JOURNAL OF BACTERIOLOGY, Sept. 2000, p. 5076–5081 Vol. 182, No. 18
0021-9193/00/$04.00+0

Recognition of Overlapping Nucleotides by AraC and the Sigma Subunit of RNA Polymerase

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Received 3 September 1999/Accepted 14 June 2000

The *Escherichia coli* promoter p_{BAD}, under the control of the AraC protein, drives the expression of mRNA encoding the AraB, AraA, and AraD gene products of the arabinose operon. The binding site of AraC at p_{BAD} overlaps the RNA polymerase −35 recognition region by 4 bases, leaving 2 bases of the region not contacted by AraC. This overlap raises the question of whether AraC substitutes for the sigma subunit of RNA polymerase in recognition of the −35 region or whether both AraC and sigma make important contacts with the DNA in the −35 region. If sigma does not contact DNA near the −35 region, p_{BAD} activity should be independent of the identity of the bases in the hexamer region that are not contacted by AraC. We have examined this issue in the p_{BAD} promoter and in a second promoter where the AraC binding site overlaps the −35 region by only 2 bases. In both cases promoter activity is sensitive to changes in bases not contacted by AraC, showing that despite the overlap, sigma does read DNA in the −35 region. Since sigma and AraC are thus closely positioned at p_{BAD}, it is possible that AraC and sigma contact one another during transcription initiation. DNA migration retardation assays, however, showed that there exists only a slight degree of DNA binding cooperativity between AraC and sigma, thus suggesting either that the normal interactions between AraC and sigma are weak or that the presence of the entire RNA polymerase is necessary for significant interaction.

The sigma subunit of RNA polymerase (referred to here as sigma) is responsible for the binding of the holoenzyme to promoters during transcription initiation (2, 46). It does this by making sequence-specific contacts with bases in hexameric sequences centered at 10 and 35 bases upstream of the transcription start site on promoters (3, 13, 18, 32, 45, 50). At the −10 hexamer, sigma makes base-specific contacts with the nontemplate strand (23, 34, 41, 42). In addition to sigma-DNA interactions during initiation, protein-protein contacts also occur between transcriptional activators and subunits of RNA polymerase (1, 11, 14, 21, 22, 36, 43).

At many promoters, the recognition sequences of transcriptional activator proteins partly overlap the 6 bases of the −35 region that are contacted by the sigma subunit of RNA polymerase (4). In these cases, does the activator substitute for sigma in the recognition of the −35 region; do both proteins read the −35 region, necessitating overlapped reading by both proteins; or does sigma read an adjacent sequence?

On one hand, direct protein-protein contacts between sigma and upstream transcriptional activators seem to occur. At the λ p_{RM} promoter, the binding site of λ*c*I overlaps the −35 region for sigma by 2 nucleotides, and genetic experiments suggest an interaction between the λ*c*I protein and the −35 recognition motif of sigma 70 (25, 31). Recently, interactions between sigma and Ada, an AraC homologue from the XylS family of proteins, have been demonstrated genetically at the *ada*, *alkA*, and *aidB* promoters (27, 28). A direct sigma-Ada interaction at the *ada* and *aidB* promoters has also been revealed biochemically with DNA migration retardation assays similar to those presented in this paper (27). On the other hand, at the PhoB-dependent *PpstS* and the CRP-dependent P1*gal* promoters, where the activator binding site completely overlaps the −35 hexamer, it appears possible that the activator can substitute entirely for recognition by sigma in the −35 region (26).

We studied the *ara* promoter, p_{BAD}, which is under the control of two activators, CRP (29, 30) and AraC (12, 15) (Fig. 1). The binding of AraC to the I_1 and I_2 half-sites is stimulated by the presence of arabinose. When these sites are occupied by AraC, and if they overlap the −35 hexamer by 2 or 4 bases, transcription is actively initiated from p_{BAD} (39).

At p_{BAD}, it is likely that the C-terminal domain of the α subunit of polymerase interacts both with CRP and with AraC (49). Two lines of reasoning suggest that AraC may also interact with the sigma subunit of RNA polymerase. First, the R596H mutation in the sigma subunit allows AraC to stimulate p_{BAD} to high levels in the absence of the normally required CRP (19). Second, although AraC can activate transcription from its position partially overlapping the −35 hexamer, it cannot activate (39) as CRP (14, 47) or OmpR (33) can when they are moved upstream by one or more helical turns.

We have examined whether sigma reads that part of the −35 region that lies outside the AraC-contacting region. If it does read this region, then AraC is not substituting for the contacts made by sigma in the region, and either sigma reads the −35 region as before, or it is only slightly displaced by the presence of AraC. We also analyzed sigma binding at the −35 hexamer at a second promoter where the AraC binding site overlaps the hexamer by only 2 bases.

Our results showed that sigma contacts the nonoverlapped bases of the −35 hexamer. Because of the close spatial placement of AraC and sigma on the promoter DNA, we then looked for an interaction between AraC and sigma that would reveal itself as cooperativity in the binding of AraC and sigma to DNA. To avoid the difficulties that would arise from the known interactions between AraC and the alpha subunit of RNA polymerase (49), we used purified sigma in the absence of the other RNA polymerase subunits. Also, to enhance the weak DNA binding affinity of sigma in the absence of core polymerase, we used a truncated variant of sigma (Δ133). This truncation rid the protein of the N-terminal acidic domain that

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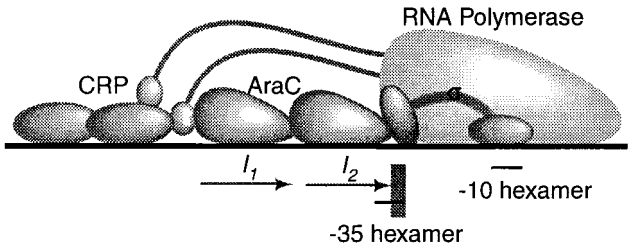

FIG. 1. Polymerase-promoter and activator-promoter interactions at p_{BAD}. The σ^{70} subunit of RNA polymerase contacts the −35 and −10 hexamers. Occupancy of the I_1 and I_2 half-sites by AraC activates transcription with the aid of the CRP protein, most likely utilizing the α-subunit–activator interactions as shown. The binding sites of σ^{70} and AraC overlap by 4 bp at p_{BAD}. The nucleotides in the −35 hexamer that lie outside the region of overlap are shaded.

interferes with binding of sigma to DNA (6, 7), and we were able to observe a slight cooperativity between AraC and sigma in binding to p_{BAD}.

MATERIALS AND METHODS

Strains and plasmids. The plasmid used for the initial construction of the I_1-I^*p_{BAD} mutants contained an I_1-I_2p_{BAD}–*galK* fusion in pES51 (20). The promoter region of the p7 plasmid, which carries an I_1-I_1–*lacZ* fusion (39), was replaced with the I_1-I^*p_{BAD} promoter region, resulting in an I_1-I^*p_{BAD}–*lacZ* fusion. Promoter activity was assayed in TR322 cells (*araC*$^+$*B*$^+$*A*$^+$*D*$^+$ *galK* Str^r) (only relevant markers are shown) (16).

The plasmid used for overexpression of the σ^{70} variants was pQE30 (QIAgen), in which the *rpoD* gene is under the control of the T5 promoter (48). This was a kind gift from Alicia Dombroski. Protein was overexpressed in XL1 Blue cells (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1*) from Stratagene.

Construction of mutant I_1-I^*p_{BAD} templates. Site-directed PCR mutagenesis was performed to modify the promoter-proximal *araI* site in p_{BAD} and to randomize the nonoverlapping bases (X) in the −35 box. The I^* half-site (TAGC GGATCCATCCATA) contained the beginning sequence of the I_2 half-site (TAGCGGATCCTACCTGA) and the later sequence of I_1 (TAGCATTTTTA TCCATA). The promoter region was amplified from pES51 with two oligo nucleotides, AAGATTAGCGGATCCATCCATA**XXXX**CTTTTTATCGCAA (containing the underlined I^* *araI* half-site and the randomized nucleotides marked **X**) and ACTTAAACTAACCACTTGTG, in PCR buffer containing 50 mM KCl, 20 mM Tris-Cl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each deoxynucleoside triphosphate, 100 ng of each oligonucleotide, 1 ng of plasmid DNA as a template, and 5 U of *Taq* polymerase with 29 cycles of 94°C for 1 min, 40°C for 1 min, and 72°C for 1 min. The amplified fragment was treated with 5 μg of proteinase K/ml in 0.01 M Tris-Cl (pH 7.8), 5 mM EDTA, and 0.5% sodium dodecyl sulfate at 56°C for 30 min. The sample was extracted with an equal volume of phenol followed by ethanol precipitation, digestion with *Bam*HI and *Hin*dIII endonucleases, and electrophoresis on a 0.8% agarose gel. The doubly digested fragment was purified from the agarose gel using the Geneclean II gel extraction kit from Bio 101 and cloned into the *Bam*HI and *Hin*dIII sites of pES51 to obtain the I_1-I^*–*galK* constructs. To transfer the mutant promoter region to a *lacZ*-containing plasmid, the promoter region of each I_1-I^*–*galK* construct was cloned into the *Ase*I and *Hin*dIII cloning sites of the p7 plasmid (39).

Assays. The promoter activity of the p_{BAD} promoter variants was quantitated in *Escherichia coli* TR322 cells (16) with either β-galactosidase or galactokinase levels. The cells were grown to an optical density at 600 nm of 0.6 in M10 minimal salts, 0.4% glycerol, 10 μg of vitamin B_1 per ml, 0.4% Casamino Acids, 1 mM $MgSO_4$, and 0.2% arabinose (44), 1 ml was withdrawn, and promoter activity was assayed for β-galactosidase, as described by Miller (37), or for galactokinase (10, 35).

Construction of promoter templates for the DNA migration retardation assay. End-labeled DNA fragments were generated by PCR using two oligonucleotides such that the I_1-I^* site was centrally located on the 100-bp product. PCR was performed using 100 ng of γ-^{32}P-end-labeled oligonucleotide (ATTTGCACGG CGTCACAC) at 10^6 cpm/ng, 300 ng of unlabeled oligonucleotide (CGTTTCA CTCCATCCAAA), and 10 ng of template plasmid with 0.4 U of *Taq* polymerase in PCR buffer for 29 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min.

The I_1-I_2p_{BAD} (Fig. 2a), I_1-I_2p_{BAD} consensus −10 (Fig. 2b), and I_1-I^*p_{BAD} (Fig. 2c) variant promoter fragments used to test for sigma binding were generated by PCR. The I_1-I_2p_{BAD} bubble (Fig. 2d) was constructed by annealing two oligonucleotides. For the I_1-I_2p_{BAD} consensus −10 template, the TATAAT sequence at the −10 box was introduced into p_{BAD} by in vitro mutagenesis as described below before PCR amplification.

For the in vitro mutagenesis reaction, 50 ng of double-stranded-DNA template was mixed with 125 ng each of the two complementary oligonucleotides containing in 50 μl of 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 20 mM Tris-Cl (pH 8.0), 2 mM $MgCl_2$, 0.1% Triton X-100, and 10 μg of nuclease-free bovine serum albumin (BSA)/ml. The extension reaction was performed with 2.5 U of *Pfu* polymerase with the following cycling parameters: 95°C for 30 s and then 18 cycles of 95°C for 30 s, 55°C for 1 min, and 68°C for 12 min per cycle. The reaction generated unmethylated complementary double-stranded DNA containing the desired mutation. Ten units of *Dpn*I endonuclease was added for 1 h at 37°C to digest the original methylated DNA template present in the reaction. This is the site-directed mutagenesis technique of the QuikChange protocol of Stratagene.

End-labeled DNA templates for the in vitro DNA migration retardation assay were prepared by PCR amplification. For PCR, 100 ng of γ-^{32}P-end-labeled oligonucleotide at 10^6 cpm/ng, 300 ng of unlabeled oligonucleotide(s), and 25 ng of template plasmid containing the required promoter were mixed in 100 μl of PCR buffer. The PCR cycle parameters were 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 29 cycles. The oligonucleotides used to amplify the I_1-I_2p_{BAD}, I_1-I_2p_{BAD} consensus −10, and I_1-I^*p_{BAD} templates were ATTTGCACGGCTC ACAC and CGTTTCACTCCATCCAAA. The I_1-I_2p_{BAD} bubble DNA was prepared by hybridizing ACTTTGCTAGCCCATAGCATTTTTATCCATAAGAT TAGCGGATCCTAC**CTGACG**CTTTTTATCGCAACTCTCTAgcacttctccATA CCCGTTTTTTTGG and CCAAAAAAACGGGTATcctcttcacgTAGAGAGTT GCGGATAAAAAG**CGTCAG**GTAGGTACCGCTATCTTATGGATAAAAA TGCTATGGGCTAGCAAAGT (the underlined sequences represent the AraC half-sites I_1 and I_2, the boldface letters represent the −35 sequence, and the lowercase letters show the bubble region around the −10 region). For this I_1-I_2p_{BAD} bubble, the two oligonucleotides were mixed in equimolar concentrations in 10 mM Tris-Cl (pH 8.0), 1 mM EDTA (pH 8.0), 5 mM $MgCl_2$, and 50 mM KCl, heated for 10 min at 94°C, and cooled slowly to room temperature over the course of an hour.

Purification of the sigma subunit. The R596H mutation was introduced into the Δ133 sigma-encoding DNA template by in vitro site-directed mutagenesis (QuikChange). The hexahistidine tag-containing Δ133 and R596HΔ133 sigma variants were overexpressed, purified from inclusion bodies by using nickel columns under denaturing conditions, and renatured as described previously (9, 48).

DNA migration retardation assay. The DNA migration retardation assay was used to measure dissociation rates of AraC from mutant I_1-I^*p_{BAD} templates as previously described (17). AraC was bound to the mutant I_1-I^*p_{BAD} templates in buffer containing 10 mM Tris-Cl (pH 7.4), 1 mM K-EDTA, 75 mM or 150 mM KCl (depending on the salt concentration required), 1 mM dithiothreitol, 5% glycerol, 50 mM arabinose, and 0.05% NP-40. The higher salt concentration was used when the binding reactions were performed in the presence of arabinose because AraC binds more tightly to DNA in the presence of its ligand and does not show any significant dissociation at lower salt concentrations. For the binding

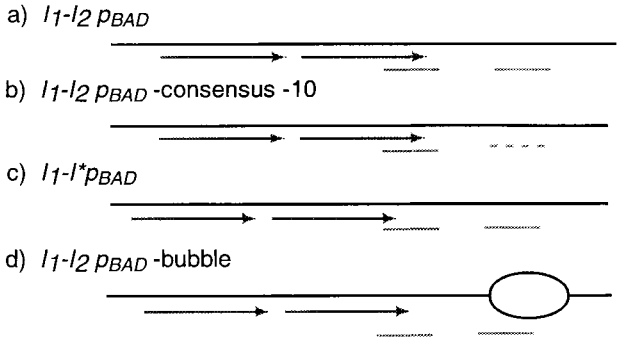

FIG. 2. DNA template variants used to test sigma binding. The two AraC binding half-sites are shown with arrows (underlined in the sequences), and the −35 and −10 hexamers for sigma are shown with solid or broken lines (boldface in the sequences). (a) I_1-I_2p_{BAD} with the wild-type *araI* half-sites for AraC and the 4-nucleotide overlap at the −35 hexamer. The sequence of the −10 hexamer was not changed. 5′GCCCATAGCATTTTTATCCATAAGATT AGCGGATCCTAC**CTGACG**CTTTTTATCGCAACTCTC**TACTGTT**TCTCC 3′. (b) I_1-I_2p_{BAD} consensus −10. The −10 hexamer in I_1-I_2p_{BAD} was changed to the consensus sequence TATAAT to generate a stronger σ^{70} binding site. 5′G CCCATAGCATTTTTATCCATAAGATTAGCGGATCCTAC**CTGACG**CTT TTTATCGCAACTCTC**TATAAT**TTCTCC3′. (c) I_1-I^*p_{BAD} containing the I^* site in place of the I_2 *araI* half site in p_{BAD}. The sequence of the nonoverlapping nucleotides in the −35 hexamer was the same as the parental 5′GACG3′. The I^* site is not as tight a binding site for AraC as I_1 but is tighter than the I_2 site. 5′GCCCATAGCATTTTTATCCATAAGATTAGCGGATCCATCCA**TAGAC GCTTTTTATCGCAACTCTC**TACTGTT**TCTCC3′. (d) I_1-I_2p_{BAD} bubble is the same as the wild-type I_1-I_2p_{BAD} with a heteroduplex mismatched stretch of DNA (lowercase letters) at the −10 region. 5′GCCCATAGCATTTTTATCCATAA GATTAGCGGATCCTAC**CTGACG**CTTTTTATCGCAACTCTC**TAgcacttctcc** ATACCCG3′.

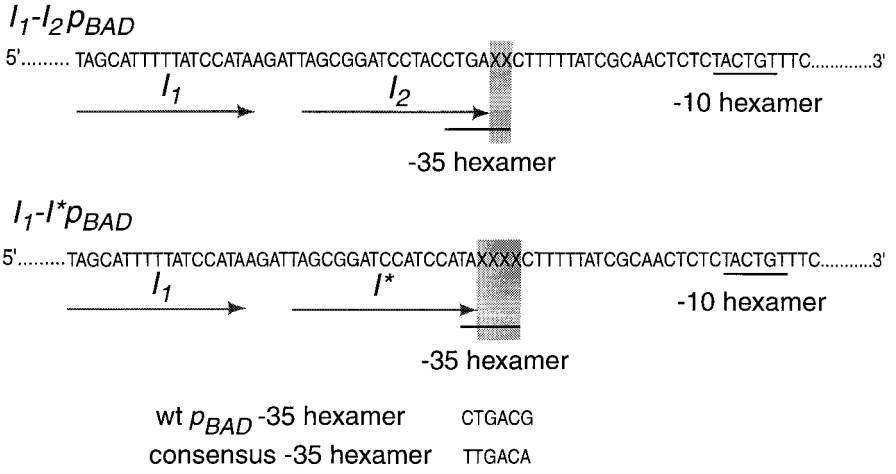

FIG. 3. Sequences of I_1-I_2p_{BAD} promoter (top) and I_1-I^*p_{BAD} promoter (bottom). Oligonucleotide-directed PCR mutagenesis was used to randomize the nonoverlapping bases (marked X and shaded) in the −35 hexamer. wt, wild type.

reaction, purified AraC was added so that just 100% of 1 ng (~10^4 cpm) of end-labeled DNA was bound. Binding of AraC to DNA was allowed to proceed for 10 min, after which an excess of a competitor containing four tandem I_1 half-sites was added. Aliquots were withdrawn at different time points and loaded onto a native 6% polyacrylamide gel cross-linked with 0.1% methylenebisacrylamide. The samples were separated by electrophoresis at 150 V for 1.5 h in 100 mM Tris-acetate (pH 7.4) and 1 mM K-EDTA. A Molecular Dynamics PhosphorImager PC was used to quantitate bound versus free DNA, and dissociation rates were determined from a plot of the DNA fraction bound by AraC as a function of dissociation time by a least-squares fit.

Sigma or variants were diluted in 10 mM Tris-Cl (pH 8.0), 10 mM KCl, 10 mM β-mercaptoethanol, 1 mM EDTA, 0.1% (vol/vol) Triton X-100, 0.4 μg of BSA/ml, and 5% glycerol. Binding reactions were performed in 25 mM Tris-acetate (pH 7.4), 14 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.03% Triton X-100, 100 μg of BSA/ml, and 5% glycerol. To look for cooperative DNA binding between AraC and sigma, sufficient AraC was added so that ~100% of 1 ng (~10^4 cpm) of γ-^{32}P-end-labeled DNA would be bound. After 10 min, sigma protein was added for 20 min before electrophoresis of the sample to separate free DNA and the various protein-bound species.

RESULTS

Do Sigma and AraC both contact the −35 region? Since the I_2 binding half-site of AraC overlaps the promoter −35 recognition region by 4 bp, it is conceivable that the sigma subunit does not contact the −35 region at all and that AraC assumes the role normally taken by part of sigma. One way to determine whether sigma makes DNA contacts in the −35 region is to vary the sequence of that part of the −35 region that is not contacted by AraC. If promoter activity is insensitive to such sequence changes, we could reasonably infer that sigma does not contact DNA in the region.

Altering the two bases of the natural *ara* p_{BAD} promoter −35 region (Fig. 3) that are not part of I_2 from CG→TT and CG→CC decreased promoter activity to 90 and 50%, respectively, of that of the parental sequence. These results suggest that sigma does read the sequence of the two bases. In the P22 *ant* promoter however, Moyle and coworkers found that C and G are equivalent at position −30 and that a C-to-T change at position −31 reduces activity to 10% (38). Most likely the difference between the modest change to 90% activity in our system and the dramatic change to 10% in the *ant* promoter results from the very different contexts in which the sequence changes occur. In the *ara* system, AraC and CRP are required for normal activation of RNA polymerase, whereas in the *ant* system, no auxiliary activators are needed by RNA polymerase.

To increase the number of bases in the −35 region that are not contained within the promoter-proximal AraC binding site, we designed a promoter variant, I_1-I^*p_{BAD} (Fig. 3), in which the AraC binding site has been moved upstream by 2 bases. The I^* site contains the beginning sequence of the I_2 site and the later sequence of the I_1 site. This promoter is still AraC dependent and is 2.3 times as active as the wild-type p_{BAD} promoter. Making such a change in the promoter permits four bases in the −35 region to be altered without affecting AraC binding. We chose to alter these four nucleotides in two ways—by directed and by random mutagenesis. If, despite its requirement for AraC and CRP for full stimulation, and despite the results obtained from the 2-base overlap, p_{BAD} possesses the same promoter sequence dependence as "bare" promoters like P22 *ant*, then a change to taGACA should have a particularly dramatic stimulatory effect because of increased homology to the consensus −35 hexamer. Table 1 shows that the activity of taGACA was not significantly different from that of the parental sequence, taGACG. The activities of most of the entries shown in Table 1 that resulted from random mutagenesis, however, were strongly dependent upon the sequence of the −35 region outside the I^* half-site, thereby indicating that sigma does contact the four bases.

Two potential factors could invalidate the conclusion that I_1-I^*p_{BAD} activity is dependent upon the identity of the −35 region nucleotides outside the I^* half-site. First, introduction of the altered nucleotides might have inadvertently altered nucleotides elsewhere in the plasmid as well, for example, within the β-galactosidase gene. To verify that the decreased levels of β-galactosidase activity we observed with some of the variants were indeed due to changes in the −35 region of the promoter and not due to extraneous mutations elsewhere on the plasmids, we changed the four randomized bases in three mutant templates back to the parental sequence by oligonucleotide-directed site-specific mutagenesis. These changes returned the β-galactosidase levels to those observed for the parental sequence, indicating that the plasmid carried no additional relevant mutations on the mutant templates.

A second possibility is that AraC binding actually is sensitive to DNA sequence outside I_1 and I^*. This possibility was excluded by measurement of the dissociation rates of AraC from the I_1-I^*p_{BAD} templates using the in vitro DNA migration retardation assay (typical data is shown in Fig. 4). Identical dissociation rates were obtained for AraC from all the I_1-I^*p_{BAD} templates (Table 1), indicating that the reduction in promoter activity from these templates was unlikely to be due

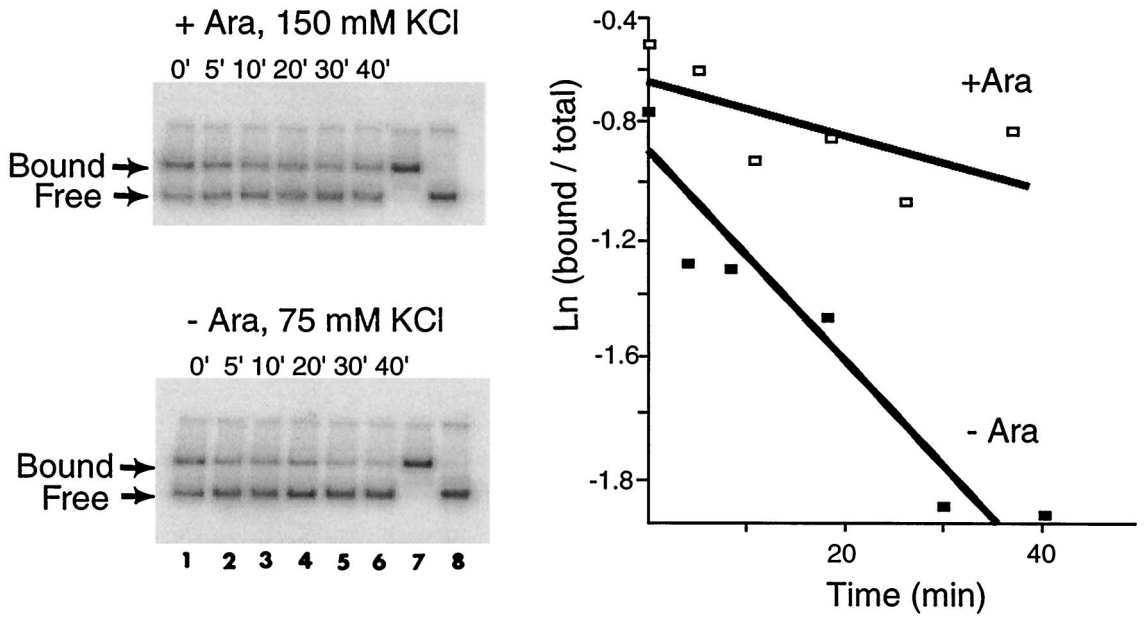

FIG. 4. In vitro DNA migration retardation assays to measure dissociation rates of AraC from the I_1-I^*p_{BAD} templates. The two gels show the bound and free DNA at each time point (lanes 1 to 6) as well as fully bound DNA (lane 7) and free DNA (lane 8). The ratio of bound to total DNA was quantitated and plotted as a function of time. + Ara, with arabinose; − Ara, without arabinose.

to altered AraC binding at the −35 region. The results suggest that altered sigma binding is the cause of the reduction.

Does sigma directly interact with AraC? On one hand, the partial interdigitation of the AraC and RNA polymerase sigma subunit binding sites on DNA suggests that the two proteins could be located very close to each other and hence might have critical interactions with one another. On the other hand, the fact that the binding site of AraC can be moved 2 bases upstream without strongly affecting promoter activity, as in I_1-I_1p_{BAD} (39) and I_1-I^*p_{BAD}, suggests that perhaps AraC and sigma do not make specific contacts with each other. To test if AraC and sigma do interact with one another, we looked for cooperativity in their binding to DNA.

TABLE 1. Properties of the I_1-I^* variant promoters

−35 sequence	Relative promoter activity[a]	AraC dissociation half-time (min)[b]	
		−Ara	+Ara
Parental taGACG	1.00	25	110
taGACA	1.00	22	110
taGCCT	0.35	25	
taTGAT	0.30	26	
taTGGT	0.30	25	110
taGGTT	0.20	26	
taCAAC	0.20		
taCAAT	0.20	22	110
taATTA	0.20	23	
taTGTC	<0.05	28	
taTTTG	<0.05	28	110
taATTG	<0.05	26	
taTTGT	<0.05	26	100
taAGGT	<0.05	28	
taAAGT	<0.05	28	
taATTT	<0.05	26	
taTTCT	<0.05	25	

[a] Relative in vivo promoter activities were quantitated from β-galactosidase assays performed in the presence of arabinose on exponentially growing TR322 cells containing the mutant promoters in the p7 plasmid. The parental I_1-I^*p_{BAD} promoter activity of 16,000 U was assigned a relative value of 1.

[b] The dissociation half-times of AraC from mutant I_1-I^*p_{BAD} DNA templates in 75 mM KCl (no arabinose [−Ara]) and 150 mM KCl (plus arabinose) were measured using the DNA migration retardation assay as described in Materials and Methods and in the legend to Fig. 4.

Purified sigma factor does not detectably bind to promoters by itself, but truncation of its acidic N-terminal domain reveals a weak promoter binding specificity (6, 7, 48). Therefore in looking for cooperativity between AraC and sigma factor in binding at AraC-activated promoters, we used a sigma variant with its N-terminal 133 amino acids deleted. The binding of sigma was examined on I_1-I_2p_{BAD} and parental I_1-I^*p_{BAD} templates, but no binding was observed on either template in the presence or absence of bound AraC protein (see Materials and Methods for a description of the DNA templates). Changing the conserved arginine at position 596 to a histidine in the sigma subunit enables RNA polymerase to be active on p_{BAD} in the absence of CRP (19). Possibly the increased activation results from a sigma-AraC interaction, either an interaction where none existed before or a stronger interaction. Therefore, we introduced the R596H mutation into the Δ133 sigma variant. We were still unable to observe sigma binding to either the I_1-I_2p_{BAD} or parental I_1-I^*p_{BAD} DNA in the presence or absence of AraC. To create a stronger sigma binding site on p_{BAD}, we changed the −10 hexamer to the consensus −10 sequence (see Materials and Methods), but still no binding was observed for either the Δ133 or the R596HΔ133 sigma variant on I_1-I_2p_{BAD} consensus −10.

In a further effort to increase sigma binding, we used a template that mimics the DNA present in the open complex (RP_o) during transcription initiation (6, 8). Such a bubble sequence provides a significant advantage for sigma binding, as shown by the preference of RNA polymerase holoenzyme for binding to premelted sequences (5). We used a heteroduplex mismatch bubble-containing template, I_1-I_2p_{BAD} bubble, that contained the AraC binding sites, I_1 and I_2, with a mismatch region spanning the −10 region (see Materials and Methods).

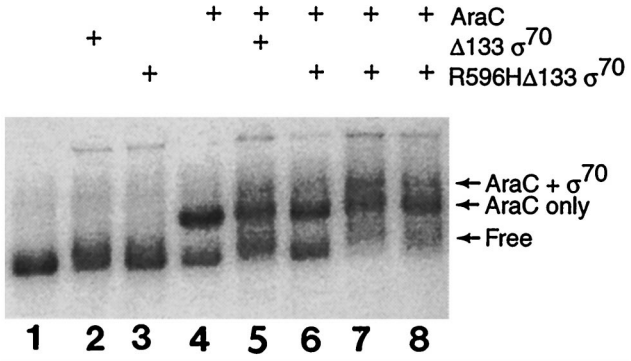

FIG. 5. DNA migration retardation assay showing weak cooperativity in the binding of AraC and σ^{70} to I_1-I_2p_{BAD} bubble DNA. The ingredients present (+) are indicated, and except in lanes 7 and 8, where the concentrations of R596HΔ133 σ^{70} were 190 nM and 130 nM, respectively, the concentrations used were 420 pM AraC, 60 nM Δ133 σ^{70}, and 380 nM R596HΔ133 σ^{70}.

With such DNA, we observed some AraC-dependent DNA binding by the Δ133 and R596HΔ133 sigma variants (Fig. 5). Using another bubble template with binding sites for AraC and the consensus −10 region on the nontemplate strand of the bubble (34) did not enhance binding by sigma in the presence or absence of AraC. We note that the AraC-dependent binding by the truncated sigma protein in all these experiments was not completely reliable, and occasional experiments failed to demonstrate any cooperativity in the binding of AraC and sigma to DNA.

DISCUSSION

Our experiments yield the following conclusions: AraC and the sigma subunit of RNA polymerase both make contacts with DNA in the −35 region of the p_{BAD} promoter, and interactions between AraC and a truncated form of sigma can be observed in their binding in vitro to DNA, but these interactions are not strong.

The −35 hexamer of p_{BAD} shares homology of four bases with the consensus −35 sequence, making it a potentially tight binding site for the sigma subunit of RNA polymerase (Fig. 3). On the other hand, because the polymerase-proximal half-site of AraC overlaps the −35 region by 4 bp, it is possible that AraC substitutes for the role taken by the domain of sigma that normally contacts the −35 region. In the experiments reported here, we found that p_{BAD} activity is strongly dependent on the identities of the bases of the −35 hexamer that are not contacted by AraC. We presume, then, that these bases are contacted by sigma. Consequently, we further presume that either the remaining bases of the −35 region are also contacted by the sigma subunit or sigma is displaced and reads the bases immediately adjacent to the AraC binding site.

It is possible that AraC and the sigma subunit sequentially contact the common four bases in their partially overlapping binding sites at p_{BAD}. We note, however, that simultaneous contact of these bases by alpha helices is also geometrically possible (Fig. 6). Our detection of cooperativity, albeit weak, in the binding of sigma and AraC to the DNA indicates a direct interaction between the two, suggesting simultaneous DNA binding. We must add that our in vitro binding studies utilized AraC and the sigma subunit alone but that the normal binding involves AraC and the RNA polymerase holoenzyme. The additional subunits of RNA polymerase could also interact with AraC or alter the structure of sigma and alter the interaction.

We first used a truncated variant of sigma (Δ133) that lacked the N-terminal acidic domain to enhance the weak DNA binding affinity of sigma in the absence of core polymerase. Because we observed no binding by the truncated sigma factor and no binding cooperativity between AraC and sigma, we then tried DNA templates that should have higher affinity than double-stranded DNA. Ultimately, we did observe binding cooperativity between AraC and truncated sigma, but this required the use of a DNA template possessing a single-stranded region in the −10 region. We are aware of only one other experiment to examine by direct biochemical means an interaction between an upstream activator and sigma factor (27). This work used the Ada protein, double-stranded DNA, and intact sigma factor. As significant binding cooperativity was observed with the Ada and sigma proteins at the *ada* and *aidB* promoters, it is possible that the Ada protein interacts significantly more strongly with sigma than does AraC. Alternatively, it is possible that in removing the N-terminal portion of sigma to enhance its DNA binding abilities, we also removed important regions for the AraC-sigma protein interaction.

Promoter recognition at p_{BAD} by sigma is intriguing because,

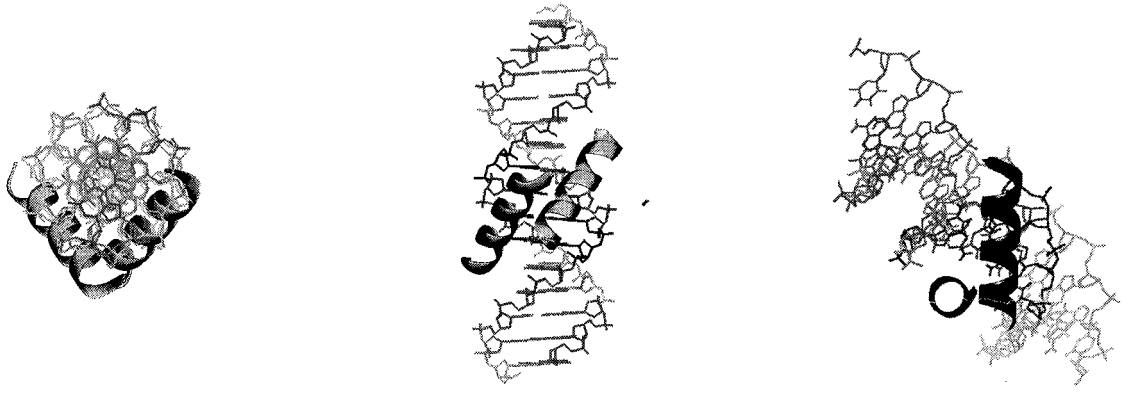

FIG. 6. Model showing simultaneous recognition of four overlapping bases by two α-helices fitted into the major groove visualized from the top (left), front (middle), and side looking down one DNA major groove directly at one α-helix (right).

while any deviation from the parental sequence causes a reduction in promoter activity, we could see no correlation between specific sequence changes and promoter activity. Similarly, at the *pmelR* promoter, positions 3 to 6 of the −35 region lie outside the CRP binding site and play an important role in activation by sigma. Some −35 hexamer sequences at *pmelR* are more tolerant of substitutions than others, and mutations that change nonconsensus bases to consensus do not necessarily increase promoter activity (40). Similar observations have been noted with the *melAB* promoter (24) and the P22 *ant* promoter (38).

ACKNOWLEDGMENTS

We thank Alicia Dombroski for helpful advice and for providing the Δ133 sigma template and the purification protocol and members of our laboratory for ongoing discussions.

This work was supported by NIH grant GM18277 to R.S.

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