit is properly positioned to interact with RNA polymerase or because a dimer bound to only one subsite affects access of RNA polymerase to an important region of the promoter DNA.

The assignment of a primary regulatory role to a single subunit of a multiprotein complex has also been proposed for other systems. Bacteriophage λ *c*I dimers bound to the three operators, O_R1, O_R2 and O_R3, have distinct functional roles in regulating two divergent promoters, P_R and P_{RM}. The dimers at O_R1 and O_R2 repress P_R, a dimer at O_R2 activates P_{RM}, and a dimer at O_R3 represses P_{RM} (Meyer *et al*., 1980; Ptashne, 1986). The location of the operator sites with respect to the promoters is a key

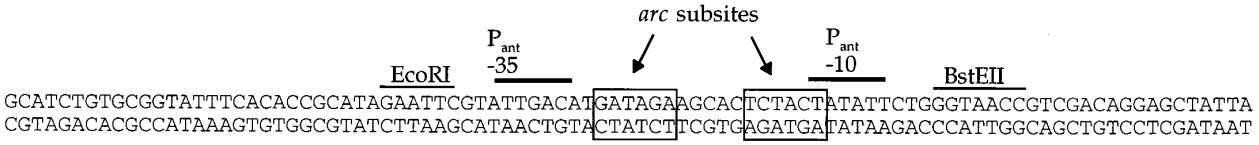

Figure 6. Flanking sequence of the P_{ant}/both variant showing the cloning sites.

determinant of regulation in this system. Further specification of the functional roles of the two monomers in the λ *c*I dimer bound to O_R2 can also be detected. Activation of the P_{RM} promoter by a dimer bound to O_R2 operator depends on protein–protein interactions between *c*I and RNA polymerase, and only the promoter proximal monomer of the λ *c*I dimer is thought to mediate the activation (Hochschild *et al*., 1983; Kuldell & Hochschild, 1994; Li *et al*., 1994; Ptashne, 1986). Regulation of promoters dependent on the cAMP receptor protein (CRP) also results from interactions between CRP and RNA polymerase (Bell *et al*., 1990; Niu *et al*., 1994; Zhou *et al*., 1993b, 1994a). At some promoters, CRP binds upstream of the –35 promoter region, whereas at other promoters, the CRP site overlaps the –35 region. In the former case the promoter-proximal monomer of the CRP dimer is responsible for activation whereas in the latter case, the promoter-distal monomer activates transcription (Zhou *et al*., 1993a, 1994b). Thus, which CRP monomer is functional in activation depends on the position of the binding site with respect to the promoter. Arabinose-induced activation by AraC involves binding of AraC to two subsites composed of direct repeats separated by less than a helical turn of DNA. The promoter-proximal subsite slightly overlaps the –35 region of the promoter, and binding of a monomer of AraC to this subsite is absolutely required for activation. Binding of an AraC monomer to the promoter-distal subsite plays a less significant role in activation. Switching the orientation of the direct repeat subsites results in no activation by AraC. Therefore, regulation by AraC mainly requires binding in a particular orientation to one subsite that overlaps the –35 region (Reeder & Schlief, 1993). Arc is an interesting addition to the set of regulators of this type because the same arc DNA subsite may be primarily responsible for regulation of two divergent promoters even though it is in a different position with respect to each promoter.

Materials and Methods

Cloning of promoter fragments

Oligonucleotides were synthesized using an Applied Biosystems 381A DNA synthesizer and were annealed and ligated to the backbones of the appropriate plasmids for cloning. Each of the promoter variants was cloned as an *Eco*RI-*Bst*EII cassette into the backbone of pSA660, a derivative of pSA600 (Milla *et al*., 1993) in which the *Eco*RI site in the arc gene was eliminated by cassette mutagenesis (without changing the amino acid sequence), and an *Eco*RI site was added next to the P_{ant} promoter to allow cloning of promoter fragments. The sequence of the region surrounding the cloning sites does not derive from the *immunity I* operon and is shown for the P_{ant} cassette in Figure 6.

Four of the P_{ant} promoter variants (P_{ant}, P_{ant}/35A, P_{ant}/10A, and P_{ant}/none) were also ligated as *Eco*RI-*Bst*EII cassettes to a promoterless trpA'-lacZ fusion in plasmid pTS300. The pTS300 plasmid was constructed in the following manner. The plasmid pACYC184 was digested with *Hin*dIII and *Eag*I and the large backbone fragment was ligated to the cassette,

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5'-AGCTTAGCCCGCCTAATGAGCGGGCTTTTTTTTGGCCGCCTAGGCCC-3',
   3'-ATCGGGCGGATTACTCGGCCGAAAAAAAACCGGCGGATCCGGGCCGG-5'
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(which includes a trpA transcriptional terminator and an SfiI restriction site), to generate pTS100. The *Sfi*I fragment from the plasmid miniTn5lacZ1 (De Lorenzo *et al*., 1990), which contains the same promoterless trpA'-lacZ fusion as pRZ5605 (Mandecki & Reznikoff, 1982), was cloned into the *Sfi*I site of pTS100 to generate pTS200. Plasmid pTS300 was generated by cloning the cassette

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5'-AATTCGTATTGACATGATAGAAGCACTCTACTATATTCTGGTAACCGACGCCATGGCACCCC-3',
   3'-GCATAACTGTACTATCTTCGTGAGATGATATAAGACCATTGGCTGCGGTACCGTGGGG-5'
```

between the *Eco*RI and *Sma*I sites upstream of the trpA'-lacZ fusion. Introduction of this cassette placed the *lacZ* gene under the control of the wild-type P_{ant} promoter variant and resulted in the proper *Eco*RI and *BstE*II sites needed to clone the additional promoter variants. The other promoter variant-*lacZ* constructs were made by cloning the appropriate cassette between these *Eco*RI and the *Bst*EII sites. These plasmids are compatible with the pBR322-derived plasmid pSA600, which carries the arc gene (Milla *et al*., 1993), allowing measurements of the effects of regulation by Arc *in vivo*.

Template preparations

Linear templates for transcription and footprinting reactions were generated by the polymerase chain reaction (PCR) using a pair of primers with 5' ends located at –87 and +85 with respect to the start site of transcription. The –87 primer was end-labeled with [γ-^{32}P]ATP using bacteriophage T4 polynucleotide kinase. PCR reactions were performed with the labeled –87 primer, the unlabeled +85 primer, and the linearized plasmid template DNA. The 173 bp PCR product was purified on a 10% polyacrylamide 0.5 × TBE (90 mM Tris/borate (pH 8.0), 2 mM Na_2EDTA) gel.

Protein purification

The cooperativity defective variant Arc-SL35 was used in all experiments. Unlike wild-type Arc, this variant does not facilitate non-specific binding of additional dimers next to specifically bound dimers (Smith & Sauer, 1995). For purification purposes, a C-terminal tail of six histidines (st6) was also added to the Arc-SL35 variant.

This tail does not affect the stability or DNA binding properties of Arc (Milla *et al*., 1993). The Arc-SL35(st6) protein was purified as described using nickel chelate chromatography (Qiagen) followed by SP-Sephadex chromatography (Milla *et al*., 1993). RNA polymerase σ^{70} holoenzyme was purified as described (Hager *et al*., 1990; Smith & Sauer, 1995).

DNase I footprinting reactions

DNA (0.2 nM) was incubated with or without Arc-SL35 (75 nM) at 37°C in the same buffer as was used for transcription assays (see below). DNase I was added to a final concentration of 18 ng/ml for one minute. Reactions were quenched by the addition of an equal volume of 2.5 M ammonium acetate, 20 mM Na_2EDTA, and 10 μg/ml sonicated salmon sperm DNA. The reactions were extracted with phenol/chloroform (1:1, v/v), precipitated with ethanol using glycogen as a carrier, washed with 70% ethanol, dried, and resuspended in 5 μl of 85% (v/v) formamide, 10 mM NaOH, 1 mM EDTA, 0.1% (v/v) bromophenol blue, and 0.1% (v/v) xylene cyanol. Products of the reactions were resolved on 6% polyacrylamide, 8.3 M urea gels run in 1 × TBE.

Run-off transcription assays

DNA (0.2 nM) was incubated at 37°C with or without Arc-SL35 (75 nM in the single-point assays) in a volume of 25 μl of buffer containing 30 mM Hepes-KOH (pH 7.5), 100 mM potassium glutamate, 10 mM $MgCl_2$, 1.5 mM $CaCl_2$, 0.1 mM Na_2EDTA, 100 μg/ml bovine serum albumin, 1 mM dithiothreitol, 0.02% NP-40 and nucleoside triphosphates (NTPs; 200 μM each of GTP, CTP and ATP, and 6 μM UTP, at a specific activity of 1.4×10^4 cpm/pmol). One unit of RNase inhibitor (Promega) was also included in each reaction. Reactions were initiated by the addition of RNA polymerase σ^{70} holoenzyme in a volume of 5 μl to a final concentration of 7.5 nM. After ten minutes, 5 μl of heparin (0.7 mg/ml) was added. After ten additional minutes, 2 μl of 0.5 M Na_2EDTA and 40 μl of 8 M urea, 0.1% xylene cyanol, 0.1% bromophenol blue and 50% glycerol were added and the reactions were heated to 90°C before loading the gel. The products of the reactions were analyzed on 12% polyacrylamide/8.3 M urea gels run in 1 × TBE. End-labeled DNA markers were used for approximate size standards. The major products of the reactions were transcripts of approximately 85 nucleotides in size. The appearance of these products was dependent on the addition of RNA polymerase, NTPs, and a promoter sequence in the appropriate position of the template. The template and transcript bands were quantified using a Phosphorimager and the ImageQuant program (Molecular Dynamics), and the transcript bands were normalized to the amount of template in each lane.

DNA mobility shift experiments for open-complex formation

DNA (0.2 nM) was incubated at 37°C with or without Arc-SL35 (75 nM in the single-point assays) in 25 μl of the same buffer used for the transcription assays plus one unit of RNase inhibitor. RNA polymerase was added in a volume of 5 μl to a final concentration of 7.5 nM. After two minutes, formation of additional open complexes was stopped by the addition of 5 μl of a solution containing heparin (0.7 mg/ml) and 36% sucrose. In control reactions, this concentration of heparin prevented any association of free RNA polymerase with the DNA (not shown).

β-Galactosidase Assays for Repression *in vivo*

The pTS300 promoter variant plasmids (P_{ant}/both, P_{ant}/35A, P_{ant}/10A, and P_{ant}/none) were transformed into *E. coli* strain XL-1 Blue either alone or with a pSA600 plasmid encoding Arc-SL35 (Smith & Sauer, 1995) or Arc-RA13 (Brown *et al*., 1994). The pTS300 plasmid was selected with 100 μg/ml chloramphenicol and the pSA600 plasmid was selected with 150 μg/ml ampicillin. Freshly transformed cells were grown overnight in LB broth with the appropriate antibiotics, subcultured the next day and allowed to grow to an A_{600} of approximately 0.4 to 0.8. β-Galactosidase assays were then performed as described by Miller (1972).

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