

Purification and Properties of RhaR, the Positive Regulator of the L-Rhamnose Operons of *Escherichia coli*

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The product of the *rhaR* gene, which regulates the level of mRNA produced from the four L-rhamnose-inducible promoters of the rhamnose operon, has been hypersynthesized and purified by a two-column procedure. The purified protein is a 33 kDa DNA-binding protein that binds to an inverted repeat structure located within the p_{sr} promoter, the promoter for the *rhaS* and *rhaR* genes. The equilibrium binding constants and kinetic constants have been determined under a variety of solution conditions. The protein binds with high affinity and its binding is sensitive to salt concentration and the presence of L-rhamnose. The nucleotides and phosphate residues contacted by RhaR were identified by chemical interference assays. All of the contacts are made to one face of the DNA and the symmetrical pattern matches the inverted repeat sequence proposed for the binding site. An unusual property of the binding site is that the two half-sites of the inverted repeat are separated from one another by 17 base-pairs of uncontacted DNA. Significant binding is retained if the 17 base-pairs are extended by insertions of integral turns of DNA, but not by half-integral turns. The complex of RhaR–DNA appears to be sharply bent, approximately 160°.

1. Introduction

The structural genes required for the metabolism of L-rhamnose are encoded by a cluster of genes at map position 87·7 on the *Escherichia coli* chromosome (Power, 1967; Bachmann, 1983). We have previously cloned the operon and partially sequenced the operon and shown that there are four L-rhamnose-inducible mRNAs, originating from promoters p_{sr} , p_1 , p_2 and p_3 (Tobin & Schleif, 1987). The p_{sr} promoter is the promoter for the regulatory genes *rhaS* and *rhaR*. The other three are apparently the promoters for the structural genes (Tobin & Schleif, 1987). The *rhaR* and *rhaS* genes are responsible for the regulation of the L-rhamnose operon. S_1 nuclease mapping data demonstrated that these two gene products regulate the level of mRNA produced from the four L-rhamnose-inducible promoters (Tobin & Schleif, 1987).

Here, we demonstrate that the *rhaR* gene encodes

a DNA-binding protein that binds specifically to the inverted repeat sequence found upstream from the RNA polymerase binding site at the p_{sr} promoter. The overproduction and purification of RhaR is described, and some of the physical properties of the protein. Measurement of the equilibrium binding constant in the presence and absence of L-rhamnose reveals that the presence of the sugar increases the affinity of the protein for the p_{sr} binding site. In addition, the salt dependence of the equilibrium binding constant shows that a net of four monovalent salt ions are released from the DNA upon binding.

Using chemical interference assays, we have identified the nucleotides and phosphate residues contacted by RhaR. The symmetrical pattern of the contacts matches well with an inverted repeat sequence there, but an unusual property of the binding site is that a gap of 17 base-pairs exists between the closest edges of the half sites of the inverted repeat. We present data that show that RhaR makes specific contacts to the half sites, while looping out the 17 base-pairs of intervening spacer DNA to form a DNA microloop.

No DNA binding activity was found associated with RhaS, and its role in transcriptional regulation of the operon remains unclear.

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2. Materials and Methods

(a) General methods

DNA manipulation, growth of cells and other general methods were performed as described by Schleif & Wensink (1981) and Maniatis *et al.* (1982).

Plasmid pJT20 was constructed by digesting plasmid pJTC9 (Tobin & Schleif, 1987) with *EcoRI* and *PvuII*, isolating the 500 base-pair fragment containing the p_{sr}

promoter, and ligating it (Maniatis *et al.*, 1982) into the *EcoRI*-*SmaI* sites of the vector pUC19 (Yanisch-Perron *et al.*, 1985). Plasmid pJT25 constructed by isolating the 500 base-pair *EcoRI*-*BamHI* fragment from pJT20 and ligating it into the corresponding sites in pES27 (*SspI*-*HindIII*; Tobin, 1989). Plasmid pJT28, containing an additional 11 base-pairs between the half sites, was constructed by annealing the following oligonucleotides and ligating them into the *BamHI* site of pUC19.

5'GATCACGCTGTATCTTGAAAAATCGACGTTTTT3'
3'TGCGACATAGAACTTTTGTAGCTGCAAAAAATGC5'

NheI

5'TACGGCTAGCCTACGTGGTTTTCCGTGCGAAAAATTTAAGGTAAGA 3'
3'CGATCGGATGCACCAAAAGGCAGCTTTTAAATTCCATTCTCTAG5'

Plasmid pJT26, containing an additional 15 base-pairs of spacer DNA, was constructed by digesting pJT28 with *NheI*, filling in the 5' recessed ends with DNA polymerase I (Klenow fragment) and religating the plasmid. The +33 base-pair construct, plasmid pJT27, was obtained acci-

dentally. We tried originally to obtain a +22 base-pair construct by ligating an 11 base-pair linker into the *NheI* site of pJT28, but we obtained a +33 base-pair insert. The sequence of the DNA is:

5'GATCACGCTGTATCTTGAAAAATCGACGTTTTTTTACGGCTAGCTAGCCTACGGTCAGT
3'CTAGTGCACATAGAACTTTTGTAGCTGCAAAAAATGCCGATCGATCGGATGCCAGTCA

5'CGAGGCCTAGGCCTCACCTAGCCTACGTGGTTTTCCGTGCGAAAAATTTAAGGTAAGAGA
3'GCTCCGGATCCGGAGTGGATCGGATGCACCAAAAGGCAGCTTTTAAATTCCATTCTCT

All DNA fragments were end-labeled with phage T4 polynucleotide kinase. The fragments used in the gel shift assays were the 461 base-pair *HindIII* to *MluI* and the 161 base-pair *EcoRI* to *MluI* fragments from plasmid pJTC8 (Tobin & Schleif, 1987), or the 132, 136 and 154 base-pair *HindIII* to *EcoRI* fragments from pJT26, pJT27 and pJT28, respectively. The 74 base-pair *HindIII* to *NheI* fragment and the 65 base-pair *NheI* to *EcoRI* fragment from pJT28 were used to determine K_{app} for the individual half sites.

Protein was measured by the method of Bradford (1976) using bovine serum albumin as a standard.

SDS/polyacrylamide gel electrophoresis was performed

as described by Laemmli (1970) using 12% (w/v) polyacrylamide slab gels (15.0 cm × 17.0 cm × 0.4 cm).

(b) Overproduction of the *RhaS* and *RhaR* proteins

Two overproducing plasmids were constructed. Both the *rhaS* and *rhaR* genes were fused to the strong bacterial promoter *tac* (DeBoer *et al.*, 1983a,b) to produce plasmid pJTC105, shown in Fig. 1(a). Briefly, the *PstI*-*PvuII* fragment of pTAC12 (Amann *et al.*, 1983), the *PstI*-*MluI* fragment, containing the *rhaS* and *rhaR* genes, of pJTC8 and a 48 base-pair linker, containing complementary ends,

5'TATATGACCGTATTACATAGTGTGGATTTTTTTCCGTCTGGTAA3'
3'ATATACTGGCATAATGTATCACACCTAAAAAAGGCAGACCATTGC5'

were ligated to produce plasmid pJTC105. The insertion of the linker permitted the initiation codon of the *rhaS* gene to be placed 8 base-pairs from the strong ribosome binding site, the optimal spacing required for efficient

translation. Fig. 1(b) outlines the steps used to fuse the *rhaR* gene downstream from the *tac* promoter. A 94 base-pair linker (made by ligating 4 oligonucleotides together)

5'TATATGGCTTTCTGCAATAACGCGAATCTTCTCAACGTATTTGTAC
3'ATATACCGAAAGACGTTATTGCGCTTAGAAGAGTTGCATAAA5'
5GCCATATTGCGAATAATCAACTTCGTTCTCTGGCCGAGGTAGCCACGG
3'CATGCGGTATAACGCTTATTAGTTGAAGCAAGAGACCGGCTCCATCGT

containing a blunt end and a *BglI* complementary end was ligated to the *BglI*-*XhoI* *rhaR*-containing fragment of pJTC8. Next, the *XhoI* sticky end was made blunt by treatment with the Klenow fragment of DNA polymerase I in the presence of the appropriate deoxynucleotides.

Finally, this blunt-ended fragment was ligated into the *PvuII* site of pTAC12 to produce plasmid pJTC114. The linker permitted the initiation codon of the *rhaR* gene to be spaced 8 base-pairs from the ribosome binding site of pTAC12.

(c) *Lysate preparation*

Strains containing the appropriate overproducing plasmids were grown in YT broth (Schleif & Wensink, 1981) to an absorbance of 0.6 at 550 nm, at which time isopropyl- β -D-thiogalactoside was added to a final concentration of 1.0 mM. After incubation for 2 h at 37°C, 5.0 ml of cells were pelleted by centrifugation and resuspended in 0.5 ml of lysate buffer (0.1 M-potassium phosphate (pH 7.4), 50 mM-KCl, 1 mM-EDTA, 10% (v/v) glycerol, 1 mM-dithioerythritol, 160 μ g phenylmethylsulfonyl-fluoride/ml). The cells were lysed by sonication (3 \times 6 s pulses with a microprobe, 60% lysis) and the cell debris removed by centrifugation at 4°C.

(d) *Gel shift assay*

Specific binding of the RhaR protein to DNA was detected using a gel shift assay initially described by Fried & Crothers (1981) and Garner & Revzin (1981), and later modified by Hendrickson & Schleif (1984). Polyacrylamide gels (6% polyacrylamide to bis-acrylamide ratio, 60:1, w/w) were soaked in running buffer (10 mM-Tris-acetate (pH 7.4), 1 mM-EDTA) for 2 h and then prerun at 10 V/cm for 15 min prior to use.

The buffer used in the binding reactions was 10 mM-Tris-acetate (pH 7.4), 1 mM-EDTA, 5% glycerol, 50 μ g bovine serum albumin/ml, 1 mM-dithioerythritol, 0.05% (v/v) Nonidet P40, 50 mM-KCl, 50 mM-L-rhamnose. We used a high concentration of L-rhamnose in the binding buffer since, in the analogous *ara* system, K_m for L-arabinose binding to AraC is 5×10^{-3} M (Wilcox, 1974). Protein was added to 20 μ l of binding buffer containing radioactive DNA and the reaction incubated at 37°C for the appropriate amount of time. Sonicated calf thymus DNA (25 μ g/ml: 4×10^{-8} M of 1000 base-pair fragments) was included in each reaction as a non-specific DNA competitor. With the gel running, the sample was loaded and electrophoresed for 1 to 1.5 h at 10 V/cm.

To elute protein from a RhaR-DNA complex, a gel shift assay was performed by incubating 1.5 μ g of p_{sr} DNA with 4.8×10^{-13} mol of RhaR in 30 μ l of binding buffer. The RhaR-DNA complexes were separated from free DNA by electrophoresis in a 6% polyacrylamide gel. Gel slices containing the samples were cut out, soaked in 1% (w/v) SDS, placed in the wells of an SDS/12% polyacrylamide gel and electrophoresed.

(e) *Purification of the RhaR protein*

Strain JM101 was transformed with plasmid pJTC105 or pJTC114. Cells (30 l) were grown to an absorbance of 0.7 at 600 nm in yeast tryptone medium containing 50 μ g ampicillin/ml. To induce synthesis of the RhaS and RhaR proteins, isopropyl- β -D-thiogalactoside was added to a final concentration of 0.1 mM, and the cells were incubated for an additional 2 h. The cells were harvested (180 g) by centrifugation and the cell pellet stored at -70°C.

A RhaR-specific DNA affinity column was prepared as described by Kadonaga & Tjian (1986). Briefly, a sticky-ended 64 base-pair oligonucleotide, containing the RhaR binding site, was ligated to produce oligomers of the binding site ranging from 3-mers to 75-mers. This mixture was linked to cyanogen bromide-activated Sepharose 4B under conditions such that each oligomer was linked only once to the column.

(i) *Step 1. Lysate preparation*

Frozen cells (40 g) were thawed at 37°C and resuspended in 150 ml of phosphate buffer (10 mM-sodium

phosphate (pH 7.8), 2 mM-EDTA, 10% glycerol, 0.1 mM-dithioerythritol, 200 mM-NaCl, 50 mM-L-rhamnose) containing 0.16 mg phenylmethylsulfonylfluoride/ml. All the subsequent steps of the purification were performed at 4°C. Cells were lysed by adding lysozyme to a final concentration of 0.4 mg/ml for 30 min. The lysed cells were centrifuged at 10,000 g for 40 min. This pellet, containing the RhaR protein, was resuspended in 200 ml of phosphate buffer, sodium deoxycholate was added to 0.05% (w/v), and blended at high speed for 2 to 3 min in a Waring Blender to shear the DNA. Solid NaCl and MgCl₂ were added to final concentrations of 4.0 M and 0.1 M, respectively (64 g of NaCl and 6.0 g of MgCl₂ to 240 g of extract). The mixture was stirred for 3 to 4 h and then centrifuged at 10,000 g for 40 min. Nucleic acid was removed from the resulting supernatant (280 ml) by a polyethylene glycol/Dextran phase partition (Schleif & Wensink, 1981). Briefly, 1/3 weight of 6.4% (w/v) Dextran, 25.6% polyethylene glycol 8000, and 4.0 M-NaCl was added to 1 weight of the extract. The mixture was stirred for 30 min at 4°C, followed by centrifugation at 5000 g for 15 min. The supernatant was removed and dialyzed overnight against 1.5 l of phosphate buffer containing 50 mM-NaCl to reduce the salt concentration to 50 mM-NaCl (buffer changed 4 times). This supernatant will be referred to as the high-salt extract.

(ii) *Step 2. Cellulose-phosphate chromatography*

Cellulose-phosphate (50 ml: Whatman), equilibrated with phosphate buffer containing 50 mM-NaCl, was added to the high-salt extract and gently stirred for 30 min. The suspension was filtered and the resin washed with 200 ml of phosphate buffer containing 50 mM-NaCl. The resin was resuspended in a small volume of phosphate buffer and packed into a 2.5 cm \times 25 cm column. The column was eluted with a linear 50 mM to 500 mM NaCl gradient in phosphate buffer (300 ml, flow-rate 0.5 ml/min).

(iii) *Step 3. DNA affinity chromatography*

The pooled fractions (12 to 14, approx. 40 ml) from the cellulose-phosphate chromatography were dialyzed against phosphate buffer containing 100 mM-NaCl to reduce the salt concentration to 100 mM-NaCl, calf thymus DNA was added to 20 μ g/ml and incubated for 10 min. The protein-DNA mixture was passed through 10.0 ml of affinity resin at a flow-rate of 0.5 ml/min. The flow-through fraction contained 95% of the protein loaded onto the column and less than 1% of the RhaR activity. The column was washed with phosphate buffer containing 0.3 M-NaCl (30 ml, 0.5 ml/min) and eluted with a linear 0.3 M to 1.0 M-NaCl gradient in phosphate buffer (50 ml/min). The pooled fractions (5 and 6) were diluted with phosphate buffer to lower the concentration of NaCl to 0.1 M and reappplied to a 1.0 ml column containing the same affinity resin. The column was washed with 4.0 ml of phosphate buffer containing 0.1 M-NaCl; 1.2 ml of phosphate buffer containing 1.0 M-NaCl was added to the column, mixed thoroughly, left for 10 min and the protein was eluted. The protein solution was diluted with phosphate buffer to reduce the salt concentration to 50 mM-NaCl and the protein concentrated by reducing the volume to 200 μ l using a Centri-prep 10 concentrator (Amicon).

(f) *Measurement of equilibrium and kinetic constants*(i) *Measurement of association rates*

RhaR protein was mixed with radioactive DNA fragment and allowed to bind for varying amounts of time.

The reaction was stopped by the addition of a large excess of competitor DNA and immediately loaded onto a 6% polyacrylamide gel. The association rate constant was calculated from the half time required for complex formation under conditions where dissociation of RhaR-DNA complexes was negligible (Kim *et al.*, 1987).

(ii) *Measurement of dissociation rates*

Radioactive RhaR-DNA complexes were formed and dissociation measurements were initiated by adding a large excess of competitor DNA (enough to bind any uncomplexed protein). The samples were loaded onto a 6% polyacrylamide gel at various times and the dissociation rate constant was determined from the half time of dissociation.

(iii) *Measurement of equilibrium constants*

RhaR protein was mixed with radioactive DNA and incubated until the system reached equilibrium (generally 5 times longer than the dissociation half time). An excess of competitor DNA was added, and the samples immediately loaded onto a 6% polyacrylamide gel. The apparent equilibrium constant K_{app} is the concentration of RhaR required to give 50% free DNA and 50% complexed DNA.

(g) *Contact experiments*

(i) *DNA preparation*

The RhaR binding site is contained on a 207 base-pair *Bst*NI-*Mlu*I fragment in plasmid pJT20. The top strand was end-labeled by first digesting with *Bst*NI followed by *Mlu*I digestion. The fragment was isolated by polyacrylamide gel electrophoresis and gel electroelution. The bottom strand was labeled at the *Mlu*I site.

(ii) *DNA premodification*

The N-7 position of guanine nucleotides in the major groove and the N-3 position of adenine nucleotides in the minor groove were methylated using dimethyl sulfate (Maxam & Gilbert, 1980). End-labeled DNA was resuspended in 50 mM-sodium cacodylate buffer (pH 8.0). Dimethyl sulfate (1 μ l) was added and allowed to react with the DNA for 1 min at 21°C. The reaction was stopped by addition of 50 μ l of 1 M-Tris·HCl (pH 7.5), 1 M-2-mercaptoethanol, 1.5 M-sodium acetate, 1 mM-EDTA. Phosphate groups were ethylated by reaction with ethylnitrosourea (Hendrickson & Schleif, 1984). The reaction was allowed to proceed for 30 min at 50°C before being stopped by precipitation with ethanol. The modified DNA was precipitated with ethanol and resuspended in binding buffer.

To perform missing contact experiments, the DNA was dephosphorylated by reaction with formate; cytosine and thymine nucleotides were removed by reaction with hydrazine (Brunelle & Schleif, 1987).

(iii) *Binding reaction*

Premodified end-labeled DNA (2.0×10^5 cts/min (3×10^{-9} M)) was added to 20 μ l of binding buffer (10 mM-Tris-acetate (pH 7.4), 50 mM-KCl, 1 mM-EDTA, 1 mM-dithioerythritol, 50 mM-L-rhamnose, 5% glycerol, 0.5% Nonidet P40, 50 μ g bovine serum albumin/ml, 4×10^{-8} M-calf thymus DNA). RhaR protein was added (enough to completely bind all of the modified DNA), and allowed to bind for 15 min at 37°C. A large excess of non-radioactive competitor DNA was added and dissociation allowed to proceed for about 15 min, 0.2 of the half time required for dissociation from unmodified DNA.

(iv) *Electrophoresis and DNA isolation*

After partial dissociation, the DNA was loaded onto a 6% polyacrylamide gel and electrophoresed for 1.5 h. Following autoradiography, the DNA in the bound and free bands was isolated by cutting out the gel slices and electroeluting the DNA (Brunelle & Schleif, 1987). The DNA was precipitated with ethanol, cleaved at the modified positions, and displayed on a 6% sequencing gel (Sanger & Coulson, 1978). The DNAs modified using the G+A and C+T reactions were cleaved by reaction with piperidine (Maxam & Gilbert, 1980). DNAs treated with dimethyl sulfate or ethylnitrosourea were cleaved with sodium hydroxide (Hendrickson & Schleif, 1984).

(h) *DNA bending assay*

The DNA bending assay was performed as described by Wu & Crothers (1984) with the following modifications. The 787 base-pair *Hind*III fragment of pJT25 was treated with calf intestinal phosphatase and end-labeled with [γ - 32 P]ATP (Maniatis *et al.*, 1982). The radioactive fragment was ligated under dilute conditions (Kramer *et al.*, 1988) to produce mainly circular products. The minicircle was digested with *Hind*III, *Eco*RI, *Mlu*I, or *Bam*HI to produce a set of DNA fragments of the same size containing the RhaR binding site at different positions within the fragment. The radioactive fragments were used in gel shift assays as described above.

3. Results

(a) *Overproduction of the RhaS and RhaR proteins*

In vivo dimethyl sulfate footprinting and DNase I footprinting, performed with crude extracts, indicated that RhaR, but not RhaS, was a DNA-binding protein capable of binding to a DNA binding site located within the p_{sr} promoter (Tobin & Schleif, 1987). To purify sizable quantities of the *rhaS* and *rhaR* proteins for *in vitro* studies, we overproduced both proteins. The strategy for overproducing the proteins was twofold. First, the weak p_{sr} promoter was replaced with the strong *tac* promoter (DeBoer *et al.*, 1983). The second part of the strategy was to increase the translation of the *rhaSR* or *rhaR* mRNA by introducing a good ribosome binding site (DeBoer *et al.*, 1983; Guarente *et al.*, 1980). With appropriate DNA linkers, the Shine-Dalgarno sequence, the initiation codon and the spacing between the two were set to a sequence likely to provide a high translation efficiency (Fig. 1). The RhaS protein was overproduced to a level of 5% and RhaR to a level of approximately 1% of the total cellular protein as assayed by SDS/polyacrylamide gel electrophoresis (data not shown). The level of RhaR expression could not be increased further since, even at 1%, the protein was lethal to the cells.

(b) *Purification of the RhaR protein*

We used the gel shift assay (Hendrickson & Schleif, 1984) to detect specific binding of RhaR to its DNA binding site within the p_{sr} promoter. We prepared extracts from cell lines that expressed either the *rhaS* gene, the *rhaR* gene, or both and

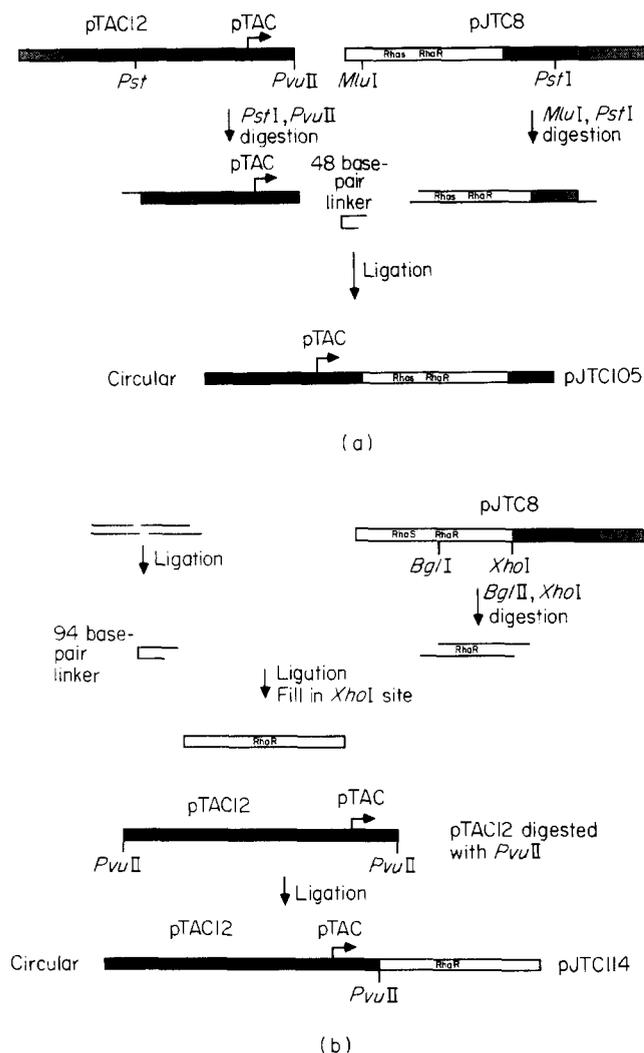


Figure 1. (a) Construction of the RhaS and RhaR overproducing plasmids, pJTC105. (b) Construction of the RhaR overproducing plasmid, pJTC114.

used them in a gel shift assay. The binding reactions contained a radioactive 161 base-pair p_{sr} fragment and large molar excess of non-specific competitor DNA. Only extracts that contained the RhaR protein showed specific band shift. Addition of a large excess of non-radioactive p_{sr} DNA completely

abolished binding. The insertion of a termination codon at position 219 of the RhaR gene also abolished DNA binding activity. Additionally, this mutant truncated protein has a *trans* dominant negative phenotype (Tobin, 1989) indicating that a fragment of the protein is produced and can oligomerize with intact subunits of RhaR encoded by the chromosomal copy of the gene but cannot bind to DNA. No RhaS-specific binding could be detected (data not shown; see Tobin, 1989).

We used the gel shift assay to develop a simple two-column procedure to purify RhaR protein from crude cell extracts. The results of the purification are shown in Table 1. Despite the fact that the RhaR protein represents 1% of the total cellular protein, it is only 0.2% of the protein in the crude high-salt cell extract. Therefore, the total yield is small, 0.1 mg RhaR per 40 g of *Escherichia coli*. These quantities were, however, sufficient for the experiments described below, due to the high affinity of RhaR for its binding site. RhaS was not purified, since an assay for its activity was not available.

The purification exploits the DNA-binding properties of the RhaR protein. The first step is elution of the protein from a cellulose-phosphate column. An elution profile of the 25% of the total protein that adsorbed to the column is shown in Figure 2(a). The bulk of the activity is eluted in a discrete peak. These fractions were pooled and applied to a RhaR-specific DNA affinity column. An elution profile of the 5% of the applied protein that bound to this column is shown in Figure 2(b). The fractions containing the activity were pooled, re-applied to the affinity column and re-eluted. The salt concentration was reduced by dilution and the protein was concentrated by filtration.

An SDS/polyacrylamide gel, Figure 3, shows that the purified preparation contains two proteins. One protein migrates at an apparent molecular weight of 33 kDa and represents 70% of the total protein. The other migrates at greater than 100 kDa and represents 30% of the protein.

We confirmed that the 33 kDa protein and not the 100 kDa protein was the RhaR protein by performing a gel shift assay and eluting protein from the protein-DNA complex. Displaying the

Table 1
Purification of RhaR

Fractionation step	Total activity (units†)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
High-salt extract	2.2×10^6	810	2778	100	---
Cellulose-phosphate chromatography	5.5×10^5	15	36666	24	13
DNA affinity chromatography I	2.5×10^5	0.22	1.1×10^6	11	410
DNA affinity chromatography II	2.0×10^5	0.09	2.2×10^6	9	800

† One unit of activity is defined as the amount of extract required to bind 1.2×10^{-14} mol of p_{sr} DNA.

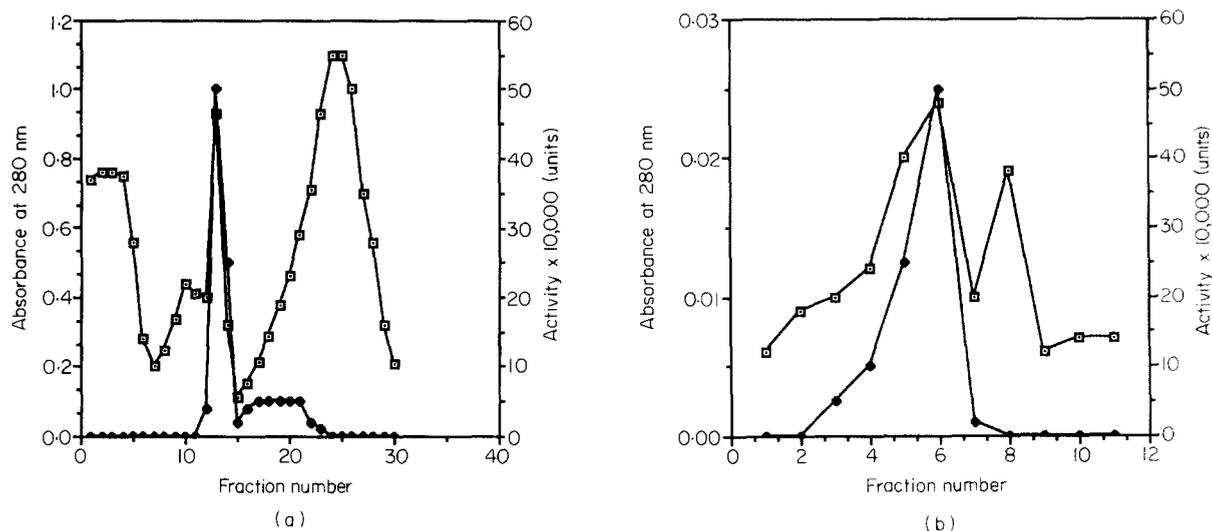


Figure 2. (a) and (b) (◆) Activity; (□) absorbance at 280 nm. (a) Cellulose-phosphate chromatography. (b) DNA affinity chromatography. The sequence of the oligonucleotides used to make the affinity column are:



eluate on an SDS/polyacrylamide gel demonstrated that the protein-DNA complex contained only the 33 kDa protein and none of the 100 kDa species.

(c) Equilibrium binding measurements

We performed equilibrium binding measurements to determine the affinity of RhaR for the p_{sr} binding site. Protein was mixed with radioactive DNA and the mixture was allowed to come to equilibrium (20 min at 150 mM-KCl and 100 min at 100 mM-KCl). The protein-DNA complexes were separated from the uncomplexed species using the

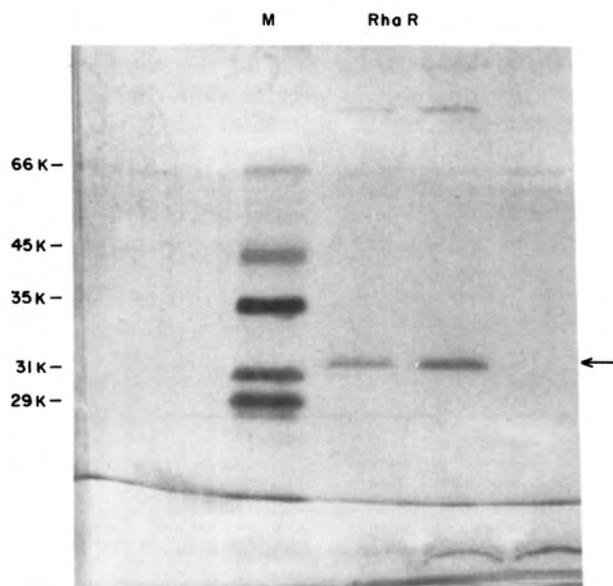


Figure 3. SDS/12% polyacrylamide gel of purified RhaR. The arrow indicates the location of the 33 kDa RhaR protein. Lane M, molecular weight standards, indicated at the left.

gel shift assay. The concentration of DNA (10^{-13} to 10^{-14} M) is well below the RhaR concentration. The apparent equilibrium constant, K_{app} , was calculated as the amount of active protein that gave 50% protein-DNA complex. The concentration of active protein (approx. 30%) was calculated from a titration experiment using a concentration of the p_{sr} binding site ten times greater than the apparent equilibrium constant for binding to DNA.

Table 2 lists the apparent equilibrium constants determined in the presence and absence of L-rhamnose and in varying concentrations of KCl assuming a simple model in which there is only a single binding site for RhaR. The K_{app} at 50 mM-KCl in the presence of L-rhamnose could not be determined, since the protein was unstable during the time needed to reach equilibrium. The higher affinity detected in the presence of L-rhamnose is specific, since the addition of the diastereomer, L-fucose, cannot mimic the effect.

(d) Kinetic measurements

We performed kinetic measurements to confirm the equilibrium measurements and to determine

Table 2
 Equilibrium dissociation constants of RhaR to p_{sr} DNA

L-Rhamnose	[KCl] (mM)	K_{app} (M)
+	50	$3.0 \times 10^{-13} \dagger$
+	100	7.5×10^{-12}
+	150	1.5×10^{-11}
-	50	7.5×10^{-12}

† Calculated from the association rate and dissociation rate constants.

Table 3

Dissociation rate constants of RhaR from p_{sr} DNA

L-Rhamnose	[KCl] (mM)	Half-time (min)	k_d (s^{-1})
+	50	240	4.8×10^{-5}
+	100	20	5.8×10^{-4}
+	150	4	2.9×10^{-3}
-	50	17	6.8×10^{-4}

whether L-rhamnose and increased salt concentrations affect the association or dissociation rates.

Dissociation rate constants were determined by first forming protein-DNA complexes with a radioactive DNA fragment, then adding a large excess of non-radioactive competitor DNA and monitoring with the gel shift assay the dissociation of the complex as a function of time. The dissociation of RhaR follows first-order kinetics and is independent of the concentration of competitor DNA added. The results in Table 3 show that increasing the concentration of KCl increases the dissociation rate constant. The presence or absence of L-rhamnose also affects the dissociation rate but not the association rate of RhaR.

To determine association rate constants, radioactive DNA is mixed with RhaR for various periods of time. The reactions are stopped by adding a large excess of competitor DNA and the percentage protein-DNA complex formed is monitored by the gel shift assay. Under most conditions, dissociation of RhaR is negligible. The association rate constant is determined from the half time required for complex formation. The results are shown in Table 4. The value obtained at 150 mM-KCl is approximate, since considerable dissociation occurs under these conditions. The values obtained at the lower KCl concentrations and in the absence of L-rhamnose demonstrate that the association rate constant does not change when these parameters are changed.

We calculated the equilibrium constants using the kinetic constants. The calculated equilibrium constants (K_{calc}) agree with the observed equilibrium constants (Table 5). Therefore, K_{calc} at 50 mM-KCl is a good approximation for the apparent equilibrium binding constant.

The apparent net number of ions displaced from a DNA binding site upon binding of a protein can be determined by plotting $-\log K_{app}$ versus $-\log$ salt

Table 4

Association rate constants of RhaR from p_{sr} DNA

L-Rhamnose	[KCl] (mM)	Half-time (min)	k_a ($M^{-1} s^{-1}$)
+	50	1	1.6×10^8
+	100	1	1.6×10^8
+	150	1	1.6×10^8
-	50	1	1.6×10^8

Table 5

Comparison of K_{app} to K_{calc}

L-Rhamnose	[KCl] (mM)	K_{calc} (M)	K_{app} (M)
+	50	3.0×10^{-13}	N.D.
+	100	3.6×10^{-12}	7.5×10^{-12}
+	150	1.8×10^{-11}	1.5×10^{-11}
-	50	4.2×10^{-12}	7.5×10^{-12}

N.D., not determined.

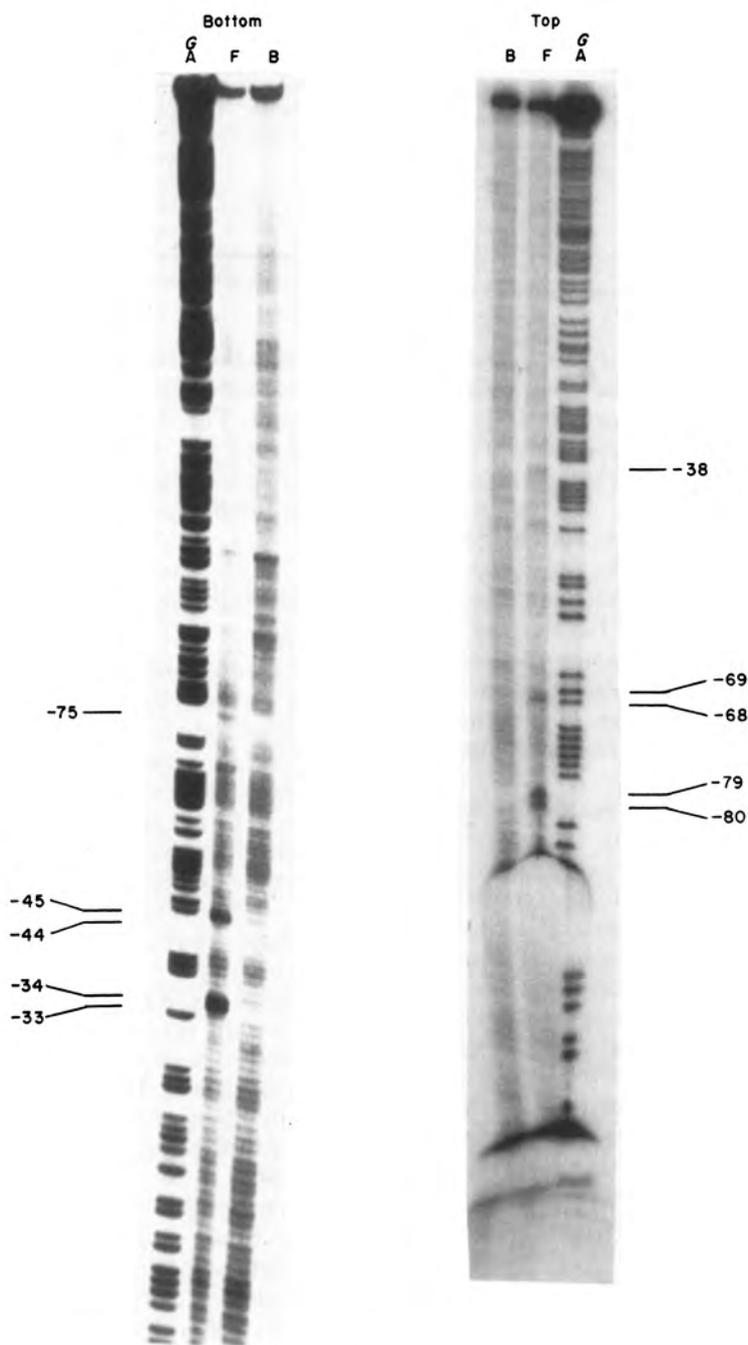
concentration (Record *et al.*, 1976, 1977). The slope of this line gives the net number of salt ions displaced. The slope of this line is -3.6 for RhaR binding to p_{sr} , indicating that a net of approximately four monovalent salt ions are displaced from the p_{sr} binding site upon RhaR binding.

(e) RhaR makes specific DNA contacts to its p_{sr} binding site

We used chemical interference assays (Hendrickson & Schleif, 1984; Brunelle & Schleif, 1987) to determine the nucleotides and phosphate residues to which RhaR makes specific contacts. The results of the contact experiments are summarized in Figure 5. RhaR apparently makes close contacts to ten phosphate groups. On the top strand, phosphate contacts are made on the 5' side of bases -80 , -79 , -69 , -68 and -38 . On the bottom strand, the phosphate groups on the 5' side of bases -33 , -34 , -44 , -45 and -75 are contacted (Fig. 4(a)). The pattern of the contacts is symmetrical and is centered within the half sites of the inverted repeat sequences. There are no phosphate contacts apparent to the spacer DNA between the half sites.

Methylation of the N-7 position of guanine nucleotides in the major groove and the N-3 of adenine nucleotides in the minor groove revealed four guanine contacts but no adenine contacts. On the top strand, RhaR makes close contact with a guanine nucleotide at position -47 . On the bottom strand, RhaR closely contacts the guanine nucleotides at position -80 , -67 and -45 (Fig. 4(b)). These contacts are restricted to the half sites of inverted repeat sequence. No strong contacts are evident in the spacer region between the sites.

The missing contact approach (Brunelle & Schleif, 1987) provides information about contacts made to all four nucleotides, whereas the premethylation experiment gives information only about close approaches to guanine and adenine bases. Additionally, a bulky methyl group on guanine could easily interfere with binding, even though a specific hydrogen bond is not made to that position. The missing contact experiment does not have this shortcoming, in that the base is completely removed. The result of an experiment examining both purine and pyrimidine contacts is illustrated by Figure 4(c). On the top strand, the removal of T81, C80, T79, T78, G47, A40, and T39, G34 and G33 has a significant effect on binding. On the bottom strand, G80, T76, T75,



(a)

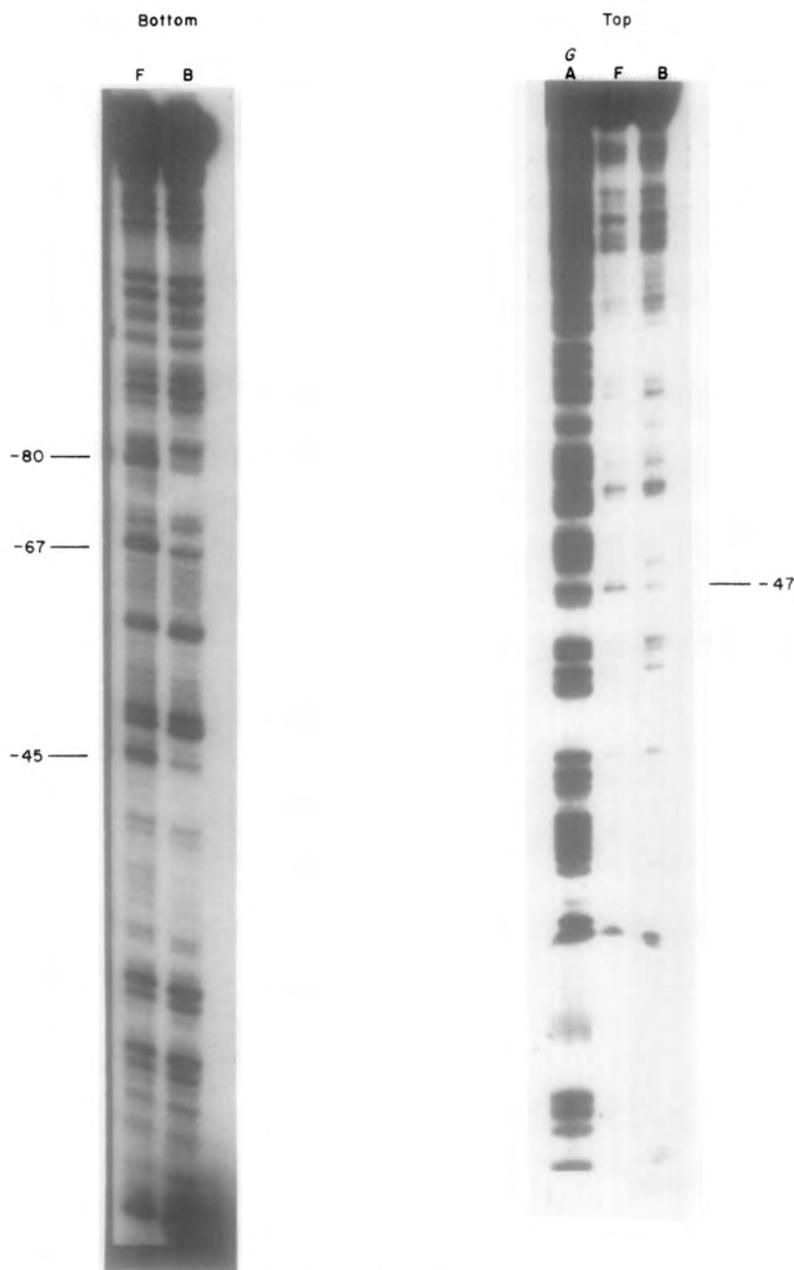
Fig. 4.

G45, T36, T35, C34 and C33 are apparently contacted by RhaR. Again, the distinguishing feature of these contacts is that they are located within the inverted repeat half sites and that no detectable contacts are made to the spacer region.

The affinity of RhaR for the p_{sr} binding site is 15-fold lower in the absence of L-rhamnose. This reduced affinity could be due to altered or missing DNA contacts made by the protein in the absence of L-rhamnose, but a contact experiment performed in the absence of L-rhamnose revealed an identical

pattern of contacts to those performed in the presence of L-rhamnose (data not shown). Therefore, within the limits of the assay, the lower affinity cannot be attributed to altered or missing contacts.

Figure 5(b) displays the contacts on a DNA helix that has a helical repeat of 10.5 base-pairs. The contacts are distributed across two major grooves at each of the 20 base-pair half sites, with the majority of the contacts found within the distal major groove at each half site. The other feature of the contacts is that they are restricted to one face of the DNA. The



(b)

Fig. 4.

symmetrical pattern of the contacts agrees well with an inverted repeat structure and cannot be fit to a direct repeat binding site.

(f) *The RhaR-DNA complex forms a DNA microloop*

The RhaR contacts to the p_{sr} site are unusual, in that the protein makes no contacts to the 17 base-pair DNA sequence between the half sites of the inverted repeat. This unusual pattern of contacts would be expected if RhaR bound to the p_{sr} binding site by making contacts to the two half sites while looping out the intervening spacer DNA. Insertion of helical turns of DNA between the respective operator sites in the *ara* (Dunn *et al.*, 1984) and

lambda systems (Hochschild & Ptashne, 1986) affects looping. Insertion of a non-integral number of helical turns drastically interferes with looping, whereas insertion of an integral number of helical turns has a much smaller effect. In both systems, the DNA sites required for looping need to be positioned on the same face of the DNA. We measured the affinity of RhaR for mutant sites containing an integral or non-integral number of helical turns between the half sites. Mutant p_{sr} binding sites were constructed by inserting an additional 11, 15 and 33 base-pairs into the 17 base-pair spacer region between the two half sites. As expected for a looped structure, RhaR binds considerably less tightly to a DNA molecule containing a non-integral number of

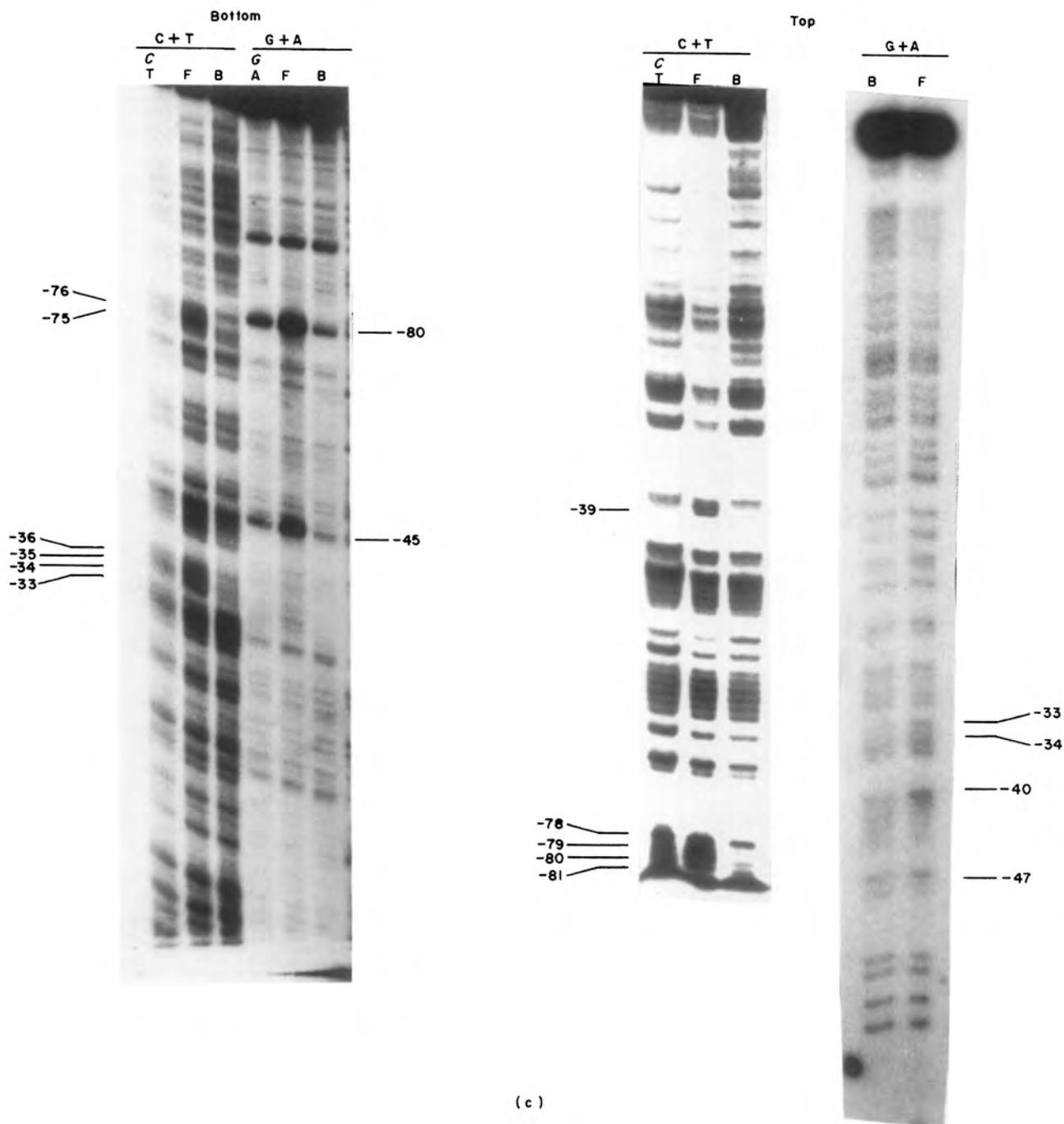
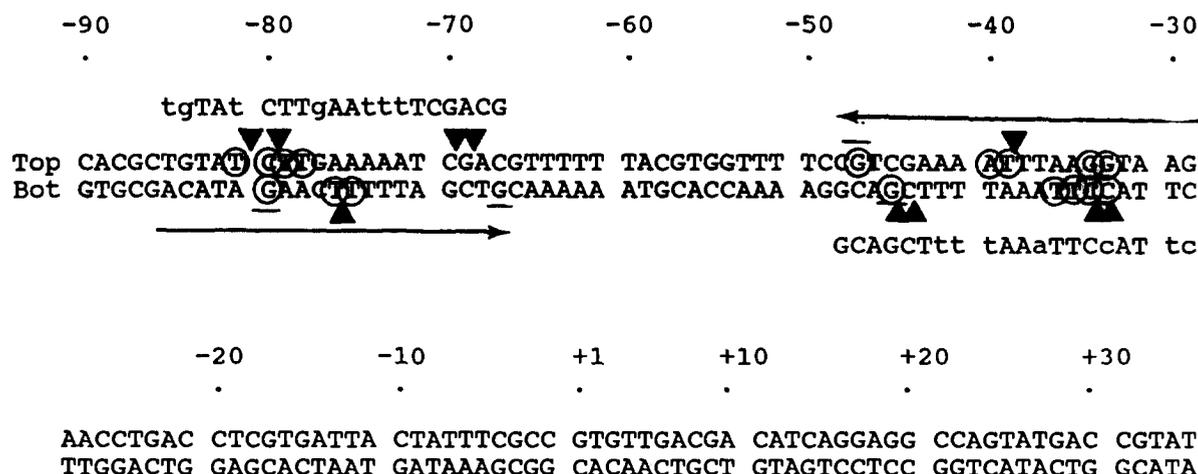


Figure 4. Phosphate, premethylation and missing contact data. (a) The phosphate contacts made by RhaR are as indicated. The top and bottom strand are as described in the text and in Fig. 5(a). B is DNA isolated from the bound, complexed, fraction. F is DNA isolated from the free, uncomplexed, fraction. G/A is cleaved depurinated DNA standard. (b) The guanine contacts detected by premethylation are as indicated. The top and bottom strand are as described in the text and in Fig. 7. B is DNA isolated from the bound, complexed, fraction. F is DNA isolated from the free, uncomplexed, fraction. G/A is cleaved depurinated DNA standard. (c) Missing contacts data. The guanine, adenine, cytosine and thymine nucleotides that RhaR contacts are as indicated. The top and bottom strand are as described in the text and in Fig. 5(a). B is DNA isolated from the bound, complexed, fraction. F is DNA isolated from the free, uncomplexed, fraction. G/A is a cleaved depurinated DNA standard, while C/T is a cleaved depyrimidated DNA standard.

helical turns (+15, Table 6) between the half sites than it does to DNA molecules containing insertions of integral numbers of helical turns (+11 and +33, Table 6). The K_{app} value for RhaR binding to the mutant site containing the non-integral number of

helical turns (+15) is higher than for the individual half sites (Table 6), indicating that half sites are capable of interacting, albeit weakly, when positioned on opposite sides of the DNA helix.

Since RhaR binding induces the formation of a



(a)



(b)

Figure 5. (a) Summary of contact data. The arrows indicate the half sites of the inverted repeat. Phosphate contacts are indicated by carets. (○) G, A, C and T contacts detected by the missing contact approach. (–) The guanine contacts detected by premethylation. (b) RhaR contacts displayed on a DNA helix with a 10.5 base-pair helical repeat. The arrows indicate the half sites of the inverted repeat. The filled circles indicate the phosphate, G, A, C and T contacts.

DNA loop, which most likely would be bent, we used the gel electrophoresis assay developed by Wu & Crothers (1984) to look for a bend in the p_{sr} binding site. Figure 6 shows that the mobility of RhaR- p_{sr} DNA complexes is dependent on the position of the binding site with respect to the ends of a set of DNA molecules of the same length. This is expected for bent DNA, and the alteration in migration velocity as a function of the position of the binding site permits estimation of the bending angle (Thompson & Landy, 1988). The DNA alone contains a static bend in the absence of bound RhaR.

The DNA molecules have a faster mobility (Fig. 6, lanes E and M) when the p_{sr} binding site is located at the end of the molecule than when the site is located in the center (Fig. 6, lanes H and B). We estimated the bending angle to be 70° using the method described by Thompson & Landy (1988). In the presence of RhaR protein, the retardation as a function of the location of the binding site is much larger, and the bending angle appears to be 160° .

4. Discussion

To obtain sufficient quantities of RhaR for study of its DNA binding activities, we hypersynthesized the protein using the strong bacterial *tac* promoter. Binding experiments performed previously (Tobin & Schleif, 1987) and those performed in this study showed that RhaR binds specifically to the inverted repeat element contained upstream from the p_{sr} promoter. The binding is independent of RhaS. A truncated RhaR protein lacking the carboxy-terminal portion of RhaR fails to bind but displays a *trans* dominant negative phenotype. This result suggests that the carboxy-terminal domain of RhaR is involved in DNA binding and that the amino-terminal portion is involved in oligomerization (Isackson & Bertrand, 1985; Nelson *et al.*, 1987). A

Table 3
Equilibrium dissociation constants of RhaR for spacing mutants

DNA construct	K_{app} (M)	Fold weaker than wild-type
Wild-type	3.0×10^{-13}	1
+11	2.4×10^{-11}	80
+15	$3.0 \times 10^{-09}\dagger$	1.0×10^4
+33	2.4×10^{-11}	80
Half site	$7.4 \times 10^{-08}\dagger$	2.5×10^5

† These values for K_{app} were obtained by fitting a single data point to a Michaelis-Menton binding curve.

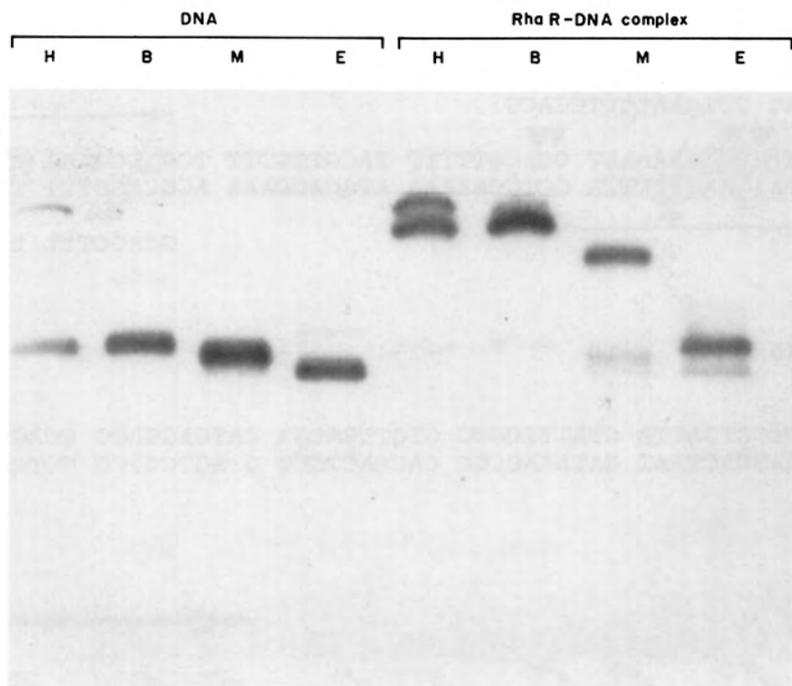


Figure 6. The relative mobility of p_{sr} DNA and RhaR- p_{sr} DNA complexes in a 6% polyacrylamide gel is dependent on the position of the DNA binding site. The 787 base-pair minicircle (Materials and Methods) was digested with (H) *Hind*III, (E) *Eco*RI, (M) *Mlu*I, or (B) *Bam*HI to produce a set of DNA fragments of the same size containing the RhaR binding site at different positions within the fragment. The bands of greater intensity are the circularly permuted fragments containing the RhaR DNA-binding site. The bands of lesser intensity arise from cleavage of the unligated (linear) 787 base-pair *Hind*III fragment. The additional band (slowest mobility) present in the H lanes is undigested minicircle DNA.

carboxy-terminal truncated RhaS protein also displays a *trans* dominant negative phenotype, indicating that its active form may be an oligomer. We have not been able to detect any binding of RhaS to DNA (data not shown; see Tobin, 1989).

The RhaR protein was purified by a simple two-column procedure using the gel shift assay to monitor the purification steps. Since no assay was available for the RhaS protein, its purification was not attempted. The RhaR purification procedure consists of three steps. The cells are lysed by the addition of lysozyme. After this step, most of the RhaR activity is associated with the cell pellet. This is not surprising, since RhaR is a DNA-binding protein and most of the cellular DNA is contained in this pellet. High-salt extraction of this pellet, followed by DNA removal, left a crude extract containing RhaR at approximately 0.2% of the total cellular protein. Cellulose-phosphate chromatography and DNA affinity chromatography resulted in a RhaR preparation that was 70% homogeneous containing predominantly a 33 kDa protein and a lesser amount of a 100 kDa protein. Further purification was not attempted because the yield was too low at this point. The 33 kDa protein was shown to be RhaR since it, and not the 100 kDa protein, was found in RhaR-DNA complexes. The nature of the 100 kDa protein that copurifies with

RhaR is not known. It does not comigrate with any of the subunits of purified RNA polymerase on an SDS/polyacrylamide gel.

We determined several of the physical properties of the purified RhaR protein using the gel shift assay. A general property of DNA-binding regulatory proteins is that they specifically bind to DNA with affinities in the nanomolar to picomolar range. Such high affinities easily allow these proteins to discriminate between non-specific and specific DNA binding sites at the *in vivo* concentrations of these proteins. The equilibrium binding constants and kinetic constants of RhaR for the p_{sr} site were determined under a variety of solution conditions. These experiments showed that RhaR binds tightly to its site with K_{app} ranging from the subpicomolar to the ten picomolar range. A direct determination of K_{app} at 50 mM-KCl could not be obtained, since the protein was not stable during the time required to make such a measurement. Its value was obtained indirectly by measuring the association and dissociation rate constants. The calculated equilibrium binding constants agree within twofold with the measured values obtained at 100 and 150 mM-KCl. Therefore, K_{calc} at 50 mM is a good approximation of K_{app} at this salt concentration. In the absence of L-rhamnose, the affinity of RhaR is reduced by approximately 20-fold.

Examination of the kinetic constants demonstrates that it is the dissociation rate constant and not the association rate constant that is affected by salt concentration and L-rhamnose. The k_a of RhaR for p_{sr} remains constant under the conditions where it can be reliably measured, whereas k_d varies greatly. Examination of the salt dependence on the equilibrium binding constant demonstrates that a net of approximately four monovalent salt ions are released from the DNA upon RhaR binding. This value seems small in light of the fact that the RhaR protein contacts at least ten phosphate residues in its p_{sr} binding site. An analogous situation has been described for the AraC protein binding to the *araI* site by Martin & Schleif (1987). These authors suggest that as AraC binds to DNA, a large number of ions are displaced from the DNA but the majority of them rebind to the protein, thereby reducing the salt dependency on the binding constant.

Methylation of guanine, ethylation of phosphate, and missing contact studies identified the nucleotides and phosphate groups contacted by RhaR. The ethylation of phosphate both removes negative charge and adds a bulky ethyl group. Therefore, charge and/or steric effects can contribute to its influence on binding. Methylation of guanine -67 interferes with RhaR binding, but removal of this base has little effect on binding as assayed by the missing contact approach. These results indicate that guanine -67 is not specifically contacted, but its methylation interferes with RhaR binding. Using *in vivo* dimethyl sulfate footprinting, we previously demonstrated that methylation of guanine -45 was increased twofold by the presence of RhaR (Tobin & Schleif, 1987). Here, we show that premethylation of guanine -45 interferes with RhaR binding. These two results need not be mutually incompatible. The enhanced methylation may be caused by higher local concentrations of dimethyl sulfate in hydrophobic pockets formed by the protein in the vicinity of guanine -45 (Ogata & Gilbert, 1978). Once guanine -45 is methylated, RhaR would dissociate more rapidly from the DNA.

The pattern of DNA contacts made by RhaR appear identical both in the presence and absence of L-rhamnose. The affinity of the protein is lower, however, in the absence of L-rhamnose. An analogous situation has been described for the AraC protein binding to the *araI* site (Hendrickson & Schleif, 1984). Suppose that in the absence of L-rhamnose RhaR binds to its DNA site but must undergo an energetically unfavorable conformational change in order to properly align each monomer for correct binding. L-Rhamnose could increase the affinity of RhaR for its DNA site if it bound to the protein and caused a similar conformational change, thereby allowing more of the energy available from DNA binding to be expressed in the binding constant.

RhaR binds to the p_{sr} binding site by contacting two major grooves of the DNA at each half site while looping out the 17 base-pairs of spacer DNA

to form a DNA microloop. Two lines of evidence support the proposed microloop structure. Firstly, RhaR makes no contacts to the 17 base-pairs of spacer DNA separating the two sites. All of the contacts are made to one face of the DNA and are located within the half sites of the inverted repeat. Secondly, the affinity of RhaR is dependent on the angular orientation of the half sites. RhaR binds tightly to mutant binding sites that contain the half sites on the same face of the DNA, but weakly to binding sites that contain the half sites on opposite faces. The pattern of contacts made by RhaR is different from those made by other well-studied prokaryotic DNA-binding regulatory proteins. These proteins contact DNA-binding sites that are smaller than the p_{sr} site. Each monomer of these dimeric proteins contacts only a single major groove of the DNA at each half site. In addition, the two half sites are adjacent to one another, there is little or no spacer DNA between the closest edges of the half sites. The formation of a RhaR induced microloop allows both half sites of the inverted repeat structure to be held in close proximity.

DNA looping plays a regulatory role in many systems (Martin *et al.*, 1986; Hochschild & Ptashne, 1986; Thompson & Landy, 1988; Kramer *et al.*, 1987; Borowiec *et al.*, 1987) and is a common mechanism that allows proteins to interact even though they are bound to DNA sites that are sometimes separated by up to 1000 base-pairs. There are two general requirements for DNA looping. The protein must bind sufficiently tightly to DNA and the protein must interact sufficiently tightly with another protein *via* protein-protein interactions. To a limited extent, strength in one type of interaction can compensate for weakness in the other. Three basic types of looping systems should exist: those with both interactions "strong"; those with strong protein-DNA interactions but weak protein-protein interactions; and, finally, those with weaker protein-DNA interactions but strong protein-protein interactions. The RhaR-DNA microloop is an example of a looped structure where the protein-DNA interactions are weak but the protein-protein interaction is strong. RhaR most likely exists as a dimer in solution (Tobin, 1989) and binds as a dimer to the complete p_{sr} site.

Using the gel electrophoresis assay developed by Wu & Crothers (1984), we demonstrated that in the absence of RhaR the fragment containing the p_{sr} binding site contains a static bend of 70°. The bend is probably caused by four naturally occurring bent DNA sequences, (A_{5-6} ; Nelson *et al.*, 1987) each contributing about 18° to the bending angle (Koo & Crothers, 1988). Once the RhaR protein is bound, the retardation as a function of the location of the binding site is much larger, and the bending angle of the DNA appears to be 160°.

There are several other palindromic sequences contained within RhaR binding site (Fig. 7). Here, we have identified an inverted repeat that contains 13 palindromic bases out of 20 at each half site (Fig. 5(a)) by aligning the phosphate contacts -75

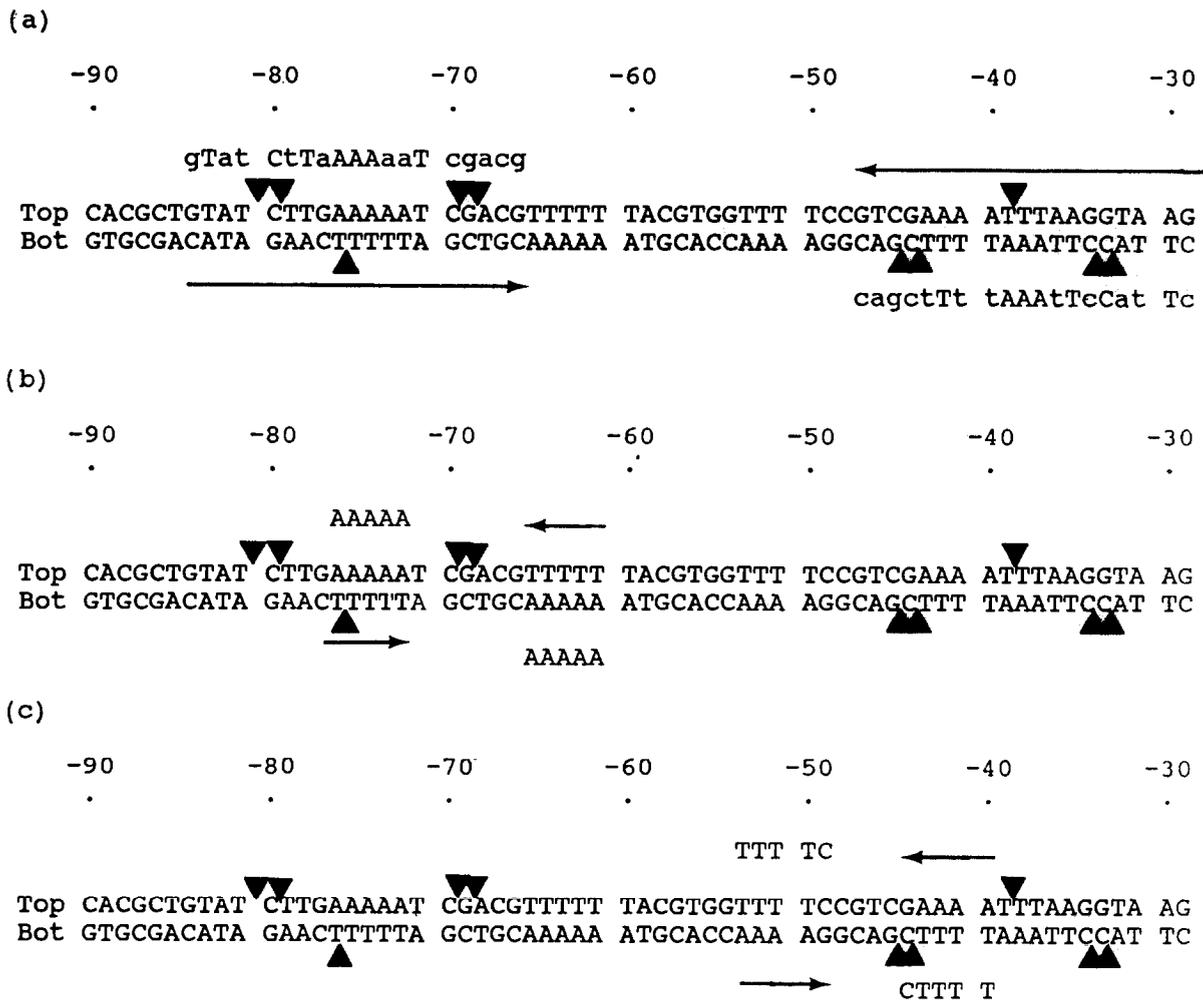


Figure 7. Summary of the palindromic sequences within the RhaR binding site. (a) The palindrome obtained by aligning the phosphate contacts -80 , -79 , -69 and -68 with phosphate contacts -45 , -44 , -34 and -33 . (b) and (c) The palindromes identified by Tobin & Schleif (1987).

and -38 . However, the alignment of the phosphate contacts -80 , -79 , -69 and -68 with contacts -45 , -44 , -34 and -33 gives a different inverted repeat containing seven palindromic bases (Fig. 7(a)). On the basis of our present data, we cannot discern which palindrome is important for binding. The two palindromes that we previously identified (Tobin & Schleif, 1987) cannot be aligned with any of the phosphate contacts (Fig. 7(b) and (c)) and are likely not part of the RhaR recognition sequence.

Figure 8 displays the phosphate contacts of the RhaR protein and the predicted phosphate contacts of RNA polymerase at the p_{sr} promoter. If the predicted RNA polymerase contacts (Bushman & Ptashne, 1986) are correct, then RhaR makes close contact to RNA polymerase at the p_{sr} promoter. In fact, phosphate -38 would be contacted by both RhaR and RNA polymerase. This may mean that RhaR stimulates transcription from the p_{sr} promoter by interacting directly with RNA polymerase.

We previously demonstrated that there is significant amino acid sequence similarity between AraC,

RhaS, and RhaR (Tobin & Schleif, 1987). We have now compiled a more complete list of bacterial regulatory proteins that have significant amino acid similarity to AraC (Fig. 9). The alignment of all these proteins reveals that most of the similarity is confined to the carboxy-terminal third of the

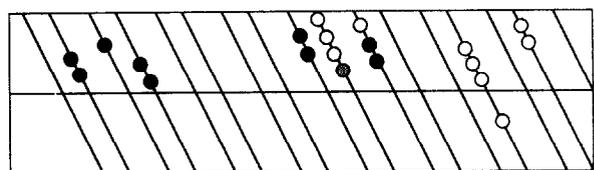


Figure 8. Planar representation of the DNA sequence (10.5 base-pairs/turn) of the p_{sr} promoter containing the predicted RNA polymerase phosphate contacts (O) and the experimentally determined RhaR phosphate contacts (●). The half tone (◐) represents the shared -38 phosphate contact. The predicted RNA polymerase phosphate contacts are those that have been determined for the bacteriophage 434 P_{RM} promoter (Bushman & Ptashne, 1986), except that the spacing between the 2 contacted regions is 3 base-pairs larger for the p_{sr} RNA polymerase binding site.

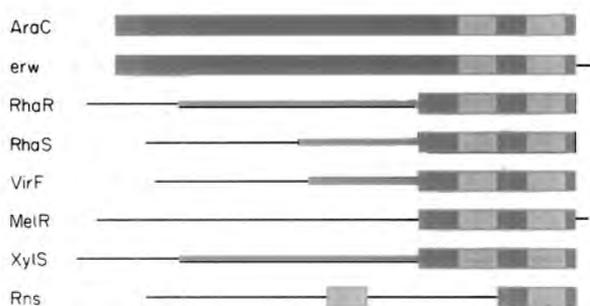


Figure 9. A representation of the primary sequence homology between the AraC protein family. The regions of greatest homology are indicated by the thickest line, the regions of least homology are indicated by the thinnest line. The grey boxes indicate the proposed DNA-binding regions. erw is the arabinose operon regulatory protein from *Erwinia carotovora*, VirF is the virulence regulon regulatory protein from *Yersina*, MelR is the melibiose regulatory protein from *E. coli*, XylS is the xylene operon regulatory protein from *Pseudomonas putida* and Rns is the adhesin regulatory protein from *E. coli*.

proteins. Mutational analysis of AraC has identified two regions located in the carboxy terminus that are important for DNA binding (Brunelle, 1988). The high degree of amino acid similarity in the carboxy terminus of these proteins likely reflects the conservation of a common DNA-binding domain. This observation is interesting in light of the fact that the AraC protein appears to recognize a DNA-binding site that contains a direct repeat structure (Brunelle & Schleif, 1989), while the RhaR protein recognizes an inverted repeat binding site. Since the carboxy termini of RhaR and AraC are so similar, it is unlikely that the structural difference that allows one protein to recognize an inverted repeat *versus* a direct repeat structure is found in this domain.

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