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Regulation of *aroL* Expression by TyrR Protein and Trp Repressor in *Escherichia coli* K-12

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The promoter-operator region of the *aroL* gene of *Escherichia coli* K-12 contains three TYR R boxes and one TrpR binding site. Mutational analysis showed that TYR R boxes 1 and 3 are essential for TyrR-mediated regulation of *aroL* expression, while a fully functional TYR R box 2 does not appear to be essential for regulation. Regulation mediated by the TrpR protein required the TYR R boxes and TrpR site to be functional and was observed in vivo only with a *tyrR*$^+$ strain. Under conditions favoring the formation of TyrR hexamers, DNase I protection experiments revealed the presence of phased hypersensitive sites, indicative of DNA backbone strain. This suggests that TyrR-mediated repression involves DNA looping. Purified TrpR protein protected the putative TrpR binding site in the presence of tryptophan, and this protection was slightly enhanced in the presence of TyrR protein. This result along with the in vivo findings implies that TyrR and TrpR are able to interact in some way. Inserting 4 bp between TYR R box 1 and the TrpR binding site results in increased tyrosine repression and the abolition of the tryptophan effect. Identification of a potential integration host factor binding site and repression studies of a *himA* mutant support the notion that integration host factor binding normally exerts a negative effect on tyrosine-mediated repression.

The *aroL* gene encodes shikimate kinase II, an enzyme involved in the common pathway for the biosynthesis of aromatic amino acids (15). *aroL* is cotranscribed with a second gene, *aroM*, which codes for a 26-kDa product of unknown function (13). The TyrR regulatory protein represses transcription from *aroL* in the presence of tyrosine or tryptophan and to a lesser extent in the presence of phenylalanine, thus making *aroL* a member of the TyrR regulon (13, 15). Three TyrR binding sites (or TYR R boxes) around the *aroL* promoter have been identified. One box overlaps the −35 region, while the other two boxes, separated by 1 bp (termed a double box), lie downstream of the −10 region (13). All units of the TyrR regulon which are repressed in the presence of tyrosine contain this double box motif (11–13, 16, 21, 30, 45). Wilson et al. (41) recently reported that TyrR can form a stable hexamer in the presence of tyrosine and ATP. This hexameric protein is thought to be the active repressing species at tyrosine-repressible promoters and could, theoretically, interact with two or three TYR R boxes at once.

Recently, it was shown that *aroL* is, in fact, under the dual control of TrpR and TyrR proteins. A putative TrpR binding site downstream of the TYR R boxes was identified, and TrpR was shown to mediate the repression of *aroL*, but only in the presence of tryptophan and in *tyrR*$^+$ strains (18).

In this paper, we describe base substitution mutations in each of the TYR R boxes and the TrpR binding site in *aroL* and discuss the regulatory roles of these operator sites. We also investigate the interaction between TyrR and TrpR regulatory proteins and their respective operator sites and postulate a potential model for *aroL* regulation, incorporating the new TyrR hexamer structure.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study were all derivatives of *Escherichia coli* K-12, and their relevant characteristics are shown in Table 1. The plasmids used are also listed in Table 1.

Media and chemicals. The minimal medium (MM) used was the half-strength 56 buffer of Monod et al. (26) supplemented with 0.2% glucose and appropriate growth factors. To study regulation, we added phenylalanine, tyrosine, and tryptophan to MM at final concentrations of 1 mM each. Trimethoprim was used in nutrient medium and MM at final concentrations of 40 and 10 μg/ml, respectively. Ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were each used at a final concentration in all media of 25 μg/ml. All of the chemicals used were obtained commercially and not purified further. [α-^{32}P]dATP (ca. 2,000 to 3,000 Ci/mmol; 10 Ci/ml) for labelling fragments to be used in DNase I footprinting was obtained from NEN-DuPont.

Recombinant DNA techniques. Standard techniques were used essentially as described by Sambrook et al. (33). DNA sequencing of operator mutants involved cloning the respective promoter-operator regions into the M13tg130 and M13tg131 vectors (22) and sequencing by the chain termination method of Sanger at al. (34) with modified T7 polymerase (38).

Plasmid construction. The translational fusion vector pMU2386 was created by modifying pMU525 (31) as follows. The coding regions of *lacY* and *lacA* were deleted as a *Dra*I fragment, and a transcriptional terminator was inserted downstream of the β-galactosidase gene to prevent readthrough from *lacZ* into the vector. The polylinker, which is fused to the eighth codon of *lacZ*, was also modified to contain six unique sites, *Pst*I, *Eco*RI, *Hin*dIII, *Bgl*II, *Sal*I, and *Bam*HI (starting with the one farthest from *lacZ*). A second transcriptional terminator was inserted upstream of the multicloning site to prevent readthrough into the *lacZ* gene from upstream sequences. DNA fragments containing the appropriate translational start signals and the amino-terminal portion of a gene can be cloned into the polylinker, resulting in the formation of a hybrid protein with β-galactosidase activity (Fig. 1).

The plasmid pMU4624 contains the regulatory region and part of the coding region of the *aroL* gene translationally fused

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TABLE 1. *E. coli* K-12 strains and plasmids used in this work

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
JP3561	*thr-1 leu-1 lacZΔM15 supE44 tonA2 gyrA379 aroL478*::Tn*10*	21
JP4822	JP3561 *tyrR366*	45
JP7218	JP3561 *trpR363*	35
JP7219	JP4822 *trpR363*	35
JP7740	*ΔlacU169 recA56*	44
JP10721	*tyrR366 trpR363 himA*::*cat ΔlacU169 recA56 srl1300*::Tn*10*	This laboratory
JP10756	JP7740 *himA*::*cat*	This laboratory
Plasmids		
M13tg131	*lacPOZ'*	22
pBR328	Ap^r Tc^r Cm^r; pMB1 replicon	37
pJPR2	Ap^r; *trpR* gene under the control of a *tac* promoter, *trp* leader, and ribosome binding site	29
pMU371	Ap^r Tc^r; *aroLM* operon in pBR322	This laboratory
pMU2386	Tp^r *lacZ'*; low-copy-number translational fusion vector	This laboratory
pMU4624	Tp^r; 361-bp *aroL* fragment in pMU2386	This work
pMU4627	pMU4624 derivative; C→G at −6 and G→T at +6 of TrpR binding site	This work
pMU4630	pMU4624 derivative; C→T at +8 and G→A at −8 of TYR R box 1	This work
pMU4632	pMU4624 derivative; C→T at +8 and G→A at −8 of TYR R box 2	This work
pMU4634	pMU4624 derivative; C→T at +8 and G→A at −8 of TYR R box 3	This work
pMU4642	pMU4624 derivative; strong box 2 ($TGTAAAAN_6TTTACA$)	This work
pMU4643	pMU4642 derivative; C→T at +8 and G→A at −8 of TYR R box 3	This work
pMU4644	pMU4642 derivative; C→A at −6 and G→T at +6 of TrpR binding site	This work
pMU4651	pMU4624 derivative; 4-bp insertion between TYR R box 1 and TrpR site	This work
pMU4653	pMU4642 derivative; 4-bp insertion between TYR R box 1 and strong box 2	This work
pMU3325	Km^r; wild-type *tyrR* cloned into pSU39	This laboratory
pMU3326	Km^r; mutant $tyrR_{EQ274}$ cloned into pSU39	This laboratory
pSU39	Km^r Ap^r; p15A replicon	5
pUC19	Ap^r; pMB1 derivative	27

[a] The genetic nomenclature is that described by Bachmann (4). Allele numbers are indicated when known.

to the *lacZ* gene. By using pMU371 as a template, a 361-bp DNA fragment extending from positions −156 to +193 (relative to the start point of transcription) and incorporating an *Eco*RI site upstream and a *Bam*HI site downstream of *aroL* was amplified by PCR and cloned into M13tg131, in which its sequence was verified. The fragment was then cloned into the low-copy-number translational fusion vector pMU2386 so that codon 23 of *aroL* was joined in phase to codon 8 of the *lacZ* structural gene. The expression of β-galactosidase from this construct is placed under the control of transcription and translation initiation signals in the *aroL* gene.

Oligonucleotide-directed mutagenesis. Oligonucleotides were synthesized on a Pharmacia GeneAssembler Plus. Mutagenesis was performed on M13tg131 derivatives containing the *aroL* promoter-operator region from pMU4624 with an oligonucleotide-directed in vitro mutagenesis system kit from Amersham. Following the screening and isolation of desired mutants, the entire *aroL* fragment was sequenced to ensure that no base changes other than those planned had occurred. The *aroL* fragments were cloned into pMU2386; the resulting plasmids were transformed into JP3561, JP4822, JP7218, and JP7219; and β-galactosidase assays were performed.

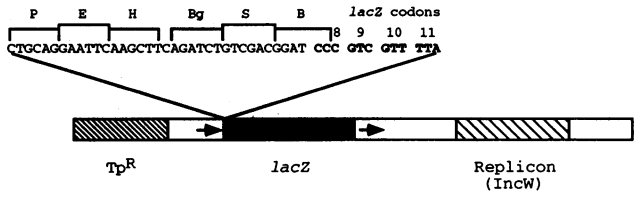

FIG. 1. Diagrammatic representation of the *lacZ* translational fusion vector pMU2386, in which codon 8 of the *lacZ* gene is linked to the polycloning site. Transcriptional terminators (arrows) terminate transcription from *lacZ* and transcription entering the polycloning site. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Tp^r, trimethoprim resistance.

Assay of β-galactosidase activity. Cultures were grown in half-strength 56 buffer containing 0.2% glucose and required growth factors at 37°C in a rotary water bath to the mid-exponential phase. β-Galactosidase activity was assayed as described by Miller (25).

DNase I footprinting of the *aroL* operator. Footprinting of the *aroL* operator was performed as previously described (2) with 361-bp *Eco*RI-*Bam*HI fragments from pUC19 derivatives of pMU4624 and various mutants. Plasmid DNA was digested with *Bam*HI and labelled with Klenow fragment in the presence of [α-^{32}P]dATP. DNA was digested with *Eco*RI, and then the resultant fragment was isolated on a 5% polyacrylamide gel. Purified TyrR repressor was produced by a method based on that developed by Argyropoulos et al. (3), and pure TrpR protein was produced from the overexpression plasmid pJPR2 by previously described methods (20, 29).

RESULTS

Regulation of *aroL* gene expression. A fragment containing the *aroL* promoter, putative operator sites, and translation initiation signals (as used by Heatwole and Somerville [18]) was cloned upstream of the *lacZ* structural gene in the low-copy-number translational fusion vector pMU2386 (see Materials and Methods). The resulting construct (pMU4624) was used to study the regulation of *aroL* gene expression in vivo.

This plasmid was transformed into the following strains:

TABLE 2. β-Galactosidase assays of strains containing an *aroL-lacZ* translational fusion vector

Strain	Background	β-Galactosidase-specific activity in[a]:					
		MM	Trp	Tyr	Phe	Tyr-Trp	Phe-Trp
JP3561	*tyrR*$^+$ *trpR*$^+$	365 (2.5)	214 (3.8)	85 (9.0)	282 (3.1)	16 (46.7)	132 (5.4)
JP4822	*tyrR366 trpR*$^+$	810 (1.1)	656 (1.2)	687 (1.1)	740 (1.2)	592 (1.3)	582 (1.2)
JP7218	*tyrR*$^+$ *trpR363*	452 (2.0)	395 (2.1)	137 (5.6)	297 (2.9)	129 (5.8)	299 (2.4)
JP7219	*tyrR366 trpR363*	921 (1.0)	813 (1.0)	766 (1.0)	871 (1.0)	747 (1.0)	717 (1.0)

[a] Trp, MM containing 1 mM tryptophan; Tyr, MM containing 1 mM tyrosine; Phe, MM containing 1 mM phenylalanine; Tyr-Trp, MM containing 1 mM tyrosine and tryptophan; Phe-Trp, MM containing 1 mM phenylalanine and tryptophan. The units of β-galactosidase-specific activity are those defined by Miller (25). Values in parentheses represent the extent of repression as the ratio of β-galactosidase activity in strain JP7219 to that in *tyrR*$^+$ or *trpR*$^+$ strains.

JP3561, haploid *tyrR*$^+$; JP4822, a *tyrR366* derivative (contains a frameshift mutation in the *tyrR* gene which leads to the formation of a truncated nonfunctional protein); JP7218, haploid *tyrR*$^+$ *trpR363*; and JP7219, a *tyrR366 trpR363* derivative. Strains were assayed for β-galactosidase activity after growth in MM in the presence or absence of various aromatic amino acids.

The expression of *aroL* was repressed approximately twofold by TyrR in the absence of effectors. In the presence of either phenylalanine or tryptophan, repression increased slightly to 3- to 4-fold, and in the presence of tyrosine, 9-fold repression was observed (Table 2). Combinations of tyrosine and tryptophan or phenylalanine and tryptophan mediated greater repression than that of each effector singly. In the absence of functional TyrR, almost no regulation was observed. In the absence of functional TrpR, however, tyrosine- and phenylalanine-mediated repression remained while tryptophan-mediated repression was not observed. In this case, combinations of tyrosine and tryptophan or phenylalanine and tryptophan showed levels similar to those obtained in the presence of tyrosine or phenylalanine, respectively. These results confirm the hypothesis that *aroL* is regulated by both TyrR and TrpR proteins, and the TrpR repressor appears to be active only in the presence of functional TyrR protein.

Analysis of operator sites. The symmetrical G · C base pairs within the TYR R box consensus sequence, TGTAAAN$_6$T TTACA, are present in all known TYR R boxes, and it is known that the alteration of either or both of these bases results in a loss of box function (30). In order to determine the regulatory role of individual boxes, we introduced changes in the invariant G and C of each box in the *aroL* operator region.

Mutations in box 1. The invariant G at position −8 and C at +8 in box 1 were changed to an A and T, respectively (pMU4630), and the β-galactosidase assay results are shown in Table 3. These changes led to a significant reduction in *aroL* repression under all conditions. In a haploid *tyrR*$^+$ *trpR*$^+$ strain, there appeared to be slight repression in the presence of tryptophan. This was abolished in a *trpR363* (Table 3) or *tyrR366* strain (results not shown), indicating that TrpR may be able to partially repress *aroL* in the absence of a functional TYR R box 1, but only in the presence of functional TyrR protein.

Mutations in box 2. TYR R box 2 was also inactivated by changing the G at position −8 to an A and changing the C at +8 to a T (pMU4632). Surprisingly, these changes did not appear to affect the regulation of *aroL* expression in any way (Table 3). The overall promoter activity of this mutant was less than that of the wild type, but the trends in regulation were very similar. This result might indicate that a functional TYR R box 2 is not essential for normal regulation of *aroL*.

Mutations in box 3. The invariant G at position −8 of box 3 was changed to an A, and the C at position +8 was changed to a T (pMU4634). In the absence of a functional box 3, the regulation of *aroL* expression by either TyrR or TrpR was completely lost under all growth conditions. This indicated that box 3 is essential for the repression of *aroL* (Table 3). A template combining mutant boxes 1 and 3 was also tested, and as expected, it was not regulated under any growth conditions (results not shown).

Mutations in the TrpR binding site. A TrpR binding site had previously been identified, and TrpR has been shown to protect an *Rsa*I restriction nuclease site within this region in the presence of tryptophan (18). Previous studies have indicated that the bases at positions +6 and −6 of a TrpR binding site may be important for TrpR binding (35). We used in vitro mutagenesis to inactivate this site in order to determine its importance in regulating *aroL*. When G→T and C→G changes were introduced at positions ±6 (pMU4627), a complete loss of tryptophan-mediated repression resulted (Table 3). TyrR-mediated repression levels were similar to those obtained for the wild type in a *trpR363* strain. These results indicate that this site is essential for tryptophan-mediated regulation of *aroL*.

Our data, which confirm the results of Heatwole and Somerville (18), show that the presence of TyrR is essential for TrpR-mediated regulation of *aroL* and that (to a lesser extent) TrpR appears to enhance TyrR-mediated repression in the presence of tyrosine (Table 2). These results suggest some form of cooperation between these two regulatory proteins. To determine the importance of the position of the TrpR binding site with respect to the TYR R boxes, we separated the TrpR binding site and TYR R box 1 by inserting 4 bp between the two sites to create pMU4651. In the absence of effectors or in the presence of tryptophan alone, the repression was similar to that of the wild type. Tyrosine-mediated repression of this mutant, however, was 35-fold rather than the 9-fold repression observed in the wild type. In addition, TrpR-mediated enhancement of repression in the presence of tyrosine and tryptophan was reduced to a very low level in this mutant (Table 3).

Is tyrosine repression affected by IHF? In trying to understand why a 4-bp insertion between TyrR box 1 and the TrpR binding site should result in enhanced tyrosine-mediated repression, we searched this region for possible binding sites of other regulatory proteins. By using the MacTargsearch program and the supplied consensus binding site sequence for integration host factor (IHF) (17, 28), a site with an overall homology of 48% on the reverse strand from bases 172 to 219 was identified. This region extends through TYR R box 1 into the TrpR binding site (Fig. 2). A 4-bp insertion between TyrR box 1 and the TrpR binding site reduces this IHF binding site to 40% homology with the consensus sequence, as well as placing TrpR and TyrR on different faces of the helix. Therefore, we repeated measurements of *aroL* repression in a *himA* mutant. These results are shown in Table 4; in a *himA* mutant,

TABLE 3. Analysis of the effects of mutations in the operator sites of *aroL* on β-galactosidase activity

Plasmid	Mutation(s)	β-Galactosidase-specific activity in[a]:							
		JP7219 (*tyrR366 trpR363*)	JP3561 (*tyrR*$^+$ *trpR*$^+$)			JP7218 (*tyrR*$^+$ *trpR363*)			
			MM	Trp	Tyr	Trp-Tyr	MM	Trp	Tyr	Trp-Tyr
pMU4624	Wild type	806	365 (2.2)	214 (3.8)	85 (9.5)	16 (50.4)	452 (1.8)	395 (2.0)	137 (5.9)	129 (6.3)
pMU4630	C·G to A·T at ±8 of box 1	525	292 (1.8)	208 (2.5)	330 (1.6)	234 (2.2)	412 (1.3)	397 (1.3)	465 (1.1)	440 (1.2)
pMU4632	C·G to A·T at ±8 of box 2	586	233 (2.5)	152 (3.9)	47 (12.5)	12 (48.8)	331 (1.8)	304 (1.9)	98 (6.0)	105 (5.6)
pMU4634	C·G to A·T at ±8 of box 3	701	711 (1.0)	611 (1.2)	635 (1.1)	563 (1.3)	692 (1.0)	750 (0.9)	662 (1.1)	672 (1.0)
pMU4627	C→G and G→T at ±6 of TrpR site	539	188 (2.9)	202 (2.7)	113 (4.8)	109 (4.9)	255 (2.1)	209 (2.6)	116 (4.7)	107 (5.0)
pMU4651	4-bp insertion between box 1 and TrpR site	728	318 (2.3)	185 (3.9)	21 (34.7)	16 (45.5)	478 (1.5)	349 (2.1)	34 (21.4)	33 (22.1)
pMU4642	TGTAAAN$_6$TTTACA in box 2	448	41 (10.9)	7 (64.0)	38 (11.8)	8 (56.0)	56 (8.0)	65 (6.9)	80 (5.6)	70 (6.4)
pMU4643	pMU4642; C·G to A·T at ±8 of box 3	831	154 (5.4)	76 (10.9)	193 (4.3)	96 (8.7)	206 (4.0)	147 (5.7)	233 (3.6)	174 (4.8)
pMU4644	pMU4642; C→G and G→T at ±6 of TrpR site	444	63 (7.0)	67 (6.6)	70 (6.3)	66 (6.7)	54 (8.2)	59 (7.5)	70 (6.3)	61 (7.3)
pMU4653	pMU4642; 4-bp insertion between boxes 1 and 2	520	202 (2.6)	50 (10.4)	112 (4.6)	17 (30.6)	283 (1.8)	635 (0.8)	222 (2.3)	188 (2.8)

[a] See Table 2, footnote *a*.

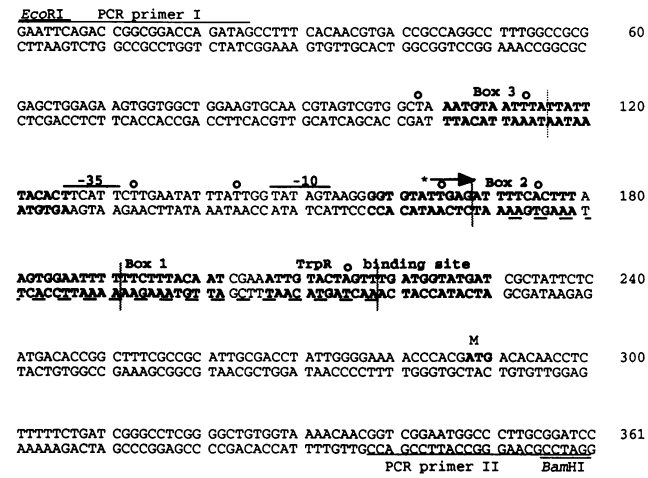

FIG. 2. Nucleotide sequence of the 361-bp *Eco*RI-*Bam*HI fragment carrying the *aroL* promoter-operator region. This fragment was generated from pMU371 by PCR and cloned into the *lacZ* translational fusion vector pMU2386 to produce pMU4624. The PCR primers and restriction sites used to generate this construct are indicated by under- and overlining. The three TYR R boxes and the Trp repressor binding site are shown in boldfaced type, and the center of symmetry for each site is indicated by a vertical line. The positions of hypersensitive sites, identified in Fig. 3D, are indicated by open circles. The −35 and −10 regions of the promoter are overlined, and the transcription start point is indicated by an asterisk. The translational initiation codon is shown in boldfaced type with M above it, and the putative IHF binding site is indicated by broken underlining.

tyrosine repression is increased twofold, while the TrpR-tryptophan enhancement of this repression remains similar to that observed in the wild type. Regulation of the mutation containing a 4-bp insertion between TYR R box 1 and the TrpR binding site was not significantly affected by the introduction of the *himA* mutation. It should also be noted that the strains used for these studies have different backgrounds from the strains described in Table 3, and as has been previously observed, apparent promoter strength and the extent of repression vary between backgrounds.

Creating a strong box 2. Box 2 is the weak box of the double box and lies over the transcriptional start point. The inactivation of this site appears to have no effect on regulation. In order to improve the binding of TyrR to this box, we altered it by a combination of two separate mutagenesis reactions so that it contained the consensus palindromic arm sequence TGTA AAN$_6$TTTACA (pMU4642). The resulting mutant was used to study the effect of TyrR binding strongly to this region on the regulation of *aroL*. In this mutant, repression was strong under all growth conditions but was strongest for cells grown in the presence of tryptophan. In contrast to the wild-type situation, repression of this mutant was not enhanced in the presence of tyrosine. The addition of tryptophan (with or without tyrosine present) resulted in around 60-fold repression in a *tyrR*$^+$ *trpR*$^+$ background, compared with 11-fold repression in the absence of effectors or 12-fold repression when tyrosine was used as the sole effector (Table 3). In a *trpR363* strain, six- to eightfold repression of *aroL* expression was observed under all growth conditions (Table 3), while no repression at all was observed in *tyrR366* strains (data not shown). These results indicate that TyrR is still essential for regulation, although the tyrosine effect has been abolished and uniform repression by TyrR protein alone is observed under all growth conditions. To

TABLE 4. Analysis of *aroL* regulation in *himA* mutants

Plasmid	Mutation	β-Galactosidase specific activity in[a]:							
		JP10721 (*tyrR366 trpR363 himA* mutant)	JP7740 (*tyrR*$^+$ *trpR*$^+$ *himA*$^+$)				JP10756 (*tyrR*$^+$ *trpR*$^+$ *himA* mutant)			
			MM	Trp	Tyr	Trp-Tyr	MM	Trp	Tyr	Trp-Tyr
pMU4624	Wild type	1,201	412 (2.9)	274 (4.4)	90 (13.3)	9 (133)	229 (5.2)	224 (5.4)	45 (26.7)	8 (150)
pMU4651	4-bp insertion between box 1 and TrpR site	960	359 (2.7)	221 (4.3)	15 (64.0)	9 (107)	200 (4.8)	171 (5.6)	12 (80.0)	8 (120)

[a] See Table 2, footnote *a*. Values in parentheses in this table represent the extent of repression as the ratio of β-galactosidase activity obtained in strain JP10721 to that in the *tyrR*$^+$ strains.

ensure that the enhanced tryptophan-mediated repression observed in a *tyrR*$^+$ *trpR*$^+$ strain was due to TrpR binding, G→T and C→A changes were introduced at positions ±6 of the TrpR binding site (pMU4644). This mutant loses enhanced tryptophan-mediated repression and shows six- to eightfold repression in the presence of TyrR, whether TrpR is present or not (Table 3).

The inactivation of box 3 completely abolished the repression of wild-type *aroL*. We wanted to see if box 3 still played an important regulatory role when TyrR was able to bind with high affinity to the box 2 site. Changing the invariant G and C of box 3 to an A and T, respectively, in a strong box 2 mutant did not abolish repression but did reduce repression levels under all growth conditions. Repression ratios in a *tyrR*$^+$ *trpR*$^+$ background in the absence of effectors or in the presence of tyrosine were two to three times lower than those observed in the strong box 2 mutant with a functional box 3 (pMU4642), while repression in the presence of tryptophan was six to seven times lower (Table 3). These results indicate that the upstream TYR R box 3 still plays an important role in the regulation of *aroL* even with two strong boxes downstream.

In the presence of tyrosine and ATP, TyrR protein has been shown to bind cooperatively to two TYR R boxes on the same face of the DNA helix (1). Previous studies have focused on strong-weak box combinations, but in the case of the strong box 2 mutant, we have a strong-strong combination. With such an arrangement, tyrosine is no longer required for repression. In order to test if box position was still important, 4 bp were inserted between box 1 and strong box 2, thus separating these sites by a half turn of the helix (pMU4653). In a *tyrR*$^+$ *trpR*$^+$ strain, this alteration reduced repression significantly. In the presence of tyrosine or tyrosine and tryptophan or in the absence of effectors, repression levels were reduced to one-half to one-third of the levels obtained when the boxes were separated by 1 bp. In the presence of tryptophan, repression was reduced from 64- to 10-fold. In a *trpR363* strain, tryptophan-mediated repression was completely abolished and maximum repression under all other growth conditions was reduced to two- to threefold (Table 3).

Regulation of *aroL* and its strong box variant by a mutant TyrR protein. A *tyrR* mutant ($TyrR_{EQ274}$) with a greatly reduced ability to repress those transcription units of the TyrR regulon normally repressed by TyrR in the presence of tyrosine has been isolated (42). The regulation of *aroL* expression by this mutant protein was tested by introducing the mutant gene into a *tyrR366* strain on a medium-copy-number plasmid (pSU39; ~10 to 15 copies per cell). In order to make a comparison between wild-type TyrR and the mutant, it was necessary to introduce the wild-type *tyrR* gene at the same copy number as the mutant in a parallel experiment. The results are shown in Table 5. Under these conditions, i.e., multicopy *tyrR*$^+$, wild-type *aroL* was repressed 3- to 4-fold in the absence of effectors, 7- to 8-fold in the presence of tryptophan, 13-fold in the presence of tyrosine, and over 200-fold in the presence of both tyrosine and tryptophan. When the multicopy plasmid carried the mutation for $TyrR_{EQ274}$, repression in the absence of effectors or in the presence of tyrosine was fivefold. The addition of tryptophan in either case only increased repression up to 11-fold. In other words, with this mutant, tyrosine-mediated repression is abolished and tryptophan enhancement of the repression seen in MM is only twofold.

Assays were also carried out with a strong box 2 variant. As previously mentioned, repression of the *aroL* promoter with the strong box 2 substitution does not require tyrosine. Cells grown in MM alone or MM supplemented with tyrosine are repressed 15- to 18-fold in multicopy *tyrR*$^+$. When tryptophan is added to the medium, repression increases to over 200-fold. Essentially identical results are obtained when the *tyrR*$^+$ gene is replaced with the $TyrR_{EQ274}$ mutant allele. Thus, the creation of a strong box 2 makes repression totally independent of any tyrosine-mediated events.

DNase I protection studies. Using purified TyrR and TrpR proteins and purified templates (see Materials and Methods), we examined the binding of purified proteins to various boxes by carrying out DNase I protection experiments. In addition to purified protein, aromatic amino acids (tyrosine and tryptophan) and ATP were used as effectors. ATP was included as it is believed that TyrR dimers are able to interact only in the presence of tyrosine and ATP. In addition, previous studies

TABLE 5. Regulation of *aroL* by the mutant $TyrR_{EQ274}$

Plasmid combination	Background	β-Galactosidase specific activity in[a]:			
		MM	Trp	Tyr	Tyr-Trp
pMU4624-pMU3325	Multicopy *tyrR*$^+$, *trpR*$^+$	266 (3.5)	105 (7.7)	60 (12.8)	3 (249)
pMU4624-pMU3326	Multicopy $tyrR_{EQ274}$, *trpR*$^+$	200 (4.6)	77 (10.6)	154 (5.0)	66 (11.3)
pMU4642-pMU3325	Multicopy *tyrR*$^+$, *trpR*$^+$	51 (18.1)	2 (407)	50 (15.3)	3 (249)
pMU4642-pMU3326	Multicopy $tyrR_{EQ274}$, *trpR*	58 (15.9)	4 (203)	71 (10.8)	4 (187)

[a] See Table 2, footnote *a*.

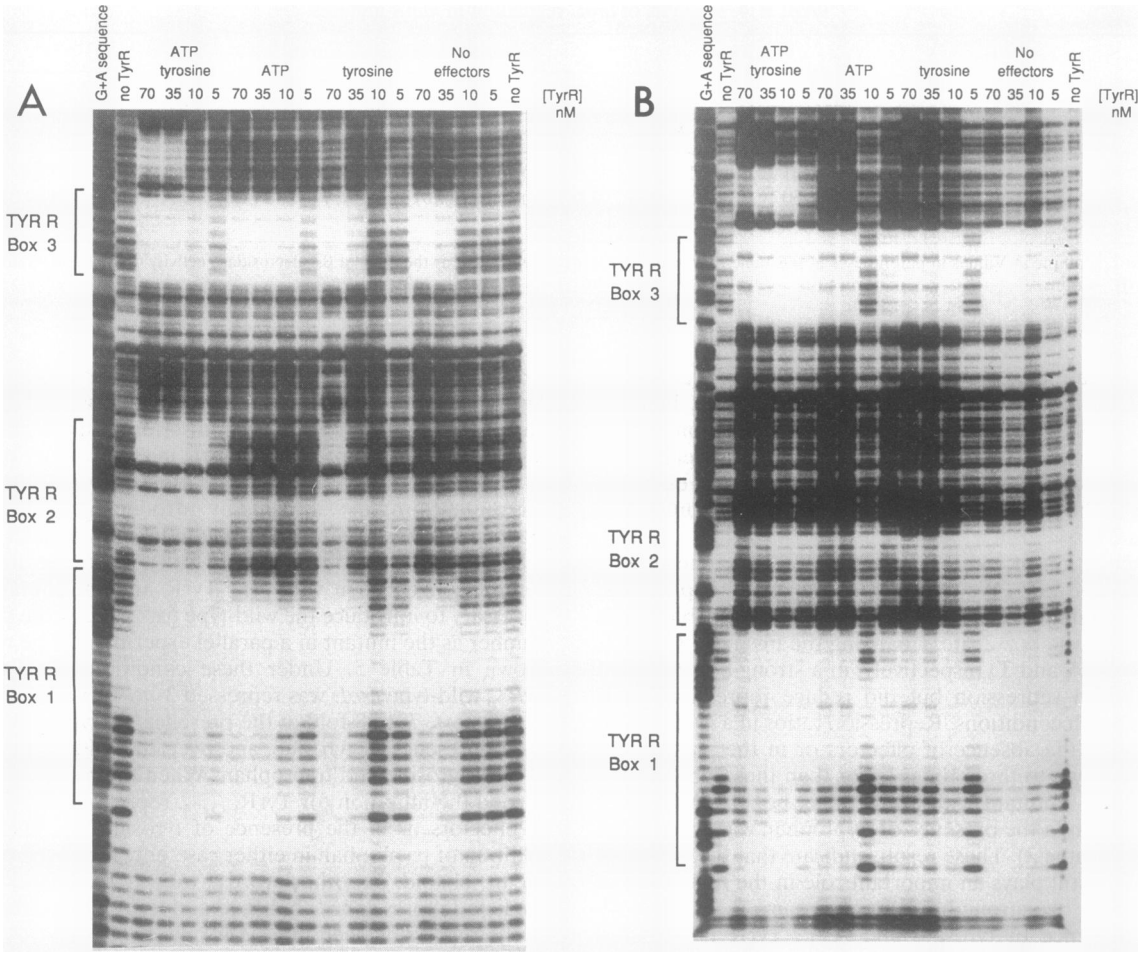

FIG. 3. DNase I footprinting of the antisense strand of *aroL* wild-type and mutant operators. A 361-bp DNA fragment containing the *aroL* regulatory region was ^{32}P labelled at the 3′ end of the antisense strand and subjected to partial DNase I digestion (see Materials and Methods) in the presence of purified TyrR protein and/or TrpR protein. Tyrosine, tryptophan, and ATP were added at final concentrations of 1, 1, and 0.2 mM, respectively, to the preincubation mixture. The concentrations of TyrR and TrpR proteins are shown above the gels. The G+A Maxam-Gilbert sequences of some operator fragments are shown, with the regions corresponding to the TYR R boxes and the Trp repressor binding site marked. (A) Wild-type operator; (B) operator from pMU4632, in which the G and C at positions ±8 in box 2 have been changed to an A and T, respectively; (C) operator from pMU4642 with a strong box 2, showing TrpR binding to its binding site; (D) wild-type and mutant box 3 operators, with hypersensitive sites indicated by arrows. The location of each hypersensitive site in Fig. 1 is indicated by a number in parentheses.

had indicated that the presence of ATP was critical for the protection of weak TYR R boxes in a double box motif (1, 2, 45). Figure 3 shows the DNase I protection patterns obtained when the wild-type *aroL* template and some derivatives with mutations in various boxes were studied.

When the wild-type template (Fig. 3A) was tested in the presence of tyrosine and ATP, all three TYR R boxes were protected with TyrR at concentrations of 5 nM or higher. In the presence of ATP only, boxes 1 and 3 were protected at above 5 nM TyrR but box 2 was not protected at all. When tyrosine was the sole effector, boxes 1 and 3 were protected at TyrR levels of above 10 nM and box 2 was protected at 70 nM. In the absence of any effectors, box 2 was not protected while boxes 1 and 3 were protected at 35 nM and above. A region of protection upstream of TYR R box 3 was observed at high levels of TyrR and in the presence of tyrosine and ATP. Analysis of the sequence around this region identified a site which contained the $GN_{14}C$ motif and could possibly act as a weak TYR R box. The binding to this site was weak, and mutations in this region had no effect on the regulation of *aroL* expression (data not shown). The protection of this region may be an artifact of the experiment, and this site is not discussed further here.

The footprints obtained with a box 2 mutant (Fig. 3B) in which the invariant G and C at positions −8 and +8 were changed to an A and T, respectively, (pMU4632) showed protection of boxes 1 and 3 in the presence or absence of effectors, but box 2 was not protected under any conditions. In vivo this mutant behaved similarly to the wild type, but whereas box 2 is protected in the wild type in the presence of tyrosine and ATP, there is no protection in the mutant. This result indicates that the binding of TyrR protein to box 2 is not involved in repression. When mutant box 1 or mutant box 3 templates were used, the footprinting patterns were those expected from the in vivo results. An inactive box 1 mutant showed protection at box 3 only (under all conditions), while a mutant box 3 template showed protection of boxes 1 and 2 in

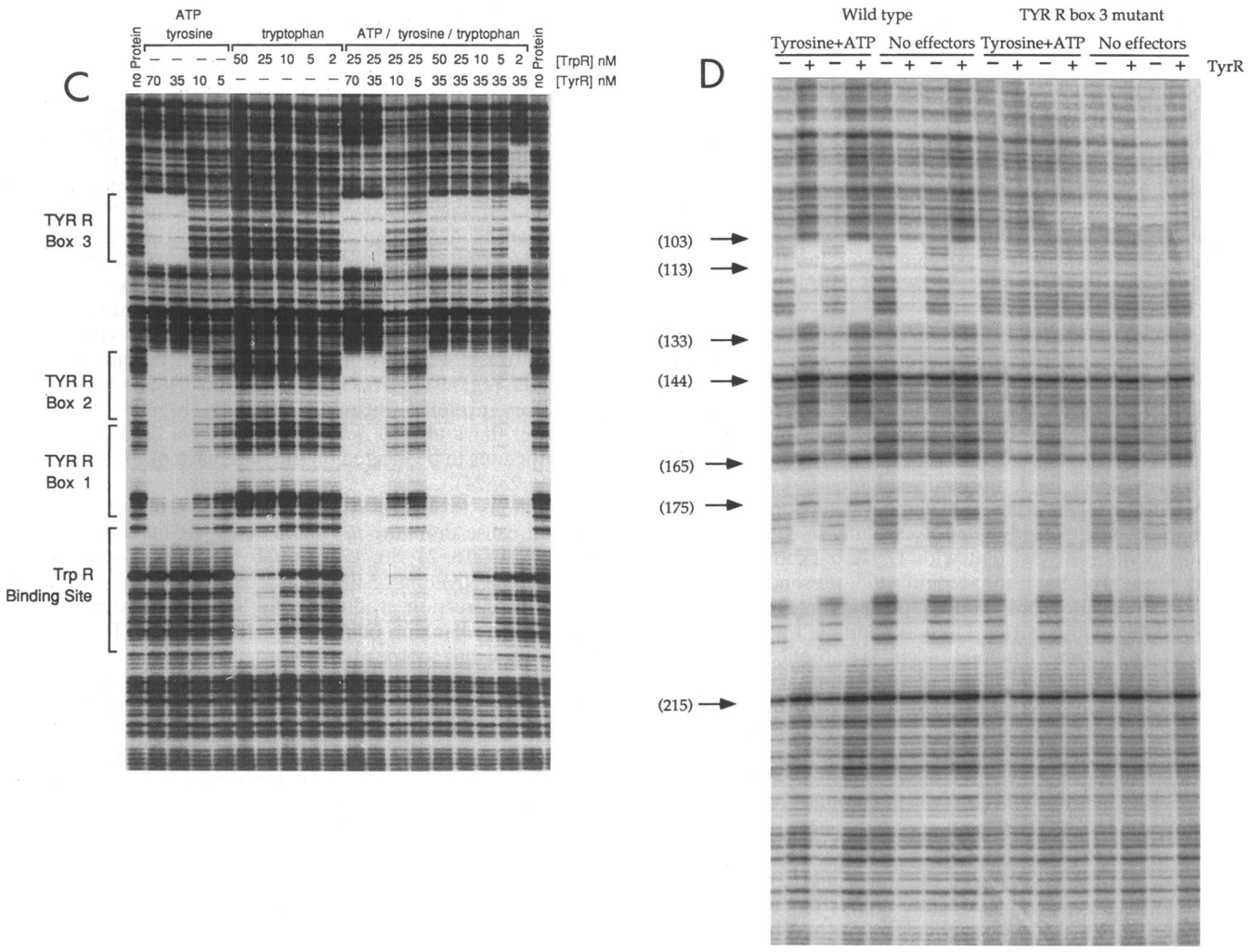

FIG. 3—*Continued.*

the presence of tyrosine and ATP and protection of only box 1 under all other conditions (results not shown).

When a strong box 2 template was used (pMU4642), boxes 1 and 2 were protected in the presence or absence of effectors and box 3 was protected in the presence of ATP (results not shown). Figure 3C shows the footprinting pattern of the strong box 2 mutant in the presence of various combinations of effectors and TrpR and TyrR proteins. In the absence of TyrR protein, the predicted TrpR site is protected; in the presence of tryptophan, the TrpR site is protected at levels of TrpR protein as low as 25 nM. When footprints are carried out in the presence of TyrR protein, tyrosine, ATP, and tryptophan, the TrpR site is protected by levels of TrpR protein as low as 10 nM. This result may suggest some form of cooperative action between TrpR and TyrR, as implied by some of the in vivo results.

Hypersensitivity sites. Analysis of the wild-type footprint revealed the presence of several hypersensitive bands when the protection experiment was carried out under repressing conditions (i.e., in the presence of tyrosine and ATP). The presence of phased hypersensitive bands has been reported for a number of other systems and is indicative of backbone strain which may be caused by the formation of DNA loops (8, 19). In order to demonstrate these hypersensitive sites, we repeated the DNase I protection experiments in the presence or absence of effectors and included TyrR protein in alternate tracks. A wild-type template and a template containing mutations in box 3 were employed, and the results are shown in Fig. 3D. In the wild-type template, hypersensitive bands can be observed at intervals of 10 or 20 bases in the presence of TyrR, tyrosine, and ATP. Some of the identified sites are quite pronounced (positions 103, 113, 165, and 175), while others are weaker but appear to show hypersensitive cutting when studied over several experiments (positions 133, 144, and 215) (Fig. 3D). This effect is not observed in the absence of tyrosine and ATP. Footprints of a template containing an inactive box 3 (pMU4634) also fail to reveal these hypersensitive sites in the presence and absence of effectors.

DISCUSSION

The role of each operator site in *aroL* has been determined by inactivating each site individually. Changes at the invariant $GN_{14}C$ bases of the TyrR boxes or the C and G bases at positions ±6 in the TrpR binding site interfere with the binding of regulatory proteins to these regions and affect the ability of the box to function. Similar results have been shown

for a number of other genes in the TyrR and TrpR regulons (7, 11, 12, 16, 21, 35, 45).

In vitro and in vivo studies of wild-type and mutant promoter-operator regions indicate that TYR R boxes 1 and 3 (the two strong boxes in *aroL*) must be functional for normal regulation of *aroL*. The fact that neither box 1 nor box 3 can function alone suggests that there may be some form of interaction between the two sites, possibly via the TyrR protein bound at the two boxes. In other TyrR-tyrosine-regulated members of the TyrR regulon, repression involves cooperative binding of TyrR protein to adjacent boxes, but the two critical sites in *aroL* are separated by 54 bp (~5 helical turns). TYR R box 3 overlaps the promoter −35 region, while box 1 lies outside and downstream of the RNA polymerase binding region. It is possible that the bending or looping of DNA would allow boxes 1 and 3 to be positioned in such a way that TyrR could bind cooperatively to both sites. The formation of such a loop would also sequester the whole RNA polymerase binding region. Hochschild and Ptashne (19) and Borowiec et al. (8) provided evidence for λ repressor- and *lac* repressor-mediated DNA looping by showing in DNase I protection experiments alternating hypersensitive and resistant sites between the bound operators, consistent with DNA backbone strain. By taking these results into account, the presence of phased hypersensitive sites observed in protection experiments on wild-type *aroL* implies that DNA is bending; this distortion is likely to play a role in regulation. In the absence of effectors, we assume that there is independent binding of TyrR protein at boxes 1 and 3. Repression in vivo is low (twofold), and no phased hypersensitive bands are seen between the boxes in vitro. In the presence of tyrosine and ATP, repression in vivo increases 10-fold and in vitro phased hypersensitive sites can be seen between the two boxes. Under these conditions, we conclude that TyrR proteins bound at each of these sites are able to interact via a DNA loop.

Recently, it has been shown that the dimeric form of TyrR protein, which exists in the absence of effectors, self-associates in the presence of tyrosine and ATP to form a hexamer. This hexameric form of TyrR protein is believed to be the active repressing species for the various tyrosine-repressible promoters of the TyrR regulon and could bind simultaneously to boxes 1 and 3 of *aroL*, thus causing DNA between the two TYR R boxes to loop, as was suggested by Wilson et al. (41). The mutant protein $TyrR_{EQ274}$ represses transcription from the *aroF*, *tyrP*, and *aroP* promoters far less efficiently than does wild-type TyrR under multicopy conditions in the presence of tyrosine (42). The regulation of *aroL* by $TyrR_{EQ274}$ protein shows similar trends. Its repression levels in MM are similar to those mediated by wild-type TyrR. In the presence of tyrosine, however, $TyrR_{EQ274}$ repression is significantly less than that of the wild type and, in fact, is similar to that seen in the absence of effectors. These results can be explained in the following way. TyrR protein is predominantly in dimeric form, rather than hexameric form, in the absence of effectors, and in vitro results show that although TyrR protein alone binds to boxes 1 and 3, it does not appear to distort DNA. Similarly, if the mutant protein $TyrR_{EQ274}$ is unable to hexamerize normally in the presence of tyrosine and ATP, it is not able to initiate the formation of a DNA loop between TYR R boxes 1 and 3. Thus, we believe that the base level of repression observed in MM and in the presence of the mutant $TyrR_{EQ274}$ is due to TyrR dimers binding separately to the two strong boxes in *aroL*. In the presence of tyrosine and ATP, we believe that three wild-type dimers interact to form a hexamer and cause DNA between the two strong TYR R boxes to loop. A similar situation exists in the *lac* operon, in which *lac* repressor dimers bind multiple operator sites before interacting to form tetramers, thus generating DNA loops (10).

Does box 2 play any role in the regulation of *aroL*? All units of the TyrR regulon which are repressed in the presence of tyrosine contain a double box, and in each case (with the exception of *aroP*), the weak box overlaps the RNA polymerase binding region. TyrR protein binds to the strong box in the absence of effectors, but in the presence of ATP and tyrosine, TyrR protein also binds to the weak box. This binding to the weak box is absolutely dependent on the presence of tyrosine and ATP, and studies carried out on the double box in *tyrP* showed that the two boxes must be on the same face of the DNA helix in order for TyrR to bind in a cooperative manner to both sites (1). It has been postulated that this cooperative binding to the weak box excludes RNA polymerase from the promoter region (30). Similar studies involving several other regulatory proteins, including AraC, λ repressor, and *lac* repressor, have also shown that cooperative binding requires operator sites to be located on the same face of the DNA helix (14, 19, 23, 24).

Mutations in the weak box of several units of the TyrR regulon cause dramatic reductions in TyrR-mediated repression (11–13, 16, 21, 30, 45). Therefore, we were surprised to find that inactivation of the weak box (box 2) in *aroL* has no effect on repression at all. Initially, we thought that the two strong boxes flanking box 2 might be directing TyrR to the mutant box, but footprints show no TyrR binding to this site. The finding that mutations in box 3 led to total derepression of *aroL* expression was also unexpected. Although box 3 is inactive in this mutant, boxes 1 and 2 should still be functional and both are bound by TyrR protein in the presence of tyrosine and ATP. These sites in *aroL* are positioned 10 bp closer to the promoter than are the two boxes in *tyrB*. While the TYR R boxes in *tyrB* appear by sequence to be weaker than the boxes in *aroL*, they are able to mediate fourfold repression of *tyrB* transcription in the presence of tyrosine (45). The fact that the double box in *aroL* is unable to mediate any repression at all is difficult to understand, unless the 10-base difference in position between the respective sites in *aroL* and *tyrB* is important.

The alteration of box 2 so that it contains the consensus TYR R box palindromic arms, $TGTAAAN_6TTTACA$, allows TyrR to bind this site in the presence or absence of effectors, as opposed to the wild-type situation in which box 2 is bound only in the presence of tyrosine and ATP. This mutant also mediates strong repression under all growth conditions, although repression is strongest in the presence of tryptophan and the tyrosine effect seen in wild-type *aroL* is gone. Thus, by changing the weak box of *aroL* into a putative strong box, we have effectively abolished tyrosine-mediated repression and created a situation in which TyrR protein can cause significant repression in the absence of effectors. Since this repression is not reduced in a strain in which wild-type TyrR protein has been replaced by a mutant form (unable to cause tyrosine-mediated repression), we postulate that TyrR protein binds independently to box 1, strong box 2, and box 3 to cause the repression observed in MM. Even though we postulate independent binding of TyrR dimers to box 1 and strong box 2, we found that moving the boxes apart by a half turn of the helix has a significant effect on repression. We do not believe that the separation of the boxes in this case affects the binding of TyrR protein unless when the boxes are adjacent, the interaction between bound dimers strengthens the overall binding of TyrR protein to DNA. Another possibility is that by moving box 1 4 bp downstream, we have affected the extent to which bound TyrR protein interferes with RNA polymerase binding or initiation of transcription. The observation that repression

in the strong box 2 mutant is still partially reduced when box 3 is inactivated also requires an explanation. One possibility is that in the absence of hexamerization and DNA looping, the binding of each TyrR dimer to each TYR R box has an additive effect, with each one increasing the difficulty for RNA polymerase to bind or initiate transcription. Alternatively, if the binding of TyrR protein to individual boxes causes some local distortion, as has been reported for a number of DNA-binding proteins (9, 32, 36, 39), the bend at each of these three boxes may create an overall change which interferes with transcription.

Regardless of the mechanism of repression in this strong box 2 mutant, the repression levels observed in the absence of effectors or in the presence of tyrosine are similar to those observed for the wild type or inactive box 2 mutant in the presence of tyrosine. Thus, the independent binding of TyrR protein to the box 2 site does not mediate any greater repression than that observed in the wild type; instead, it abolishes the need for tyrosine in this repression. It is of interest that there are no examples in the TyrR regulon of two strong boxes located adjacent to one another. The creation of a double strong box in *tyrP*, however, abolished phenylalanine-mediated activation from the *tyrP* promoter (2), and the insertion of a second strong box adjacent to the single TYR R box upstream of *aroG* led to the loss of effector-mediated regulation from the *aroG* promoter. In the case of *aroG*, tyrosine-mediated repression was observed when the inserted strong box was replaced by a weak box (6). These results, along with the *aroL* double strong box result, suggest that the weak binding site has evolved as a fine-tuning mechanism which allows effector-sensitive regulation of these genes.

TrpR-mediated regulation of *aroL*. Previous studies predicted a TrpR binding site downstream of TYR R box 1 and showed that TrpR protected an *Rsa*I restriction site within this region (18). In this study, DNase I footprinting incorporating purified TrpR protein showed protection of the predicted binding site in the presence of tryptophan. This protection appears to be slightly stronger when TyrR is bound to TYR R boxes. In vivo results indicate that TrpR regulation is significant only in the presence of TyrR protein and is greatest when TyrR is bound to all three TYR R boxes, i.e., when the wild-type strain is grown in the presence of tyrosine (and tryptophan) or in the strong box 2 mutant (in the presence of tryptophan). These results indicate some form of physical interaction or cooperativity between TrpR and TyrR; alternatively, the binding of TyrR may cause a conformational change in DNA which allows TrpR to bind more easily. Interestingly, when the TrpR binding site is inactivated in the wild-type *aroL* template, TyrR-mediated repression in the presence of tyrosine drops from 10- to 6-fold. Also, when the TrpR site is inactivated in a strong box 2 mutant, repression in the presence or absence of any effector drops to six- to sevenfold. Thus, TrpR may also play a role in stabilizing the binding of TyrR, although TyrR is able to repress *aroL* transcription in the absence of TrpR.

In an attempt to look for possible interactions between TrpR and TyrR proteins, we inserted 4 bp between the TrpR binding site and TYR R box 1. Unexpectedly, this resulted in a three- to fourfold increase in tyrosine-mediated repression (compared with wild-type levels) which was independent of tryptophan and TrpR protein. Consequently, in this mutant, the enhancement of tyrosine-mediated repression by TrpR-tryptophan is almost abolished, although the overall levels of repression in the presence of tyrosine and tryptophan are unaffected. We identified a putative IHF binding site which extended through TYR R box 1 to the middle of the TrpR box (bases 172 to 219 on the reverse strand [Fig. 2]). It seems possible that the 4-bp insertion could disrupt this binding site as well as move the TrpR protein to a different face of the helix. When we tested *aroL* repression in a *himA* mutant, we found a twofold increase in tyrosine repression, whereas tryptophan enhancement of repression was similar to that observed in the wild type. On the basis of these results, we propose that when IHF binds to its binding site, it has a negative effect on tyrosine-mediated repression which is relieved in +4 and *himA* mutants. Further experimentation is under way to test this hypothesis.

Although in vivo *aroL-lacZ* fusions and in vitro binding studies suggest that TrpR protein plays an important role in the regulation of the *aroL* gene, previously reported studies on the regulation of shikimate kinase activity indicated that there were no changes in the regulation of shikimate kinase synthesis in a *trpR* mutant strain (15). Recently, these enzyme assays were repeated with a strain with 5 to 6 copies of the *aroL* gene and 10 to 15 copies of the *tyrR*$^+$ gene. In this case, three- to fourfold derepression of *aroL* expression was observed when comparing shikimate kinase activities in *trpR*$^+$ and *trpR363* strains grown in the presence of tryptophan and tyrosine (40). These results indicate that TrpR does play a role in regulating the expression of wild-type shikimate kinase II.

In addition to *aroL*, the *mtr* gene is regulated by TyrR and TrpR, and it has been suggested that these two proteins may interact at the *mtr* operator sites (35, 43). In this case, however, TrpR is the dominant regulator and cooperative binding between TyrR and TrpR has not been shown. Thus, there may be two different situations in which TyrR and TrpR proteins interact to regulate one promoter, although further work is required to determine the precise nature of these interactions.

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