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A Regulatory Cascade in the Induction of *rhaBAD*

Susan M. Egan and Robert F. Schleif

Biology Department
The Johns Hopkins University
3400 N. Charles Street, Baltimore, MD 21218, U.S.A.

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The RhaS and RhaR regulatory proteins are encoded in the *Escherichia coli* L-rhamnose gene cluster. We used complementation analysis and DNA mobility shift assays to show that RhaR is not the direct activator of the L-rhamnose catabolic operon, *rhaBAD*. An in-frame deletion of *rhaS* (*rhaS*$^-$ *rhaR*$^+$) eliminated expression from the *rhaBAD* promoter, p_{BAD}, while overexpression of *rhaS* greatly speeded the normally slow induction of transcription from p_{BAD}. Expression from p_{BAD} in a coupled transcription–translation assay was only detected when *rhaS*$^+$ DNA was added to allow synthesis of RhaS protein. RhaS thus appears to be the direct L-rhamnose-specific activator of *rhaBAD* expression. Deletion mapping located the binding site for the L-rhamnose-specific regulator to a region overlapping position −70 relative to the *rhaBAD* transcription start site. Deletion mapping and DNA mobility shift assays located a CRP binding site just upstream from the binding site for the L-rhamnose-specific regulator. Quantitative primer extension analysis showed that induction of both the *rhaBAD* and *rhaSR* messages was unusually slow, requiring 40 to 50 minutes to reach a steady-state level. Induction of *rhaBAD* apparently involves a regulatory cascade in which RhaR first induces *rhaSR* expression, then RhaS accumulates and induces *rhaBAD* expression.

Keywords: rhamnose; rhaBAD; RhaS; CRP; transcriptional activation

1. Introduction

We have analyzed the regulation of the operon encoding the L-rhamnose catabolic enzymes, *rhaBAD*, to determine the structure of the regulatory region and to identify the regulatory proteins involved. These experiments can now be rigorously performed since all of the known genes involved in L-rhamnose catabolism in *Escherichia coli* have been cloned and sequenced (Tobin & Schleif, 1987; Moralejo *et al.*, 1993; Baldomá *et al.*, 1990; Tate *et al.*, 1992; García-Martín *et al.*, 1992, see Fig. 1). The regulatory locus, originally designated *rhaC*, consists of two genes, *rhaS* and *rhaR* (Tobin & Schleif, 1987), one of which is likely to be the direct inducer of *rhaBAD*. The *rhaS* and *rhaR* gene products show a high degree of sequence similarity with AraC, the regulator of the *E. coli ara* genes, and even greater similarity with each other (Tobin & Schleif, 1987; Ramos *et al.*, 1990; Gallegos *et al.*, 1993).

Tobin and Schleif found that RhaR protein directly activates *rhaSR* transcription in response to L-rhamnose (Tobin & Schleif, 1990*a*). A RhaR protein dimer bound an inverted repeat upstream of *rhaSR*, with 17 bp of uncontacted DNA between the two half-sites (Tobin & Schleif, 1990*b*). While each RhaR half-site is the same length as an AraC binding half-site, the binding sites differ in that AraC binds a direct repeat with only 4 bp between the half-sites (Brunelle & Schleif, 1989; Carra & Schleif, 1993). The structure of the RhaR and AraC sites gives an idea of the expected structure of the L-rhamnose regulator binding site upstream of *rhaBAD*.

Before the DNA sequence of *rhaBAD* was determined, Tobin & Schleif (1987, 1990*a*) identified two L-rhamnose-inducible transcripts which we now believe result from cleavage of a complete *rhaBAD* transcript between *rhaB* and *rhaA* (Moralejo *et al.*, 1993). Using complementation analysis in a strain (ECL343) they believed was deleted for both *rhaS* and *rhaR*, Tobin and Schleif found that RhaR was required for induction of the transcripts, but that RhaS$^-$ had only a small affect on induction of the transcripts.

A number of questions concerning *rhaBAD* regulation are now accessible. First, what are the positions of regulatory protein binding sites upstream of *rhaBAD*? Based on the similarities between the *rha*

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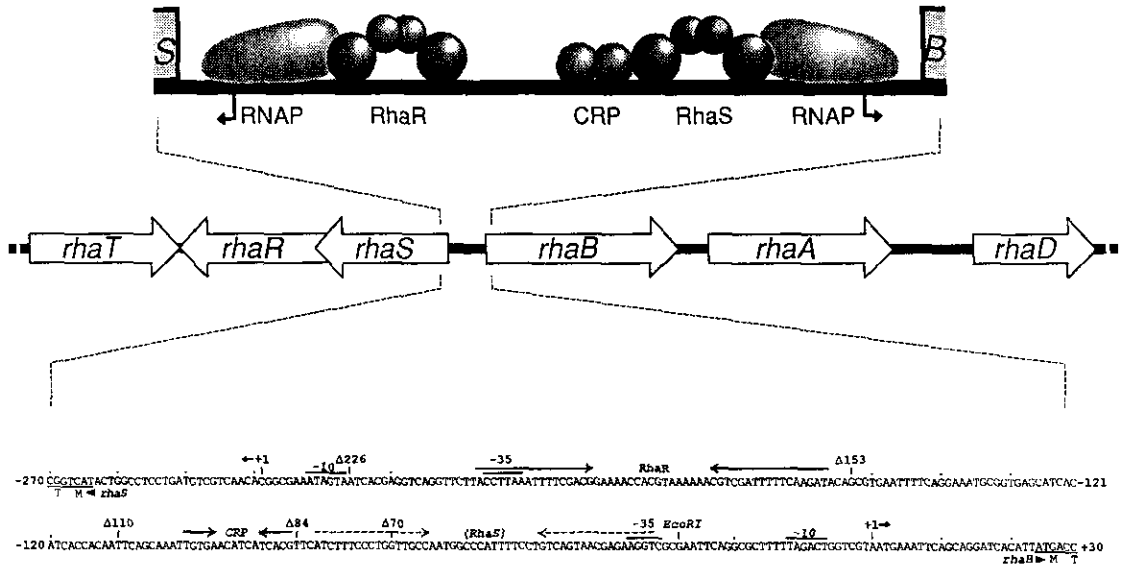


Figure 1. Schematic representation of the *E. coli rha* region. Top: The *rhaS-rhaB* regulatory region with the proposed activator proteins and RNA polymerase illustrated. The RhaS and CRP positions are based upon the results of this work. RhaS and RhaR are illustrated as dimers consisting of 2-domain monomers based on the structure of AraC (Bustos & Schleif, 1993). Middle: The order and orientation of the genes in the *rha* region. *rhaT* encodes the L-rhamnose transport protein (Baldomá *et al.*, 1990; Tate *et al.*, 1992). Bottom: DNA sequence of the *rhaS-rhaB* regulatory region illustrating the RhaR binding site at p_{SR}, the proposed CRP and RhaS binding sites at p_{BAD}, and the upstream deletion endpoints.

and *ara* systems, the following binding site order might be expected: CRP-Rha regulator-RNA polymerase. Second, what is the direct L-rhamnose regulator of *rhaBAD* expression? We wish to determine whether the RhaR requirement for *rhaBAD* induction is direct or indirect, and to further analyze whether RhaS plays a role in *rhaBAD* induction.

In the deletion analysis reported here, we identified two *cis*-acting regulatory sites upstream of *rhaBAD*. A site centered at −92·5 is likely a CRP protein binding site, while a site immediately downstream of this is likely the binding site for the L-rhamnose regulator. Our results also indicate that RhaS is the L-rhamnose regulator of *rhaBAD* expression (Fig. 1). These results include the following: (1) deletion of *rhaS* indicated that *rhaS*$^+$ was required for L-rhamnose induction of p_{BAD}; (2) RhaR was not directly required for *rhaBAD* transcription and, (3) an *in vitro* coupled transcription–translation assay indicated that RhaS was sufficient for L-rhamnose induction of *rhaBAD*. Finally, we investigated the kinetics of mRNA accumulation from both *rhaBAD* and *rhaSR* and found both to be unusually slow.

2. Materials and Methods

(a) *General methods*

Standard methods were used for restriction endonuclease digestion, ligation, and transformation of DNA (Davis *et al.*, 1980). RNAzol (Chomczynski & Sacchi, 1987) was from Cinna/Biotecx (Friendswood, TX). Oligonucleotide primers (listed in Table 2) were made on an Applied Biosystems 381A synthesizer. Oligonucleotides were cleaved from the column with 30% NH_4OH for 15 min, deprotected at 55 °C for 6 to 18 h, and precipitated directly from this solution with 10 vol. *n*-butanol, not water-saturated (Sawadogo & Van Dyke, 1991). The resulting single phase solution was centrifuged at 12,000 ***g*** for 1 min. The pellet was dried and resuspended in TE (Sambrook *et al.*, 1989). Oligonucleotides for primer extension analysis were purified on a Pharmacia FPLC Mono-Q anion exchange column (Cubellis *et al.*, 1985).

(b) *Culture media and conditions*

For primer extension experiments and enzyme assays, cells were grown in M10 salts (Schleif & Wensink, 1981) plus 0·4% (v/v) glycerol, 50 μM thiamine, 0·2% Casamino acids, and when added, 0·2% (w/v) L-rhamnose, unless otherwise indicated. Cultures were grown at 37 °C with shaking, in flasks no more than one-tenth full. Ampicillin, kanamycin and chloramphenicol were used at 200, 75 and 25 μg/ml, respectively. For other experiments (cloning, screening, etc.), cells were grown in yeast extract–tryptone (YT) medium (Sambrook *et al.*, 1989).

(c) *Strains, plasmids and phage*

All strains were derivatives of ECL116 (Backman *et al.*, 1981, Table 1). A derivative of ECL116 with a chloramphenicol-resistance insertion in *recA* (ECL116 *recA938*::Cm) was constructed using bacteriophage P1 *kc*-mediated transduction (Miller, 1972). ΔrhaS was constructed using "long-way-around the plasmid", or inverse, PCR† (Arnheim, 1990) with primers 853 and 854 (Table 2), and pJTC6 as the template (Tobin, 1989). This deleted approximately two-thirds of the *rhaS* coding region, but retained the translational frame across the deletion.

† Abbreviation used: PCR, polymerase chain reaction.

Table 1
Bacterial strains, plasmids and phage

Strain, plasmid or phage	Genotype	Source or reference
E. coli strains		
ECL116	F $\Delta lacU169$ *endA hsdR thi*	Backman *et al.* (1981)
ECL715	As ECL116 but *rhaA502*	Chen *et al.* (1987)
SME1048	As ECL116 but *recA938*::Cm	This work
SME1053	As ECL116 but Δ*rhaS recA938*::Cm	This work
SME1085	As ECL116 but Δ(*rhaSR*)::Km *recA938*::Cm	This work
Plasmids		
pUC4K	Ap^r Km^r	Vieira & Messing (1982)
pMAK705	Cm^r, *'lacZ rep(ts)*	Hamilton *et al.* (1989)
pJT5	Ap^r, *rhaBAD*$^+$ *rhaSR*$^+$ in pUC12	Tobin & Schleif (1987)
pJTC6	Ap^r, *rhaS*$^+$ *rhaR*$^+$ in pEMBL19	Tobin (1989)
pJTC105	Apr, *rhaS*$^+$ *rhaR*$^+$ in pTAC12	Tobin & Schleif (1990*b*)
pJTC108	Ap^r, *rhaS*$^+$ in pTAC12	Tobin (1989)
pJTC114	Ap^r, *rhaR*$^+$ in pTAC12	Tobin & Schleif (1990*b*)
pTZ18R	Ap^r, *'lacZ* T7 ϕ10 promoter	US Biochemicals
pSME101	Ap^r, *rhaB*$^+$ *rhaSR*$^+$ in pTZ18R	This work
pRS414	Ap^r, *'lacZ lacY*$^+$ *lacA*$^+$	Simons *et al.* (1987)
pSME104	Ap^r, pRS414 Φ(*rhaB-lacZ*)Δ153	This work
pSME114	Ap^r, pRS414 Φ(*rhaS-lacZ*)896	This work
Phage		
λ RS45	*bla'-lacZ*$_{SC}$ *att*$^+$ *int*$^+$ *imm*^{21}	Simons *et al.* (1987)
λ SME101	λ RS45 Φ(*rhaB-lacZ*)Δ226	This work
λ SME102	λ RS45 Φ(*rhaB-lacZ*)Δ153	This work
λ SME103	λ RS45 Φ(*rhaB-lacZ*)Δ110	This work
λ SME104	λ RS45 Φ(*rhaB-lacZ*)Δ84	This work
λ SME105	λ RS45 Φ(*rhaB-lacZ*)Δ70	This work
λ SME106	λ RS45 Φ(*rhaS-lacZ*)896	This work

A deletion of most of *rhaS* and *rhaR* (Δ(*rhaSR*)::Km) was constructed using inverse PCR (Arnheim, 1990) with primers 773 and 777, and pSME101 as the template. This deleted the last two-thirds of *rhaS* through the first three-quarters of *rhaR*. A kanamycin-resistance cassette (from pUC4K, Vieira & Messing, 1982) was cloned between the deleted genes. The DNA sequence of the fusion junctions was determined for each deletion.

The Δ*rhaS* and Δ(*rhaSR*)::Km alleles were subcloned into pMAK705, which has a temperature-sensitive origin of replication, and recombined into the chromosome of ECL116 by the method of Hamilton *et al.* (1985). Briefly, growth at high temperature selected for recombination of the plasmid into the *rha* region, then growth at a permissive temperature allowed the unstable cointegrate to cross back out of the chromosome. PCR analysis indicated that on the order of 20% of the resulting strains had the new allele at the chromosomal *rha* locus.

(d) *Translational fusions of* lacZ *with* rha *genes*

Translational fusions of *lacZ* to *rhaB* and *rhaS* were constructed on plasmids and transferred to λ phage by *in vivo* recombination (Simons *et al.*, 1987). To construct the *rhaB* fusions, DNA fragments were amplified by PCR using primer 744 with either Δ226, Δ153, Δ110, Δ84, or Δ70, then digested with *Bam*HI and *Sma*I. The fragments were cloned between the *Sma*I and *Bam*HI sites of pRS414 to generate *lacZ* fusions (Φ) named for their upstream deletion end-point (see Table 2). Each fusion contained the first 15 codons from *rhaB* fused to the 9th codon of *lacZ*.

The DNA fragment for the *rhaS* fusion was amplified by PCR using primers 896 and 744. The fragment was digested at the *Bam*HI site in 896 and a natural *Eco*RI site upstream of *rhaS* (see Fig. 1), and cloned between the *Eco*RI and *Bam*HI sites of pRS414. This fusion contained the first 19 codons of *rhaS* fused to the 9th codon of *lacZ*.

PCR reactions were performed as described for the DNA mobility shift assays below, except that the primers were not labeled with ^{32}P. In each case, the DNA sequence of the entire cloned region was determined. The 6 resulting fusions were recombined onto λ RS45 (Simons *et al.*, 1987; Table 1). Single copy lysogens of the fusions at the λ attachment site of SME1048, SME1053 and SME1085 were isolated and verified as described previously (Stewart & Yanofsky, 1985).

(e) *β-Galactosidase assay*

For the Φ(*rhaB-lacZ*) induction time course, L-rhamnose was added at A_{600} nm of approximately 0·1. At time points, 5 ml. were taken and added into 5 ml of 1 × M10 salts with 125 μg/ml chloramphenicol, on ice. β-Galactosidase activity was determined as described by Miller (1972), except that incubation with substrate was at room temperature, approximately 21 °C. Activities are expressed in terms of cell density (A_{600}), using the formula of Miller (1972). Each culture was assayed in duplicate. Reported values are averaged from 3 independent experiments, except for the induction kinetics experiment. In that case, values from a single experiment are reported, but the experiment was representative of at least 2 other experiments.

Table 2
Oligonucleotide primers

Primer no.	Primer sequence†, 5′→3′	Use	Position‡
773	GGTAAGATCTATGAGGCATCGTGCACTGAGC	Construct Δ(*rhaSR*)::Km	*rhaR* +1534→+1554
777	GGTAAGATCTATCCGGCGAGCGATACAGCAC	Construct Δ(*rhaSR*)::Km	*rhaS* +293→+272
853	GGTAAGATCTAAAAAAATCCACACTATGTAATACGGTCAT	Construct Δ*rhaS*	*rhaS* +55→+25
854	GGTAAGATCTCATCGGCAGCTTAAGCAGCAAACGGGACTTG	Construct Δ*rhaS*	*rhaS* +636→+665
Δ226	TCACCCGGGATCACGAGGTCAGGTTCTTAC	Construct Φ(*rhaB-lacZ*)Δ226	*rhaB* −226→−206
Δ153	TCACCCGGGGCGTGAATTTTCAGGAAATGC	Construct Φ(*rhaB-lacZ*)Δ153	*rhaB* −153→−133
Δ110	TCACCCGGGTTCAGCAAATTGTGAACATCA	Construct Φ(*rhaB-lacZ*)Δ110	*rhaB* −110→−90
Δ84	TCACCCGGGTTCATCTTTCCCTGGTTGCCA	Construct Φ(*rhaB-lacZ*)Δ84	*rhaB* −84→−64
Δ70	TCACCCGGGGTTGCCAATGGCCCATTTTCC	Construct Φ(*rhaB-lacZ*)Δ70	*rhaB* −70→−50
896	CGCGGATCCTCTATCGCCACCGGACGGCGTT	Construct Φ(*rhaS-lacZ*)896	*rhaS* +84→+65
744	CGCGGATCCCCACTGGATGCGCCGAGATCG	Construct all *lacZ* fusions	*rhaB* +71→+51
897	CCGGAATTCATGACCGTATTACATAGTGTG	PCR analysis of *rhaS*	*rhaS* +26→+46
898	TGAGTAAAGCTTTTATTGCAGAAAGCCATCCCG	PCR analysis of *rhaS*	*rhaS* +860→+840
580	AAACGATGGATTTCGCGCAGCGTCAGGCT	*rhaBAD* primer extensions	*rhaB* +138→+109
664	CGGACGCGTTACCAGACGGA	*rhaSR* primer extensions	*rhaS* +74→+55
674	GCAGTCCACCGCCAAAGCTCG	*araBAD* primer extensions	*araB* +90→+70

† The sequences capable of hybridizing to *rha* or *ara* DNA are underlined.
‡ The nucleotide position of the hybridizing region of the primer within the indicated gene is given. The positions within *rhaS* and *rhaR* are relative to the *rhaSR* transcription start, while those in *rhaB* and *araB* are relative to the *rhaBAD* and *araBAD* transcription starts, respectively. Positions are listed in a 5′→3′ orientation.

With the exception of the time-course experiment, assays of samples with basal level activity were incubated for 17 to 19 h, and included a no cells control. In each case, the A_{420} value for the no cells control was subtracted from the sample values. The sample values were at least 2-fold higher than the no cell control from the same experiment, and ranged from A_{420} of 0·021 to 0·040.

(f) *DNA mobility shift assay*

DNA molecules for the mobility shift assays were generated *in vitro* using PCR with plasmid pJT5 (Tobin & Schleif, 1987) as template. For each reaction, primer 744 was ^{32}P-5′-end-labeled and the other primer was unlabeled. The reactions were performed, and the products purified as described by Carra & Schleif (1993). 30 cycles of amplification were performed with 1 min at 93 °C, 1 min at 60 °C and 1 min at 72 °C. DNA mobility shift assays were performed essentially as described by Hendrickson & Schleif (1984).

(g) *Primer extension analysis of* in vivo rha *mRNA levels*

L-Rhamnose was added to cultures at A_{600} of approximately 0·2. At various time points, 5 ml samples were taken, and added into 5 ml of 1 × M10 salts with 10 mM sodium azide, 125 μg/ml chloramphenicol and 400 μg/ml rifampicin on ice. RNA was isolated as previously described (Reeder & Schleif, 1993) except that 5 ml cells were used and the concentrated cells were treated with lysozyme before addition of the RNAzol reagent. Also, an ethanol precipitation was performed following the isopropanol precipitation.

Total cellular RNA, 1 μg, was mixed with 2·5 ng of ^{32}P-labeled primer 580, 664, or 674. Primer extension reactions were performed as described (Reeder & Schleif, 1993). Samples were run on a 6% (w/v) denaturing polyacrylamide sequencing gel. To account for losses during processing of the samples, gels were loaded with equivalent counts for each sample within a time course. The relative band intensities were quantified using a Molecular Dynamics PhosphorImager (Johnston *et al.*, 1990; Reichert *et al.*, 1992).

(h) *Coupled transcription–translation*

Coupled transcription–translation reactions (Zubay, 1973) were performed using the *E. coli* S30 Coupled Transcription Translation Kit (Promega Corp., Madison WI), following the suggested conditions. The S30 extract was from a strain that was wild-type at the *rha* locus, but not induced with L-rhamnose. A sample of the S30 extract was mixed with [^{35}S]methionine, $CsCl_2$-purified plasmid DNAs, the amino acid mix lacking methionine, and 0·2% L-rhamnose, when added, then incubated at 37 °C for 1 h. One-half of the reaction was acetone precipitated, dried and resuspended in SDS loading buffer (Schleif & Wensink, 1981). A 12% SDS/polyacrylamide gel (Laemmli, 1970) was loaded with 10 μl of the sample. The gel was fixed in 25% isopropanol, 10% acetic acid, dried, and exposed to X-ray film at −70 °C. Another sample of the reaction (not acetone precipitated), was used to determine the relative β-galactosidase activity. The values presented represent the total units per 50 μl reaction. The plasmids used in the reactions were: pSME104, pSME114,

pJTC105, pJTC108 and pJTC114 (Table 1). The level of RhaS and RhaR proteins synthesized from pJTC105, pJTC108 and pJTC114 varied, perhaps due to differences in the exact positioning of the genes relative to the plasmid-borne promoters.

3. Results

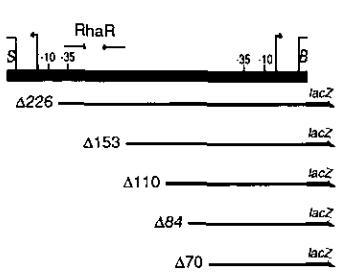
(a) *Analysis of the* rhaBAD *promoter region*

(i) *Construction of* Φ(rhaB-lacZ) *gene fusions with promoter deletions*

To dissect the *rhaBAD* regulatory region, we first constructed translational fusions between *rhaB* and *lacZ*. This permitted convenient and precise quantitation of *rhaBAD* promoter activity as increasing amounts of the *rhaBAD* upstream sequence were deleted. Using the system of Simons *et al.* (1987), the fusions were constructed on plasmids and then transferred onto λ phage by homologous recombination. All fusions were assayed as single copy lysogens to avoid potential artifacts due to multiple copies of the fusions in each cell. The fusions were named for their 5′ deletion end points (see Fig. 1 and Table 2). For example, Φ(*rhaB-lacZ*)Δ226 had a 5′ end-point at −226 relative to the *rhaBAD* transcription start site, and thus carried *rha*-specific nucleotides −226 to +70.

(ii) *Expression of* Φ(rhaB-lacZ) *gene fusions in a wild-type background*

Expression from Φ(*rhaB-lacZ*) fusions Δ226, Δ153 and Δ110, in a wild-type background, were all essentially the same (Fig. 2). Interestingly, their expression in the absence of L-rhamnose was extremely low (0·02 unit, compared to the *lacZYA* operon with 1 unit in the absence of lactose), resulting in an induction ratio on the order of 30,000-fold. Deletion of 26 bp beyond Δ110 (Δ84) led to 60-fold lower expression in the presence of L-rhamnose, but no significant change in the basal expression. While the induced level of 11 units was relatively low, it was approximately 1000-fold higher than the uninduced level. The 26 bp deleted region includes a site with sequence similarity to the CRP protein consensus binding site (Berg & von Hippel, 1988). Deletion of 14 bp beyond Δ84 (Δ70) nearly eliminated L-rhamnose inducibility. It seems likely that a CRP protein binding site lies upstream of Δ84, and an L-rhamnose responsive regulatory element overlaps the upstream endpoint of Δ70.



	β-Galactosidase sp. act.		Summary of binding results	
	-Rha	+Rha	RhaR	CRP
Δ226	0.02	650	+	+
Δ153	0.02	670	-	ND
Δ110	0.02	610	-	+
Δ84	0.01	11	ND	ND
Δ70	0.01	0.06	ND	-

Figure 2. Deletion analysis of the *rhaBAD* regulatory region. Expression levels of Φ(*rhaB-lacZ*) gene fusions with deletions of upstream sequences are shown. β-Galactosidase activity of cultures grown in the absence or presence of L-rhamnose, as indicated, was determined as described in Materials and Methods. Values from 3 independent experiments varied from the mean by 30% or less for the low basal levels, and 15% or less for all others. A summary of DNA mobility shift results is included for comparison. ND, not determined.

(iii) *The role of RhaS and RhaR in* rhaBAD *expression*

Tobin & Schleif (1987) used complementation analysis in strain ECL343, which they believed was deleted of both *rhaS* and *rhaR*, to determine the role of RhaS in induction of *rhaBAD* transcripts. They concluded that RhaS⁻ had only a very small effect on induction of the transcripts. We used PCR analysis with primers 897 and 898 to test the extent of the chromosomal deletion in ECL343, and found that it actually carries the entire *rhaS* gene (data not shown). As controls, a wild-type strain (ECL116) generated a band of identical size as ECL343, while Δ*rhaS rhaR*⁺ generated a smaller band, and Δ(*rhaSR*::Km) generated no band. The role of RhaS in *rhaBAD* induction, therefore, remains an open question.

To determine whether RhaS or RhaR or both are required for *rhaBAD* expression, we assayed three of the Φ(*rhaB-lacZ*) fusions in a strain deleted for both *rhaS* and *rhaR* (Δ(*rhaSR*::Km)), or carrying an in-frame deletion of most of *rhaS* (Δ*rhaS rhaR*⁺). An in-frame *rhaS* deletion was used to prevent polarity on *rhaR* expression. We did not assay expression in a strain deleted for only the chromosomal *rhaR* gene since the *rhaR* gene product is the direct inducer of *rhaSR* expression (Tobin & Schleif, 1990*a*,*b*), and therefore *rhaS* would be expressed at only low basal levels in such a strain.

Each of the three Φ(*rhaB-lacZ*) fusions tested, Δ226, Δ84 and Δ70, gave the same results. The fusions expressed approximately 0·01 to 0·02 Miller units in each of the deletion strains, both in the absence and presence of L-rhamnose. This level represents the low basal level expression of the L-rhamnose promoter p_{BAD}, and demonstrates that the fusions were not inducible in the deletion backgrounds. These results indicate that *rhaS*⁺ is required for *rhaBAD* expression.

The loss of induction in the Δ(*rhaS*) strain appears to be directly due to loss of RhaS activity and not due to decreased transcription of *rhaR*, which lies downstream of the in-frame *rhaS* deletion. This conclusion is based on the ability to induce high-level expression from p_{SR} (which directly requires RhaR) in the *rhaS* deletion strain (Table 3, see below).

(b) *Analysis of* rhaSR *expression*

To provide a quantitative measure of p_{SR} expression, we constructed a *lacZ* translational fusion with *rhaS*, as described in Materials and Methods. Again, to avoid artifacts due to multiple

Table 3
Expression of Φ(rhaS-lacZ) *gene fusion*

rhaSR alleles	β-Galactosidase specific activity†	
	−Rha	+Rha
rhaS⁺ *rhaR*⁺	0·25	110
Δ(*rhaS*) *rhaR*⁺	0·26	360
Δ(*rhaSR*)::Km	0·25	0·25

† β-Galactosidase specific activity was assayed as described in Materials and Methods and is reported in Miller units. Cultures were grown with or without L-rhamnose, as indicated. Values from 3 independent experiments varied from the mean by 10% or less.

copies of the fusion, the Φ(*rhaS-lacZ*) fusion was recombined onto a λ phage and then assayed as a single copy lysogen.

Expression of the Φ(*rhaS-lacZ*) fusion in a strain wild-type at the normal chromosomal *rha* locus was induced approximately 400-fold by L-rhamnose, but to a relatively low level of 110 Miller units (Table 3). In the Δ*rhaS* strain, fusion expression was inducible by L-rhamnose, in fact, to a level three-fold higher than in the wild-type strain. Deletion of both *rhaS* and *rhaR* abolished L-rhamnose induction of fusion expression. These results agree with the previous finding that RhaR is the inducer of *rhaSR* expression, and verify that the Δ*rhaS* strain (SME1053) is *rhaR*⁺.

(c) *RhaR binding to* rhaBAD *promoter fragments*

To determine whether RhaR might directly regulate *rhaBAD* expression, we tested whether purified RhaR protein would bind to various *rhaBAD* promoter fragments. The fragments had the same endpoints as those used to construct the Φ(*rhaB-lacZ*) fusions (Figs 1 and 2), and the binding reactions contained 50 mM L-rhamnose.

A DNA mobility shift assay showed that RhaR readily bound to DNA fragment Δ226 (Fig. 3), which contains the RhaR binding site that serves p_{SR} (Tobin & Schleif, 1990*b*). The binding site at p_{SR} has a K_D of approximately 3×10^{-13} M at 50 mM KCl. RhaR binding was not detected with fragments Δ153 or Δ110 (Fig. 3). If we estimate that 5% bound DNA could be easily detected, the K_D for RhaR binding to a site on these fragments must be greater than or equal to 2×10^{-8} M. This experiment suggests that no additional RhaR binding sites are likely to exist in the *rhaSR-rhaBAD* regulatory region. As Φ(*rhaB-lacZ*) fusions Δ226, Δ153 and Δ110 all have similar levels of β-galactosidase expression, it is unlikely that the RhaR binding site in the *rhaS-rhaB* regulatory region plays any direct role in *rhaBAD* regulation.

(d) *CRP binding to* rhaBAD *promoter fragments*

Sequence analysis suggested that deletion of a CRP binding site may have caused the decreased expression of Φ(*rhaB-lacZ*) fusion Δ84 compared with Δ110 (see Fig. 2). To test this we examined CRP binding to the same *rhaBAD* promoter fragments used to construct the Φ(*rhaB-lacZ*) fusions (Figs 1 and 2). The binding reactions contained 100 μM cAMP.

The DNA mobility shift assay showed that CRP protein bound to fragments Δ226 and Δ110 with a K_D less than 10^{-9} M, and generated only a single shifted species (Fig. 4). No CRP binding to fragment Δ70 was detected. If we again estimate that 5% bound DNA could be easily detected, the K_D of any sites on Δ70 must be greater than or equal to

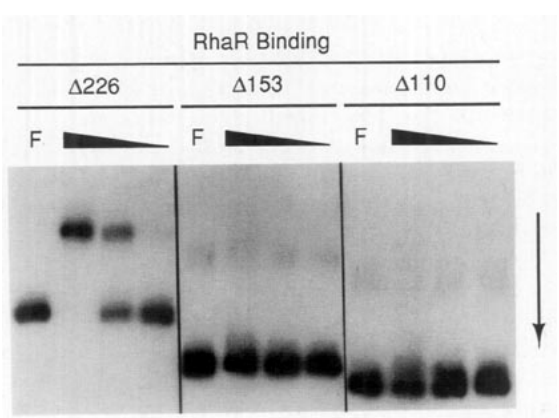


Figure 3. DNA mobility shift assays of RhaR binding to DNA fragments Δ226, Δ153 and Δ110. The vertical arrow indicates the direction of electrophoresis. The faint band in all Δ153 and Δ110 lanes (including free DNA lanes) most likely is single-stranded DNA. DNA concentration was approximately 10^{-9} M. Approximate active RhaR concentration per reaction was: 1st lane in each set, F, no RhaR added; 2nd lane in each set, $1{\cdot}1 \times 10^{-9}$ M RhaR; 3rd lane in each set, $3{\cdot}8 \times 10^{-10}$ M RhaR; 4th lane in each set, $1{\cdot}1 \times 10^{-10}$ M RhaR.

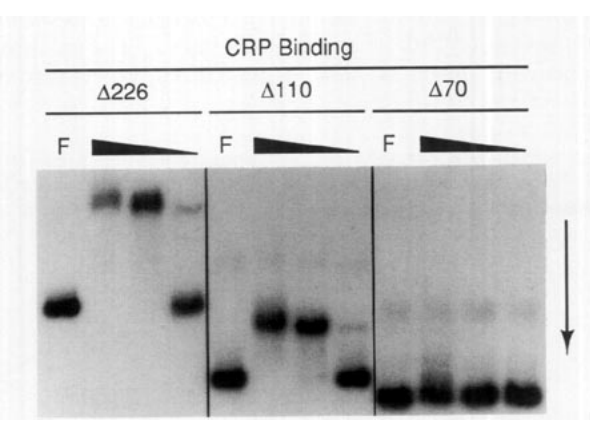


Figure 4. DNA mobility shift assays of CRP binding to DNA fragments Δ226, Δ110 and Δ70. The vertical arrow indicates the direction of electrophoresis. The faint band in all Δ110 and Δ70 lanes (including free DNA lanes) most likely is single-stranded DNA. DNA concentration was approximately 10^{-9} M. Approximate active CRP concentration per reaction was: 1st lane in each set, F, no CRP added; 2nd lane in each set: $7{\cdot}5 \times 10^{-9}$ M CRP; 3rd lane in each set, $7{\cdot}5 \times 10^{-10}$ M CRP; 4th lane in each set, $7{\cdot}5 \times 10^{-11}$ M CRP.

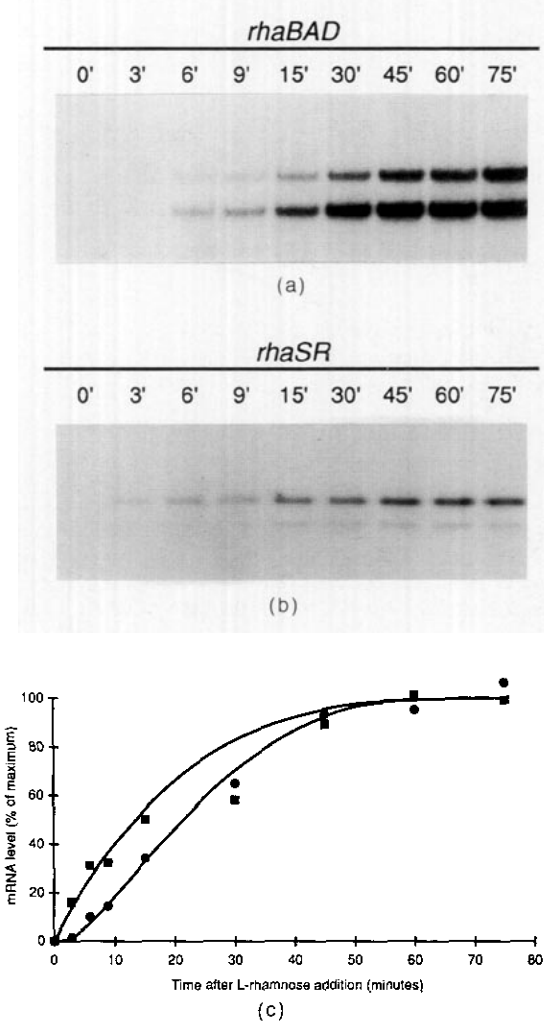


Figure 5. Induction time course of mRNA accumulation from chromosomal *rhaBAD* and *rhaSR* operons. mRNA accumulation was measured by primer extension assay. (a) *rhaBAD* mRNA levels at the indicated time points after L-rhamnose addition. The 2 bands are 138 and 133 nt long. The lower band represents either a secondary start site, or a degradation product of the upper band. Quantitation included both bands. (b) *rhaSR* mRNA levels at the indicated time points after L-rhamnose addition. The 2 bands are 75 and 73 nt long. (c) Plot of (●) *rhaBAD* and (■) *rhaSR* mRNA levels *versus* time after L-rhamnose addition. The levels of the specific transcripts were determined using a Molecular Dynamics PhosphorImager, and the curves were drawn by inspection.

10^{-7} M. These results indicate that there is a single CRP binding site in this region. This binding site likely corresponds to the region centered at −9·25 with sequence similarity to the CRP consensus binding site (see Fig. 1).

(e) rhaBAD *and* rhaSR *mRNA induction kinetics*

Tobin & Schleif (1987) previously reported that L-rhamnose induction from promoters identified as p_1, p_2 and p_3, was unusually slow, while induction of p_{SR} mRNA was faster. We now believe that p_1 and p_2 are not actually promoters, but sites at which the full length *rhaBAD* transcript is cleaved between *rhaB* and *rhaA* (Moralejo *et al.*, 1993). The origin of the p_3 transcript is not currently understood. We performed a more detailed analysis of L-rhamnose-induced mRNA accumulation from p_{BAD} and p_{SR} using quantitative primer extension analysis on total RNA. To eliminate potential artifacts due to catabolism of L-rhamnose, a *rhaA*$^-$ strain, ECL715 (Chen *et al.*, 1987), was used.

Message accumulated unusually slowly from both the *rhaBAD* and *rhaSR* operons (Fig. 5), requiring 40 to 50 minutes until steady-state levels were reached. In contrast, only three minutes was required to reach steady-state levels of *araBAD* mRNA (data not shown). The kinetics for the two *rha* operons were very similar, except that there was a slight lag in the accumulation from *rhaBAD* that was not seen from *rhaSR*. This difference was reproduced in at least three independent experiments.

Similar experiments were also performed with an isogenic wild-type strain, ECL116, grown in minimal media either with or without Casamino acids, or with Casamino acids, but 10-fold higher (2%) L-rhamnose concentration (data not shown). The kinetics of mRNA accumulation from *rhaBAD* were essentially the same as those shown in Figure 5 under all of these conditions. Slow induction even in the presence of 2% L-rhamnose suggests that L-rhamnose transport did not limit the induction rate.

It is likely that Tobin & Schleif's (1987) interpretation of fast induction of *rhaSR* message was in error due to their widely spaced time points and the relatively high cell density (A_{600} of approximately 0·75, Tobin, 1989) at which their culture was induced. We have found that the level of *rhaSR* message decreases as an induced culture enters stationary phase (unpublished results). Our results suggest that their 30-minute sample was too early to represent full induction, and that their 120-minute sample may have entered stationary phase, and therefore not represented full induction either. As a result, their culture reached an apparent, but probably not true, fully induced level more quickly than ours.

(f) *Effect of* rhaS *overexpression on* rhaBAD *induction kinetics*

Our analysis of Φ(*rhaB-lacZ*) expression in Δ*rhaS* and Δ(*rhaSR*)::Km strains indicated that *rhaS*$^+$ was required for *rhaBAD* expression. Since limiting availability of RhaS might be a factor in the normally slow speed of *rhaBAD* induction (see Fig. 5), we compared the β-galactosidase expression of four strains, each carrying Φ(*rhaB-lacZ*) fusion Δ226 on a λ specialized transducing phage. Three strains, Δ*rhaS*, Δ(*rhaSR*)::Km and wild-type at *rhaSR*, each carried a plasmid (pJTC108) which overexpressed *rhaS* from a *tac* promoter (Amann *et al.*, 1983; deBoer *et al.*, 1983). The fourth strain was wild-type at *rhaSR* and did not carry the plasmid.

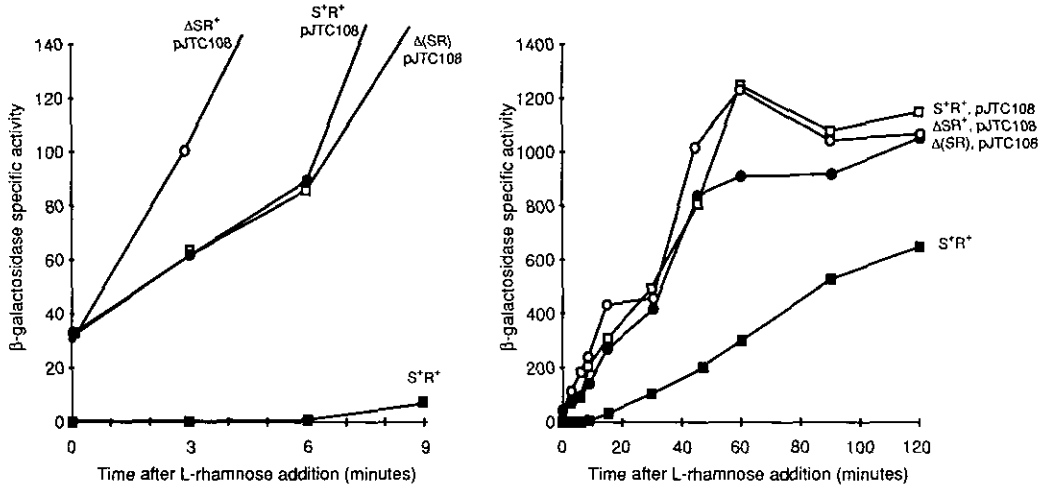


Figure 6. Induction time course of β-galactosidase expression from Φ(*rhaB-lacZ*)Δ226 in various backgrounds. β-Galactosidase activity is plotted *versus* time after induction with L-rhamnose. The left graph shows only the early time points, while the right graph shows the entire time course. Plasmid pJTC108 overexpresses *rhaS*. The relevant genotypes of the strain backgrounds are (■) $rhaS^+R^+$, (□) $rhaS^+R^+$, pJTC108, (○) Δ*rhaS*, $rhaR^+$, pJTC108, (●) Δ(*rhaSR*)::Km, pJTC108.

In the wild-type strain background with no plasmid, β-galactosidase accumulation from Φ(*rhaB-lacZ*) was slow (Fig. 6), consistent with the mRNA induction kinetics (Fig. 5). As shown in Figure 6, overexpression of *rhaS* in any of the three strain backgrounds resulted in a dramatically faster β-galactosidase accumulation. In addition, the strains overexpressing *rhaS* significantly induced transcription in the absence of inducer (30 units compared with the normal 0·01 units). These results indicate that RhaS protein is a limiting factor in the normal induction of *rhaBAD* expression, and also suggest that RhaS may be directly involved in *rhaBAD* induction.

This experiment also showed that when *rhaS* was expressed from a plasmid with a heterologous promoter, RhaR was not required for p_{BAD} induction. We believe that this was not simply due to cross-reactivity between RhaS and RhaR when overproduced since *rhaR* overexpression was unable to restore any activation to either deletion strain (data not shown). This suggests that the only RhaR requirement for *rhaBAD* induction is indirect, through *rhaSR* induction.

(g) In vitro *regulation of* rhaSR *and* rhaBAD

As we have thus far been unable to test whether RhaS binds DNA at p_{BAD} due to insolubility of the protein, we sought a different method to determine whether RhaS is sufficient to mediate L-rhamnose induction of *rhaBAD* expression. Previous work had shown that, in an S30 coupled transcription-translation system, the *araBAD* operon could be induced either by adding purified AraC protein, or by inclusion of $araC^+$ DNA in the reaction (see Zubay, 1973). In the case where *araC* DNA was added, the $araC^+$ DNA was first transcribed and translated, and the resulting protein activated *araBAD* transcription. We performed a similar experiment to test whether RhaS alone could induce *rhaBAD* expression.

We tested whether inclusion of $rhaS^+$ DNA in the S30 extract could allow L-rhamnose-inducible expression from p_{BAD} by assaying expression from fusion Φ(*rhaB-lacZ*)Δ153. Fusions of *lacZ* to the *rha* promoters were used to simplify both enzymatic assay and SDS/polyacrylamide gel separation of the reaction products. The plasmid-encoded *rhaS* and *rhaR* genes were under the control of the *tac* promoter (Amann *et al.*, 1983; deBoer *et al.*, 1983), which allowed expression of *rhaS* even in a $RhaR^-$ context. The S30 extract was made from a crp^+ strain, and the reaction mix contained cAMP, so cAMP-CRP activation of Φ(*rhaB-lacZ*) could occur.

To test the S30 system, we first demonstrated that inclusion of $rhaR^+$ DNA was necessary and sufficient to allow L-rhamnose-inducible expression from p_{SR}, by assaying expression from Φ(*rhaS-lacZ*)896 (Fig. 7(a)). This is consistent with the finding of Tobin & Schleif (1987, 1990*a*,*b*) that RhaR protein is the direct L-rhamnose inducer of *rhaSR* expression, and demonstrates that normal regulation can be duplicated in the S30 system.

We next examined the regulation of the *rhaBAD* promoter in the S30 system. There was detectable expression from p_{BAD} ony when the *rhaS* gene and L-rhamnose were present in the reaction (Fig. 7(b)). When the p_{BAD} plasmid or the $rhaS^+$ plasmid were added independently, in the presence of L-rhamnose, only basal level β-galactosidase activity was detected (data not shown). Expression from p_{BAD} was undetectable if a second plasmid carried $rhaR^+$, or under any condition where L-rhamnose was not added. These results also strongly support the idea that RhaS protein, in the presence of L-rhamnose, is sufficient for *rhaBAD* expression.

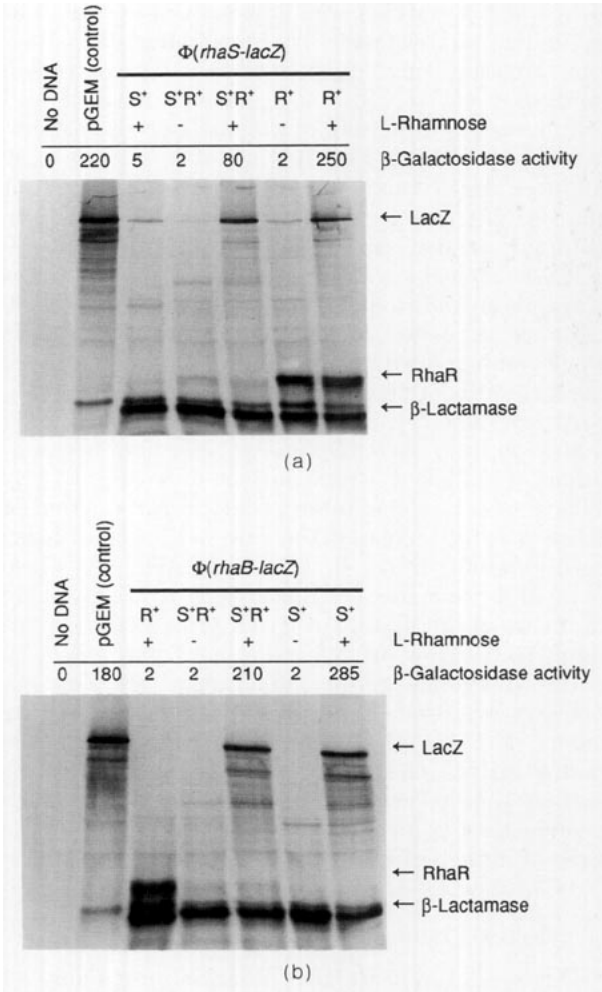


Figure 7. *In vitro* regulation of *rhaSR* and *rhaBAD*. Coupled transcription–translation reactions in an *E. coli* S30 extract were performed to detect β-galactosidase expression from either Φ(*rhaS-lacZ*) or Φ(*rhaB-lacZ*) fusions. *rhaS*, *rhaR*, or both were added to the reactions on plasmids as indicated. L-Rhamnose was also added as indicated. pGEM is a control plasmid that expresses β-galactosidase from a *lac* promoter, and β-lactamase. L-Galactosidase activity is indicated above each lane, and the [^{35}S]methionine-labeled LacZ, RhaR and β-lactamase proteins produced from the plasmids are indicated to the right. (a) Expression from pSME114 (Φ(*rhaS-lacZ*)896). (b) Expression from pSME104 (Φ(*rhaB-lacZ*)Δ153).

4. Discussion

Our experiments were designed to address the following questions concerning the regulation of *rhaBAD* expression. (1) What is the location and relative contribution of regulatory protein binding sites at p_{BAD}? (2) What are the proteins that bind these sites at p_{BAD} to directly influence *rhaBAD* transcription? (3) Why is the kinetics of *rhaBAD* transcription so slow? Based on the results of our experiments, we propose that *rhaBAD* induction involves a cascade in which RhaR first induces *rhaSR* expression, then RhaS accumulates and induces *rhaBAD* expression. This proposal is further described below.

(a) *CRP protein activates* rhaBAD *expression*

Our promoter deletions of Φ(*rhaB-lacZ*) fusions suggest that there are two *cis*-acting elements involved in normal *rhaBAD* regulation. Deletion of the upstream element resulted in a 60-fold decrease in the L-rhamnose-induced level of p_{BAD} transcription (Fig. 2). Within this deleted region, at −92·5 relative to the start of transcription, there is a site (TGTGA-N_6-TCACG) with identical spacing, and a 9 out of 10 bp match with the core consensus for CRP protein binding: TGTGA-N_6-TCACA (Berg & von Hippel, 1988). DNA mobility shift assays showed that CRP protein binds to the DNA region containing the proposed CRP binding site (Fig. 4). Thus, we conclude that CRP protein contributes to *rhaBAD* activation by binding to the site centered at −92·5.

(b) *An* L-*rhamnose responsive regulatory element is downstream of the CRP binding site*

Promoter deletions of Φ(*rhaB-lacZ*) fusions suggested a second *cis*-acting element, downstream of the CRP site, involved in *rhaBAD* regulation. Deletion of this region virtually eliminated L-rhamnose activation, suggesting that it is the L-rhamnose-responsive regulatory element. Fusion Δ70 showed a residual fourfold L-rhamnose induction. This may indicate that part of the regulatory site remained, and was capable of a low level of activator binding. The position of a potential binding site is discussed below.

(c) *RhaR does not directly activate* rhaBAD *expression*

Tobin & Schleif (1987) previously concluded that L-rhamnose induction of *rhaBAD* expression requires RhaR, but not RhaS. RhaR was, therefore, the most likely candidate for the direct activator of *rhaBAD* expression. We performed two experiments which showed that RhaR is not the direct activator.

A DNA mobility shift assay (Fig. 3) indicated that the only site in the *rhaSR-rhaBAD* regulatory region capable of binding RhaR was the site at p_{SR} previously identified by Tobin & Schleif (1990*b*). Deletion of this site did not affect Φ(*rhaB-lacZ*) expression (Fig. 2, compare Δ226 and Δ153), indicating that RhaR is not directly involved in *rhaBAD* induction.

Complementation analysis showed that L-rhamnose induction of p_{BAD} in a Δ(*rhaR*) background required a *rhaS*$^+$ plasmid (Fig. 6), but was unaffected by a *rhaR*$^+$ plasmid (data not shown). These results further indicate that RhaR is not directly required for *rhaBAD* induction.

(d) *RhaS directly activates* rhaBAD *expression*

We have thus far been unable to obtain either purified RhaS protein that remains soluble, or a cell extract with overproduced, soluble RhaS protein, to test directly whether RhaS binds at p_{BAD}. We have

instead accumulated a variety of pieces of evidence that, taken together, strongly suggest that RhaS is the direct L-rhamnose regulator of *rhaBAD* transcription.

First, there was no induction of Φ(*rhaB-lacZ*) fusions in a Δ*rhaS* strain, indicating that RhaS is required for *rhaBAD* expression. Second, evidence described above indicates that RhaR is not directly involved in *rhaBAD* expression. Third, compared with a wild-type strain alone, overexpression of *rhaS* resulted in greatly speeded induction of Φ(*rhaB-lacZ*) in wild-type, Δ*rhaS*, and Δ(*rhaSR*) strain backgrounds (Fig. 6). This indicates that RhaS protein is a limiting factor in *rhaBAD* induction and is consistent with the idea that RhaS is the direct L-rhamnose activator.

Finally, results of *E. coli* S30 coupled transcription–translation assays (Zubay, 1973) showed that p_{BAD} was expressed only when the reaction contained *rhaS*$^+$ DNA and L-rhamnose (Fig. 7(b)). This experiment indicates that RhaS is sufficient to allow L-rhamnose-induced expression of *rhaBAD*. Since the S30 reactions did not contain any unidentified DNA, this experiment ruled out the possibility that RhaS is required to activate transcription of a gene encoding some unknown regulatory protein.

While Tobin & Schleif (1987) concluded that RhaS had only a small affect on induction of *rhaBAD* transcripts, we now know that the strain used in their analysis actually carried the entire *rhaS* gene (data not shown). Taking into account that the strain was *rhaS*$^+$, Tobin and Schleif's data are entirely consistent with ours, and support the conclusion that RhaR is only directly required for *rhaSR* induction, and that RhaS is directly required for *rhaBAD* induction.

If RhaS is the direct L-rhamnose activator of *rhaBAD* transcription, then the RhaS binding site must overlap the Δ70 upstream endpoint (Fig. 2). There is an inverted repeat upstream of *rhaBAD* which overlaps the −35 sequence and has repeated sequences of 17 bp in length separated by 16 bp (Fig. 1). Δ70 would delete 11 bp from the upstream end of this sequence. This inverted repeat is a good candidate for a RhaS binding site based on the likelihood that the RhaS binding site would have features in common with RhaR and AraC binding sites. RhaR and AraC binding sites both overlap the −35 sequence, and include two 17 bp half-sites, but differ in separation of the half-sites (17 and 4 bp, respectively) and symmetry of the half-sites (inverted and direct repeat, respectively). Interestingly, while the spacing of the proposed RhaS binding site is more like the RhaR site, the position of the CRP site, −92·5, is essentially the same as an AraC regulated operon, −91·5 (Reeder & Schleif, 1993).

(e) p_{BAD} *and* p_{SR} *turn on slowly*

Tobin and Schleif (1987) previously reported, based on S_1 nuclease mapping, that the induction of p_1, p_2 and p_3 mRNA was unusually slow, but that p_{SR} induction was faster. We re-examined the induction kinetics using quantitative primer extension analysis.

Transcripts from each of the *rha* operons accumulated quite slowly (Fig. 5). In both cases, 40 to 50 minutes after L-rhamnose addition was required for the mRNA to reach a steady-state value. In contrast, under the same conditions, *araBAD* mRNA reached steady-state in three minutes after L-arabinose addition (data not shown). The 40 to 50 minutes to reach steady-state is especially long when compared with the 45-minute doubling time of the strain under the conditions of the experiment.

At least one factor in the slow kinetics of mRNA induction from *rhaBAD* appears to be slow accumulation of sufficient RhaS protein to saturate p_{BAD}. This is based on the finding that overproduction of RhaS greatly increased the rate of β-galactosidase accumulation from Φ(*rhaB-lacZ*) (Fig. 6). Since *rhaS* and *rhaR* are co-transcribed, it seems likely that slow accumulation of RhaR protein also contributes to the slow mRNA induction from *rhaSR*.

One difference in the induction of the two *rha* operons was that there was a several-minute lag before *rhaBAD* mRNA began to accumulate, while *rhaSR* mRNA accumulation was detectable at the earliest time point. This suggests that active RhaS protein may be even more severely limiting at the time of induction than RhaR protein.

(f) rhaBAD *induction involves a regulatory cascade*

Our results indicate that RhaS is most likely an L-rhamnose-responsive, DNA-binding protein, capable of activating transcription from *rhaBAD* (Fig. 1). They further indicate that *rhaBAD* induction is a multistep process, as described in the following scheme.

When L-rhamnose becomes available to the cell, basal-level RhaR protein binds the sugar, and becomes activated to induce expression from p_{SR}. Basal level RhaS protein is apparently capable of promoting only an extremely low level of *rhaBAD* expression. As *rhaSR* is expressed, higher levels of both RhaS and RhaR accumulate. RhaS then begins to activate *rhaBAD* expression, while RhaR further activates *rhaSR* expression. Full induction of *rhaBAD* transcription also requires binding of the CRP-cAMP complex.

It appears that the extraordinary 30,000-fold L-rhamnose induction of *rhaBAD* expression is due to the two-step induction process. First, L-rhamnose activation of RhaR protein most likely increases the level of RhaS protein by approximately 400-fold (Table 3). Based on experiments where RhaS was produced at a constant high level in the absence and presence of L-rhamnose, we estimate that the activity of RhaS protein for transcriptional activation is increased approximately 300-fold by L-rhamnose (unpublished results). The 400-fold increase in RhaS level, multiplied by the 300-fold increase in RhaS activity, would lead to a 120,000-fold induc-

tion, which is on the order of the 30,000-fold observed. At least two other members of the AraC family, XylS and SoxS, are also part of two-step regulatory cascades (Inouye *et al.*, 1987; Wu & Weiss, 1992).

In principle, two mechanisms could account for the slow accumulation of *rhaBAD* mRNA. The slow rate could reflect slow induction in each cell, or it could reflect slow induction over the population. It is possible that the basal level of RhaR is so low that only some of the cells are capable of immediately inducing *rhaSR* expression, while others require an accidental firing of p_{SR} before RhaR is available and induction can occur. This seems unlikely since overproduction of RhaR resulted in only a small increase in the rate of induction of a Φ(*rhaB-lacZ*) fusion (unpublished results). It seems likely, therefore, that slow induction occurs in each cell.

Interestingly, RhaS seems to have a negative effect on *rhaSR* expression. This is supported by the finding of threefold increased expression of Φ(*rhaS-lacZ*) in a *rhaS* deletion strain (Table 3), and 1·5-fold decreased expression of Φ(*rhaS-lacZ*) in a strain that overexpressed *rhaS* from a *tac* promoter (unpublished results). It is possible that once sufficient RhaS protein accumulates, it feeds back to decrease expression of *rhaSR*, perhaps by formation of inactive RhaS-RhaR heterodimers, interaction of RhaS and RhaR from their binding-sites, or competition with RhaR for binding at p_{SR}.

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References

Amann, E., Brosius, J. & Ptashne, M. (1983). Vectors bearing a hybrid *tryp-lac* promoter useful for regulated expression of cloned genes in *Escherichia coli*. *Gene*, **25**, 167–178.

Arnheim, N. (1990). The polymerase chain reaction. In *Genetic Engineering* (Setlow, J. K., ed.), vol. 12, pp. 115–137, Plenum Press, New York.

Backman, K., Chen, Y.-M. & Magasanik, B. (1981). Physical and genetic characterization of the *glnA-glnG* region of the *Escherichia coli* chromosome. *Proc. Nat. Acad. Sci., U.S.A.* **78**, 3743–3747.

Baldomá, L., Badía, J., Sweet, G. & Aguilar, J. (1990). Cloning, mapping and gene product identification of *rhaT* from *Escherichia coli* K12. *FEMS Microbiol. Letters*, **72**, 103–108.

Berg, O. G. & von Hippel, P. H. (1988). Selection of DNA binding sites by regulatory proteins. II. The binding specificity of cyclic AMP receptor protein to recognition sites. *J. Mol. Biol.* **200**, 709–723.

Brunelle, A. & Schleif, R. (1989). Determining residue–base interactions between AraC protein and *araI* DNA. *J. Mol. Biol.* **209**, 607-622.

Bustos, S. A. & Schleif, R. F. (1993). Functional domains of the AraC protein. *Proc. Nat. Acad. Sci., U.S.A.* **90**, 5638–5642.

Carra, J. H. & Schleif, R. F. (1993). Variation of half-site organization and DNA looping by AraC protein. *EMBO J.* **12**, 35–44.

Chen, Y.-M., Tobin, J. F., Zhu, Y., Schleif, R. F. & Lin, E. C. C. (1987). Cross-induction of the L-fucose system by L-rhamnose in *Escherichia coli*. *J. Bacteriol.* **169**, 3712–3719.

Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocynate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159.

Cubellis, M. V., Marino, G., Maynol, L., Piccialli, G. & Sannia, G. (1985). Use of fast protein liquid chromatography for the purification of synthetic oligonucleotides. *J. Chromatogr.* **329**, 406–414.

Davis, R. W., Botstein, D. & Roth, J. R. (1980). *Advanced Bacterial Genetics, A Manual for Genetic Engineering*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

deBoer, H. A., Comstock, L. J. & Vasser, M. (1983). The *tac* promoter: a functional hybrid derived from the *trp* and *lac* promoters. *Proc. Nat. Acad. Sci., U.S.A.* **80**, 21–35.

Gallegos, M.-T., Michán, C. & Ramos, J. L. (1993). The XylS/AraC family of regulators. *Nucl. Acids Res.* **21**, 807–810.

García-Martín, C., Baldomá, L., Badía, J. & Aguilar, J. (1992). Nucleotide sequence of the *rhaA-sodA* interval specifying *rhaT* in *Escherichia coli*. *J. Gen. Microbiol.* **138**, 1109–1116.

Hamilton, C. M., Aldea, M., Washburn, B. K., Babitze, P. & Kushner, S. (1985). New method for generating deletions and gene replacements in *Escherichia coli*. *J. Bacteriol.* **171**, 4617–4622.

Hendrickson, W. & Schleif, R. F. (1984). Regulation of the *Escherichia coli* L-arabinose operon studied by gel electrophoresis DNA binding assay. *J. Mol. Biol.* **174**, 611–628.

Inouye, S., Nakazawa, A. & Nakazawa, T. (1987). Expression of the regulatory gene *xylS* on the TOL plasmid is positively controlled by the *xylR* gene product. *Proc. Nat. Acad. Sci., U.S.A.* **84**, 5182–5186.

Johnston, R. J., Pickett, S. C. & Barker, D. L. (1990). Autoradiography using storage phosphor technology. *Electrophoresis*, **11**, 355–360.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)*, **227**, 680–685.

Miller, J. H. (1972). *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Moralejo, P., Egan, S. M., Hidalgo, E. & Aguilar, J. (1993). Sequencing and characterization of a gene cluster encoding the enzymes for L-rhamnose metabolism in *Escherichia coli*. *J. Bacteriol.* **175**, 5585–5594.

Ramos, J. L., Rojo, F., Zhou, L. & Timmis, K. N. (1990). A family of positive regulators related to the *Pseudomonas putida* TOL plasmid XylS and the *Escherichia coli* AraC activators. *Nucl. Acids Res.* **18**, 2149–2152.

Reeder, T. & Schleif, R. (1993). AraC protein can activate transcription from only one position and when pointed in only one direction. *J. Mol. Biol.* **231**, 205–218.

Reichert, W. L., Stein, J. E., French, B., Goodwin, P. & Varanasi, U. (1992). Storage phosphor imaging technique for detection and quantitation of DNA adducts measured by the ^{32}P-postlabeling assay. *Carcinogenesis*, **13**, 1475–1479.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sawadogo, M. & Van Dyke, M. W. (1991). A rapid method for the purification of deprotected oligodeoxynucleotides. *Nucl. Acids Res.* **19**, 674.

Schleif, R. & Wensink, P. (1981). *Practical Methods in Molecular Biology*, Springer-Verlag, New York.

Simons, R. W., Houman, F. & Kleckner, N. (1987). Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. *Gene*, **53**, 85–96.

Stewart, V. & Yanofsky, C. (1985). Evidence for transcription antitermination control of tryptophanase operon expression in *Escherichia coli* K-12. *J. Bacteriol.* **164**, 731–740.

Tate, C. G., Muiry, J. A. R. & Henderson, P. J. F. (1992). Mapping, cloning, expression, and sequencing of the *rhaT* gene, which encodes a novel L-rhamnose-H^+ transport protein in *Salmonella typhimurium* and *Escherichia coli*. *J. Biol. Chem.* **267**, 6923–6932.

Tobin, J. F. (1989). PhD Dissertation. Biochemistry Dept., Brandeis University, Waltham, MA 02254.

Tobin, J. F. & Schleif, R. F. (1987). Positive regulation of the *Escherichia coli* L-rhamnose operon is mediated by the products of tandemly repeated regulatory genes. *J. Mol. Biol.* **196**, 789–799.

Tobin, J. F. & Schleif, R. F. (1990*a*). Transcription from the *rha* operon p_{SR} promoter. *J. Mol. Biol.* **211**, 1–4.

Tobin, J. F. & Schleif, R. F. (1990*b*). Purification and properties of RhaR, the positive regulator of the L-rhamnose operons of *Escherichia coli*. *J. Mol. Biol.* **211**, 75–89.

Vieira, J. & Messing, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene*, **19**, 259–268.

Wu, J. & Weiss, B. (1992). Two-stage induction of the *soxRS* (superoxide response) regulon of *Escherichia coli*. *J. Bacteriol.* **174**, 3915–3920.

Zubay, G. (1973). *In vitro* synthesis of protein in microbial systems. *Annu. Rev. Genet.* **7**, 267–287.

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