

Integration Host Factor and Cyclic AMP Receptor Protein Are Required for TyrR-Mediated Activation of *tpl* in *Citrobacter freundii*

QING BAI† AND RONALD L. SOMERVILLE*

Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907

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The *tpl* gene of *Citrobacter freundii* encodes an enzyme that catalyzes the conversion of L-tyrosine to phenol, pyruvate, and ammonia. This gene is known to be positively regulated by TyrR. The amplitude of regulation attributable to this transcription factor is at least 20-fold. Three TyrR binding sites, designated boxes A, B, and C, centered at coordinates -272.5 , -158.5 , and -49.5 , respectively, were identified in the upstream region of the *tpl* promoter. The results of mutational experiments suggest that TyrR binds in cooperative fashion to these sites. The nonavailability of any TyrR site impairs transcription. Full TyrR-mediated activation of *tpl* required integration host factor (IHF) and the cAMP receptor protein (CRP). By DNase I footprinting, it was shown that the IHF binding site is centered at coordinate -85 and that there are CRP binding sites centered at coordinates -220 and -250 . Mutational alteration of the IHF binding site reduced the efficiency of the *tpl* promoter by at least eightfold. The proposed roles of CRP and IHF are to introduce bends into *tpl* promoter DNA between boxes A and B or B and C. Multimeric TyrR dimers were demonstrated by a chemical cross-linking method. The formation of hexameric TyrR increased when *tpl* DNA was present. The participation of both IHF and CRP in the activation of the *tpl* promoter suggests that molecular mechanisms quite different from those that affect other TyrR-activated promoters apply to this system. A model wherein TyrR, IHF, and CRP collaborate to regulate the expression of the *tpl* promoter is presented.

Tyrosine phenol lyase (TPL; EC 4.1.99.2), formerly known as β -tyrosinase, catalyzes a reversible pyridoxal phosphate-dependent α , β -elimination reaction that degrades L-tyrosine to phenol, pyruvate, and ammonia (22, 23, 25). In the reverse direction, TPL can catalyze the synthesis of L-tyrosine or 3,4-dihydroxyphenylalanine from pyruvate, ammonia, and phenol or catechol. Detailed studies, including the cloning and characterization of the structural gene for TPL, have been carried out for *Citrobacter freundii* (3, 24), *Erwinia herbicola* (14, 54), and *Escherichia intermedia* (26).

In *E. herbicola*, TPL is induced by L-tyrosine and repressed when cells are grown in glucose (13). The regulatory mechanism(s) that controls the expression of the TPL gene (*tpl*) is not well understood. A previous study on the regulation of transcription from the *tpl* promoter of *C. freundii* demonstrated a role for the TyrR protein in regulating the *tpl* system (50).

The TyrR protein of *Escherichia coli* regulates the expression of a number of genes involved in the biosynthesis and transport of aromatic amino acids known as the TyrR regulon (for a review, see reference 39). Seven of the genes of the regulon are repressed by the TyrR protein (39), one gene (*mtr*) is activated (18, 46), and another (*tyrP*) is regulated either positively or negatively, depending on whether phenylalanine or tyrosine is present (21, 58). In general, tyrosine serves as the effector for repression, while phenylalanine is the cofactor for activation (39). However, in the TyrR-mediated activation of the *mtr* promoter, either tyrosine or phenylalanine can function as an inducer (18, 46). The detailed mechanisms of TyrR-mediated repression and activation are unknown.

To investigate possible mechanisms for the regulation of TPL, we employed a variety of genetic and biochemical procedures to analyze transcription from the *tpl* promoter of *C. freundii*. The present work enlarges our understanding of the TyrR-regulated *tpl* system. Two proteins, integration host factor (IHF) and cyclic AMP (cAMP) receptor protein (CRP) were shown to participate in the TyrR-mediated activation of the *tpl* promoter. The most likely role of these two proteins is to bend DNA in the region upstream of the *tpl* promoter, thereby enhancing the oligomerization of TyrR dimers, thus increasing the local concentration near the *tpl* promoter, thereby increasing its ability to contact σ^{70} RNA polymerase, but also to facilitate the formation of a complex containing at least two TyrR dimers that is required for transcriptional activation. The requirement for additional protein factors in the TyrR-mediated activation of the *tpl* promoter sets this system apart from other TyrR-activated systems.

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophages, and oligonucleotides. The biological materials used in this study are described in Table 1, along with the chemically synthesized oligonucleotides used in site-directed mutagenesis.

Media. The liquid minimal medium used was salt mix E of Vogel and Bonner (56) containing vitamin B₁ (1 mg/liter), biotin (0.1 mg/liter), and either glucose (0.2%) or glycerol (0.2%). Luria broth (LB) medium (30) contained Bacto Yeast Extract (5 g/liter), Bacto Tryptone (10 g/liter), sodium chloride (5 g/liter), and glucose (1 g/liter). Solid medium was Bacto Nutrient Agar (Difco) (31 g/liter). The following compounds were included when appropriate: L-tyrosine (50 μ g/ml), 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal), 40 μ g/ml; ampicillin, 50 μ g/ml; chloramphenicol, 25 μ g/ml; streptomycin, 30 μ g/ml; and kanamycin, 25 μ g/ml.

DNA preparation. Plasmid DNA was isolated with the Wizard plus miniprep DNA purification system (Promega). Cultures for plasmid preparation were grown to saturation in L broth supplemented with appropriate antibiotics. The preparation of competent cells and their transformation were carried out by the procedure of Mandel and Higa (32). When thermosensitive lysogens were being transformed, the heat step was omitted.

* Corresponding author. Mailing address: Department of Biochemistry, Purdue University, West Lafayette, IN 47907. Phone: (765) 494-1614. Fax: (765) 494-7897. E-mail: somerville@biochem.purdue.edu.

† Present address: Department of Medical Genetics and Biochemistry, University of Pittsburgh, Pittsburgh, PA.

TABLE 1. *E. coli* strains, phages, plasmids, and oligonucleotides

Strain, phage, plasmid, or oligonucleotide	Relevant genotype, description, or sequence	Reference or source
Strains		
SP1312	F ⁻ <i>zah-735::Tn10 Δ(argF-lac)U169</i>	18
SP1313	F ⁻ <i>zah-735::Tn10 Δ(argF-lac)U169 ΔtyrR</i>	18
SP1626	As SP1312 but with <i>Δcrp-39 rpsL136</i>	This work
SP1627	As SP1313 but with <i>Δcrp-39 rpsL136</i>	This work
SP1628	As SP1312 but with <i>himD::Cm</i>	This work
SP1629	As SP1313 but with <i>himD::Cm</i>	This work
SP1630	As SP1626 but with <i>himD::Cm</i>	This work
SP1631	As SP1627 but with <i>himD::Cm</i>	This work
NK5031	<i>Δ(lacIZY)MS265 gyrA supF</i>	34
CA8439	λ ⁻ <i>relA1 rpsL136 Δcrp-39 spoT1 ΔcyaA854 thi-1</i>	44
CA8445	<i>Δcrp-45 Δcya-854 strA (=rpsL) thi</i>	44
CSH26(λRZ11)	<i>ara Δ(lac-pro) thi (λplac5 c1857 Sam7)</i>	63
BW12848	<i>lac-169 hip(himD)::cat pho-510 thi</i>	B. Wanner
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacI^qΔM15 Tn10 (Tet^r)]</i>	Stratagene
BL21(DE3)	F ⁻ <i>ompT (lon) hsdS_B (r_B⁻ m_B⁻)</i> with DE3, a λ prophage carrying the T7 RNA polymerase gene	52
Phages		
λHQS10	<i>tpl-lacZ</i> reporter; <i>Ptpl-lacZ</i> ⁺	50
λHQS10mut1	<i>tpl-lacZ</i> reporter with G→A mutation in box A	50
λHQS10mut2	<i>tpl-lacZ</i> reporter with G→A mutation in box B	50
λHQS10mut3	<i>tpl-lacZ</i> reporter with G→A and C→T mutations in box C	This work
λHQS10mut2,3	<i>tpl-lacZ</i> reporter with G→A mutation in box A and with G→A and C→T mutations in box C	This work
λHQS10Δ64	<i>tpl-lacZ</i> reporter with 64-nucleotide deletion	50
λHQS10Δ130	<i>tpl-lacZ</i> reporter with 130-nucleotide deletion	50
λHQS10Δ64C	<i>tpl-lacZ</i> reporter with 64-nucleotide deletion; box C replaced by box A	This work
λHQS10Δ179C	<i>tpl-lacZ</i> reporter with 179-nucleotide deletion; box C replaced by box A	This work
λHQS10IHFmut	<i>tpl-lacZ</i> reporter; T→C and A→C mutations in IHF site	This work
Plasmids		
pHA5	Derived from pBR322; insert of 3.6-kb <i>Bam</i> HI fragment containing <i>crp</i> gene and promoter	R. Ebright
pXZCRP	Derived from pHA5 by inserting an <i>Eco</i> RI fragment of 500 bp containing the fl1 origin	R. Ebright
pJC100	pET3a- <i>tyrR</i> ⁺	51
pJC136	pACYC184- <i>tyrR</i> ⁺	This work
pET3a	T7 vector	43
pHNβα	Derivative of pHX3-8 by conditional digestion of <i>Hind</i> III which yield incomplete digests	28
pHNβ ₂ α	Derivative of pHNβα, containing two copies of <i>himD</i> gene	28
pUC19	Cloning vector	63
pUC19tpl	Wild-type <i>tpl</i> promoter in pUC19	50
pUC19tplmut1	G→A change in box A of the <i>tpl</i> promoter in pUC19	50
pUC19tplΔ64	64-nucleotide deletion derivative of pUC19tpl	This work
pUC19tplΔ64C	64-nucleotide deletion; box C is replaced by box A of <i>tpl</i> promoter in pUC19tpl	This work
pUC19tplΔ179	179-nucleotide deletion derivative of pUC19-tpl	50
pUC19mut3	G→A and C→T changes in box C of <i>tpl</i> promoter	This work
pUC19mut1,3	G→A in box A; G→A and C→T changes in box C of <i>tpl</i> promoter	This work
pUC19mut2,3	G→A in box B; G→A and C→T changes in box C of <i>tpl</i> promoter	This work
pUC19tplIHFmut	T→C and A→C mutations in IHF site	This work
pMLB1034	Promoterless <i>lacZ</i>	6
pMLB1034tplmut3	G→A and C→T changes in box C of <i>tpl</i> promoter in pMLB1034	This work
pMLB1034tplmut2,3	G→A in box B; G→A and C→T changes in box C of <i>tpl</i> promoter in pMLB1034	This work
pMLB1034tplIHFmut	T→C and A→C mutations in IHF site	This work
pMLB1034tplΔ64C	64-nucleotide deletion; box C is replaced by box A of <i>tpl</i> promoter in pMLB1034	This work
pMLB1034tplΔ179C	179-nucleotide deletion; box C is replaced by box A of <i>tpl</i> promoter in pMLB1034	This work
Oligonucleotides		
351K	GCTGTAAATATAGGTGATGCAAATTACACGTC	
352K	GACGTGTAATTTGCATCACCTATATTTACAGC	
488K	CGGGTAAAAAATGACGTGTGTAAGCATCATTTTACATTTACAGC	
489K	CCTGTAAATGTAATGATGCTTTACACACGTCATTTTTCACCCG	
IHFc	CACGCAAAAAAACTTGTCGACTATGAACGGG	
IHFd	CCCGTTCATAGTCGACAAGTTTTTTTTTTCGCTG	

Chemicals and reagents. Restriction endonucleases, T₄ DNA ligase, and DNA polymerase I large (Klenow) fragment were purchased from New England Biolabs. [α -³⁵S]dATP and [α -³²P]dATP were purchased from Amersham. Special-purpose oligonucleotides for use in PCR and site-directed mutagenesis were synthesized in the Laboratory for Macromolecular Structure, Purdue University. The protein markers and reagents for sodium dodecyl sulfate-polyacryl-

amide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting) were purchased from Bio-Rad. Anti-TyrR antibodies were prepared as previously described (9). Dimethyl pimelimidate (DMP) was purchased from Pierce. *o*-Nitrophenyl- β -D-galactopyranoside (ONPG) was purchased from Sigma. All other chemicals were of the highest quality that was commercially available.

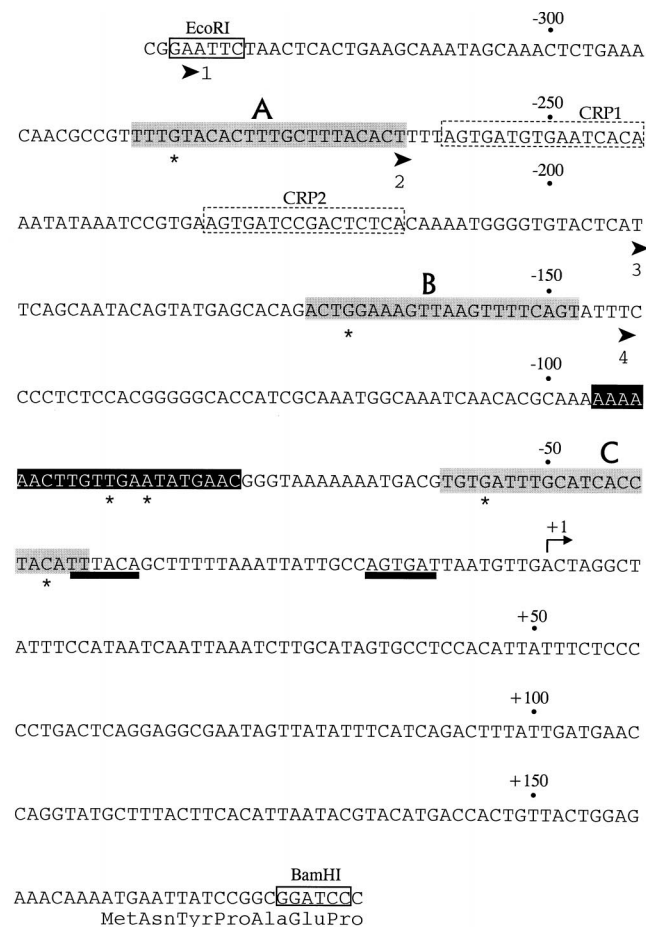


FIG. 1. The *tpl* promoter and associated regulatory elements. Only the nucleotide sequence of the messenger-equivalent strand is shown. The coordinate system has been changed from the system in the original publication (3) by assigning +1 to the start point of transcription. DNA fragments used in the functional analysis of the *tpl* promoter were synthesized by PCR, with pRVT1 (3) as the template. Cleavage sites for restriction endonucleases *EcoRI* and *BamHI* were installed at the indicated locations (coordinates -329 and +180) in order to facilitate the construction of single-copy reporter systems. The *EcoRI*-*BamHI* fragment was cloned into pUC19, which contains a *PstI* site downstream of the *BamHI* site. The fragment from pUC19-*tpl* generated by *EcoRI*-*PstI* digestion was used for DNase I footprinting experiments. The 5' end points of three truncated derivatives of the *tpl* promoter are shown as broad arrowheads labeled 2, 3, and 4 at coordinates -262, -193, and -144. The -10 and -35 recognition elements are underlined with heavy black lines. The target sites for cAMP-CRP (CRP1 and CRP2) are outlined with broken lines. TyrR boxes, named A, B, and C, are shaded. Residues marked by asterisks (-280, -166, -55 and -40) are the locations of TyrR operator mutations or IHF mutations (see text). The IHF binding site is presented in white letters on black.

Strain construction. The construction of *tyrR*⁺/*tyrR* isogenic strains and λ lysogens containing a *lacZ* reporter system specific for the *tpl* promoter was described in reference 50. High-titer lysates of *Ptpl-lacZ*⁺ λ phages were used to lysogenize the Lac⁻ strains SP1312 (*tyrR*⁺) and SP1313 (*tyrR*) to generate SP1312 *Ptpl-lacZ*⁺ and SP1313 *Ptpl-lacZ*⁺. Strains SP1626 (*tyrR*⁺) and SP1627 (*tyrR*) are Δ *crp* derivatives of the aforementioned lysogens that were constructed by standard P1 transduction methods with CA8439 as the donor (50). These strains were λ resistant, streptomycin resistant, and temperature sensitive. Strains SP1628 (*tyrR*⁺) and SP1629 (*tyrR*) are IHF-negative derivatives of SP1312 *Ptpl-lacZ*⁺ and SP1313 *Ptpl-lacZ*⁺ that were constructed with P1 grown on BW12848. This P1 lysate was also used to construct Δ *himD* mutants of SP1626 (*Ptpl-lacZ*⁺) and SP1627 (*Ptpl-lacZ*⁺). *tpl* promoter variants containing mutations in either the IHF site, box C, or boxes B and C (Fig. 1) were generated in derivatives of pUC19 as described below. Segments of *tpl* promoter DNA were inserted as *EcoRI*-*BamHI* fragments into pMLB1034. The resulting pMLB1034 derivatives were transformed into CSH26(λ RZ11). Lac⁻ λ recombinants were isolated as described in reference 50, with NK5031 as the plating indicator.

Enzyme assays. β -Galactosidase was assayed by the method of Miller (35). Cells were grown to early mid-log phase at 30°C in liquid minimal medium containing L-tyrosine (50 μ g/ml). Assays, carried out in triplicate, had standard errors of <10%. The enzyme assay values are reported in Miller units.

Proteins used in footprinting studies. TyrR protein was purified as previously described (10). CRP was purified by a modification of a method developed by R. Ebright, Rutgers University, using cAMP-Sepharose affinity chromatography. An affinity column was prepared by coupling 8-(6-aminoethyl)amino-adenosine 3',5'-cyclic monophosphate (AHAcAMP; Sigma) to CNBr-activated Sepharose 4B gel (Pharmacia). *E. coli* CA8445/pXZCRP, which carries a multicopy plasmid encoding the CRP protein, was grown in L-broth medium at 37°C for 15 h and harvested. Crude extracts were prepared using a French pressure cell (Aminco) operated at 1,000 lbs/in² and then loaded on a 6-ml cAMP affinity column. The column was washed with 2 bed volumes of wash buffer (20 mM sodium phosphate [pH 7.0], 2 mM EDTA [pH 8.0], 5 mM β -mercaptoethanol, 300 mM NaCl, 6% glycerol) and then with 2 bed volumes of wash buffer plus AMP (5 mM 5' AMP and 5 mM 3' AMP). After the washes, CRP was eluted with wash buffer containing 500 mM NaCl and 5 mM cAMP. About 90% pure CRP was obtained. A further purification step was carried out by fast protein liquid chromatography with a Mono S column (Pharmacia). The purity of CRP, by SDS-PAGE, was at least 99%. The IHF protein was a gift from Steven D. Goodman, University of Southern California. All protein concentrations were determined by Bio-Rad protein assay reagent with bovine serum albumin as a standard.

DNase I footprinting. A DNA fragment of 527 bp carrying the *tpl* promoter was released from pUC19-*tpl* by digestion with *EcoRI* plus *PstI*, isolated by electrophoresis on 1% agarose, followed by purification with a QIAEX II gel extraction kit (Qiagen). Elution was carried out with TE buffer (20 mM Tris [pH 8.0], 0.1 mM EDTA). This fragment (0.5 μ g) was selectively labeled at the *EcoRI* site by treatment with DNA polymerase I (Klenow fragment) in the presence of [α -³²P]dATP by the method of Brenowitz et al. (7). The unincorporated radiolabel was removed with a G-25 spin column (Boehringer Mannheim), and residual protein was removed with a QIA spin column (Qiagen). The optimal concentration of DNase I in footprinting analyses was established to be 1.5 to 2.0 μ g/ml. For CRP footprinting analyses, radiolabeled DNA (1 nmol/tube) was incubated with CRP (10 to 100 nM) at room temperature in assay buffer containing 10 mM Tris-Cl (pH 8.0), 5 mM MgCl₂, 1 mM CaCl₂, 2 mM dithiothreitol, 100 mM KCl, 50 μ g of bovine serum albumin per ml, 2 μ g of calf thymus DNA per ml, and 100 μ M cAMP. The total volume of each binding reaction mixture was 200 μ l. After 30 min, each tube was treated with 5 μ l of DNase I (1.5 μ g/ml) for 2 min. Each reaction was stopped by the addition of 40 μ l of 50 mM EDTA (pH 8.0), followed by a single treatment with 200 μ l of phenol-chloroform-isoamyl alcohol (24:25:1). The DNA was precipitated with ethanol containing 5 μ g of tRNA per ml and 0.1 volume of 3 M sodium acetate. The DNA was resuspended in 5 μ l of loading dye, heated for 5 min at 90°C, and loaded onto an 8% acrylamide-6 M urea gel. The products of the A+G cleavage reactions (33) were coelectrophoresed with sample to identify protected nucleotides. After electrophoresis, the gel was exposed overnight at -70°C to Kodak XAR-5 film. For the TyrR and IHF footprinting reactions, the procedure was the same as described as above. Unless otherwise stated, the assay buffer for TyrR also contained 0.2 mM L-tyrosine and 0.2 mM ATP. DNA fragments containing G-to-A changes in box A were prepared by *EcoRI*-*PstI* digestion of pUC19-*tplmut1*. DNA fragments from the deletion mutant Δ 64 or Δ 179 were generated by *EcoRI*-*PstI* cleavage of pUC19-*tpl* Δ 64 or pUC19-*tpl* Δ 179, respectively.

Mutagenesis of TyrR and IHF binding sites. The introduction of the G-to-A changes in box A or box B and the deletion of segments of the *tpl* promoter is described in reference 50. Mutations in box C (G-to-A and C-to-T) were introduced by using a Quick-change site-directed mutagenesis kit (Stratagene) with the oligonucleotide pair 351K and 352K (Table 1). The template DNA was either pUC19-*tpl* or pUC19-*tplmut2*. The consensus sequence of the TyrR target is TGTAAN₆TTTACA (N is any nucleotide). Box C DNA (TGATTTGCATCACTACA) was replaced by the box A sequence (TGTACATTTGCTTTACA), except for the six nonconserved central nucleotides. These mutational changes were introduced into either pUC19-*tpl* Δ 64 or pUC19-*tpl* Δ 179 using the oligonucleotide pair 488K and 489K. Mutations in the IHF site (A₆CTTGTTGAATATGAAC \rightarrow A₆CTTGTCGACTATGAAC) were introduced in similar fashion with the oligonucleotide pair IHFc and IHFd (Table 1). The template DNA was pUC19-*tpl*. Each mutational change was verified by DNA sequencing.

Chemical cross-linking. Reactions were allowed to proceed at room temperature (25°C) in 0.2 M triethanolamine buffer at pH 8.0 containing 1 mg of TyrR protein and 2 mg of DMP per ml. Samples were removed at different times. Each reaction was stopped by the addition of an equal volume of 2 \times loading buffer (10) and then frozen at -20°C. Circular DNA from pUC19-*tpl* was prepared with a mega kit from Qiagen. In the presence of DNA, cross-linking was carried out with TyrR and CRP in a buffer containing 200 μ M cAMP, 200 μ M L-tyrosine, 5 mM MgCl₂, 1 mM CaCl₂, and 200 μ M ATP. The molar ratio of DNA to TyrR to CRP was 1:3:1. After incubation for 20 min, 2 mg of DMP per ml was added. Successive steps were performed as described above. Cross-linking mixtures were analyzed either by 0.7% SDS-PAGE or Western blotting. The primary polyclonal anti-TyrR antibody was a 1:3,000 dilution of high-titer antiserum (9). Peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad) was employed as the second antibody (1:3,000 dilution). Staining was carried out with H₂O₂ and 4-chloronaphthol by standard procedures (17).

TABLE 2. Regulation of the *tpl* promoter by TyrR, cAMP-CRP, and IHF

Strain	Relevant genotype	β-Galactosidase activity ^a	
		Glucose	Glycerol
SP1312 (<i>P_{tpl}-lacZ</i> ⁺)	<i>tyrR</i> ⁺	783	2,540
SP1313 (<i>P_{tpl}-lacZ</i> ⁺)	Δ(<i>tyrR</i>)	35.5	68
SP1313 (<i>P_{tpl}-lacZ</i> ⁺)/pJC100	Δ(<i>tyrR</i>) <i>tyrR</i> ⁺	1,530	2,180
SP1626 (<i>P_{tpl}-lacZ</i> ⁺)	<i>tyrR</i> ⁺ Δ(<i>crp</i>)	828	812
SP1627 (<i>P_{tpl}-lacZ</i> ⁺)	Δ(<i>tyrR</i>) Δ(<i>crp</i>)	61	73
SP1626 (<i>P_{tpl}-lacZ</i> ⁺)/pXZCRP	<i>tyrR</i> ⁺ Δ(<i>crp</i>) <i>crp</i> ⁺	446	780
SP1627 (<i>P_{tpl}-lacZ</i> ⁺)/pXZCRP	Δ(<i>tyrR</i>) Δ(<i>crp</i>) <i>crp</i> ⁺	53	158
SP1628 (<i>P_{tpl}-lacZ</i> ⁺)	<i>tyrR</i> ⁺ Δ(<i>himD</i>)	19.5	ND
SP1629 (<i>P_{tpl}-lacZ</i> ⁺)	Δ(<i>tyrR</i>) Δ(<i>himD</i>)	16.3	ND
SP1312 (<i>P_{tpl}-lacZ</i> ⁺ IHF-mut)	<i>tyrR</i> ⁺ ; altered IHF site	89.2	ND
SP1313 (<i>P_{tpl}-lacZ</i> ⁺ IHF-mut)	Δ(<i>tyrR</i>); altered IHF site	39.0	ND
SP1630 (<i>P_{tpl}-lacZ</i> ⁺)	<i>tyrR</i> ⁺ Δ(<i>crp</i>) Δ(<i>himD</i>)	ND	5.6
SP1631 (<i>P_{tpl}-lacZ</i> ⁺)	Δ(<i>tyrR</i>) Δ(<i>crp</i>) Δ(<i>himD</i>)	ND	5.3
SP1628/pHNβ ₂ α	<i>tyrR</i> ⁺ Δ(<i>himD</i>) <i>himD</i> ⁺	708.5	ND

^a β-Galactosidase activity is expressed in Miller units. Cultures were grown in liquid minimal medium with L-tyrosine and with either glucose or glycerol as a carbon source. The values shown are the averages of triplicate assays. The variation was never more than ±10%.

RESULTS

Regulation of the *tpl* promoter by TyrR and cAMP-CRP. SP1312 (*P_{tpl}-lacZ*⁺) and SP1313 (*P_{tpl}-lacZ*⁺) are isogenic *tyrR*⁺/*tyrR* strains that carry single-copy *lacZ* reporter genes driven by the *tpl* promoter of *C. freundii* (50). When these strains were grown in the presence of L-tyrosine, the β-galactosidase levels in the *tyrR*⁺ strain were at least 20-fold higher than in the Δ(*tyrR*) strain (Table 2, lines 1 and 2). When SP1313 (*P_{tpl}-lacZ*⁺) was transformed with a *tyrR*⁺ plasmid (pJC100 [51]) that led to haploid level expression of a functional TyrR protein, the reporter enzyme levels were restored to control values. These results confirm that the *tpl* promoter, like the *mtr* and *tyrP* promoters, is subject to positive control by the TyrR protein (Table 2, lines 1 to 3).

Previous studies in *E. herbicola* showed that the formation of *tpl* mRNA was drastically curtailed when cells were grown in the presence of glucose (53). It is also known that a null mutation in *cya* causes about a 50-fold drop in *tpl* promoter activity (50). Inspection of the *tpl* promoter of *C. freundii* suggested that one or more potential CRP binding sites was present. To extend our understanding of catabolite repression via the CRP system as it applies to the *tpl* promoter of *C. freundii*, reporter enzyme levels were measured on cells cultivated in glycerol-containing liquid minimal medium. With glycerol as a carbon source, there is little or no catabolite repression and the intracellular levels of cAMP are elevated. In the presence of L-tyrosine, the *tpl* promoter was slightly more active (twofold) when Δ(*tyrR*) cells were grown in glycerol-based medium than in glucose-containing medium (Table 2, line 2). Also, significantly more TyrR-specific activation, approximately 37-fold, was observed in glycerol-grown *tyrR*⁺ cells compared to a 22-fold effect of TyrR when cells were grown on glucose (Table 2, lines 1 and 2). This supports previous studies (50, 53) which suggested that the cAMP-CRP system can modulate the *tpl* promoter.

The *crp* gene was inactivated in the *tyrR*⁺ and Δ(*tyrR*) strains used in the previous experiment. The resulting Δ(*crp*) derivatives [SP1626 (*P_{tpl}-lacZ*⁺) and SP1627 (*P_{tpl}-lacZ*⁺)] were grown in liquid minimal medium containing either glycerol or glucose and L-tyrosine. In the Δ(*crp*) background, the differ-

ential activation of the *tpl* promoter that was seen when glycerol-grown cells were compared to glucose-grown cells was no longer evident (Table 2, lines 4 and 5). Introduction of a Crp⁺ plasmid (pXZCRP) into the Δ(*crp*) strains partially restored the glycerol-specific induction of the *tpl* promoter (Table 2, lines 6 and 7). In the absence of the TyrR protein, cAMP-CRP had no effect on reporter enzyme levels (Table 2, lines 2 and 5). These data suggest that the role of cAMP-CRP is to potentiate the TyrR protein-mediated stimulation of transcription from the *tpl* promoter and that in *tyrR*⁺ cells, the contribution of each transcription factor to promoter strength is independent and additive.

Chemical identification of TyrR and CRP binding sites in the *tpl* promoter. DNase I footprinting of *tpl* promoter DNA was carried out to define with precision the locations of the TyrR binding sites. A fragment of DNA identical to the one that had been used to construct the *tpl* reporter systems was isolated from pUC19-*tpl* and ³²P labeled at one end as described in Materials and Methods. In DNase I footprinting studies, three TyrR binding sites, designated A, B, and C were identified (Fig. 2). Boxes A and B, centered at coordinates -272.5 and -158.5 respectively, are very similar in sequence to the consensus TyrR operator sequence (TGTAAN₆TTT ACA) (39). Box C, centered at coordinate -49.5, bore little resemblance to the consensus TyrR targets other than the two invariant G and C residues (GN₁₄C). According to the classification scheme of Pittard (39), boxes A and B are likely to be strong TyrR boxes, while box C would appear to be a weak TyrR box. The approximate affinity of TyrR for each operator target was estimated. Box A was fully protected by 1 to 5 nM TyrR (Fig. 2, lane 3 of left gel). Under the same conditions, 20 nM (lane 5 of right gel) or 80 nM (lane 7 of right gel) TyrR were required to fully protect box B or C. The apparent affinity of the TyrR protein for each target site decreased in relation to the proximity of the site to the transcription start point of the *tpl* promoter.

To explore whether TyrR binds cooperatively to the three sites, the effect of a disabling G-to-A change in box A on the binding ability of TyrR was examined. As expected, this mutation decreased the affinity of TyrR for box A by at least 20-fold (Fig. 2, lane 8 of left gel). A slight effect of this box A mutation on the binding of TyrR to boxes B and C (approximately twofold reduction) was detected (Fig. 2, lane 8 of right gel). These results suggest that the binding of TyrR to the operator targets upstream of *tpl* is slightly cooperative, in the sense that the binding of protein to box B would be favored when box A was occupied. However, these results must be interpreted with caution, given the fact that this experiment used linear DNA as the target molecule, whereas cellular DNA is negatively supercoiled.

Based on a computer search, which suggested two putative CRP boxes upstream of the *tpl* promoter, as well as the in vivo results, CRP is predicted to bind to *tpl* promoter DNA. This hypothesis was investigated by DNase I footprinting. A 500-bp *EcoRI-PstI* fragment, containing the *tpl* promoter region, was labeled at one end with [α-³²P]dATP (Materials and Methods). The DNase I footprinting results identified two regions upstream of the *tpl* promoter that were protected by CRP in the presence of cAMP (Fig. 3 and 4). The target designated CRP1 (Fig. 1), centered at coordinate -250 of the *tpl* promoter, was well protected by cAMP-CRP. Complete protection was observed at a CRP concentration of approximately 10 nM. A second target, CRP2, situated 30 bp downstream of the CRP1 site, was incompletely protected by CRP (Fig. 1 and 3). In the CRP2 region, the cleavage of certain phosphodiester bonds by DNase I was enhanced as the concentration of

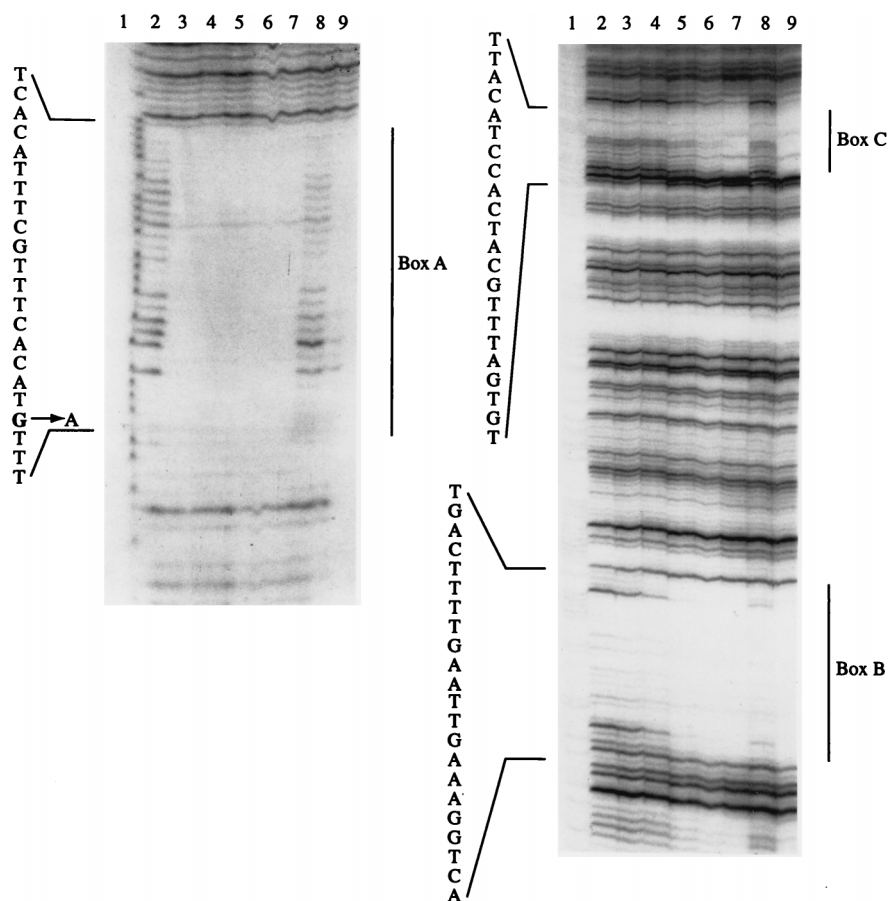


FIG. 2. DNase I footprints of TyrR bound to the promoter regions of *tpl* or *tpl-mut1*. The DNA fragments were generated by *EcoRI-PstI* digestion of pUC19-*tpl* (lanes 2 to 7, both panels), or pUC19-*tplmut1* (lanes 8 and 9, both panels). The latter construct contains a G-to-A change in box A. Both fragments were labeled with ^{32}P at the 5' ends as described in Materials and Methods. Treatment with DNase I was carried out in the presence (lanes 3 to 9, both gels) or absence (lanes 2, both gels) of TyrR. All reaction mixtures contained 0.2 mM L-tyrosine, 0.2 mM ATP, and 0.7 nM DNA. The concentrations of TyrR used were as follows: lanes 3, 5 nM; lanes 4, 10 nM; lanes 5 and 8, 20 nM; lanes 6, 40 nM; lanes 7 and 9, 80 nM. Lanes 1 contain the A+G sequence of *tpl* DNA. The regions protected by TyrR are indicated. Box A is shown in the left gel, and boxes B and C are shown in the right gel. Each of the DNA sequences shown reads from the 5' end (bottom) toward the 3' end (top) as shown in Fig. 1.

cAMP-CRP was increased. The reason for this is not clear. The sequences of the two CRP binding sites are quite similar to each other and to the other known CRP consensus sequences (31).

Because CRP site 1 and TyrR box A are separated by only 3 nucleotide pairs, it seemed possible that TyrR and cAMP-CRP, both bound at adjacent sites in the *tpl* promoter, might interact in a cooperative fashion. When a DNA fragment containing a G-to-A change in box A that drastically reduced the affinity for TyrR was used as a target in DNase I footprinting studies in the presence of TyrR, DNase I protection by cAMP-CRP at the CRP1 and CRP2 sites was unaffected (Fig. 4, compare lanes 6 to 8 with lanes 2 to 5). This suggests that cAMP-CRP and TyrR bind independently to their respective target sites within the *tpl* promoter which is consistent with the reporter enzyme experiments (Table 2).

To confirm that cAMP plays a role in the regulation of the *tpl* promoter, DNase I footprinting was carried out in the absence or presence of cAMP (Fig. 4, lanes 1 to 5). There was no protection by CRP (100 nM) alone (Fig. 4, lane 2). Increasing the concentration of cAMP from 25 to 100 μM (Fig. 4, lanes 3 to 5) afforded complete protection by CRP.

Effects of ATP and tyrosine on DNase I protection. In order to assess the roles of ligands in the binding of TyrR to its

respective target sites, a series of DNase I footprinting assays were carried out under a variety of different conditions (Fig. 5). Box A was protected by unliganded TyrR. The pattern of protection was unaltered by the addition of ATP and tyrosine, alone or in combination. Box B was partially protected by unliganded TyrR; full protection was observed when ATP or ATP plus tyrosine were present. Box C was protected only when TyrR, ATP, and tyrosine were present. When cAMP-CRP was present, there were no changes in the protection of the TyrR boxes.

Regulation of the *tpl* promoter by IHF. The center-to-center distance between boxes A and B is almost the same as the distance between boxes B and C (Fig. 1). Two CRP binding sites are located between boxes A and B. The binding of cAMP-CRP to its target site may serve to bend DNA in this region, thereby mediating interactions between TyrR dimers bound to boxes A and B. This raised the possibility that a binding site for an unknown factor lay between boxes B and C and that its role was to facilitate interactions between TyrR dimers bound to boxes A, B, and C. A putative IHF binding site was detected by a computer search of the region upstream of the *tpl* promoter. An approach similar to that used in the investigation of the role of cAMP-CRP was chosen to explore a possible role for IHF. The *himD* gene, encoding the β sub-

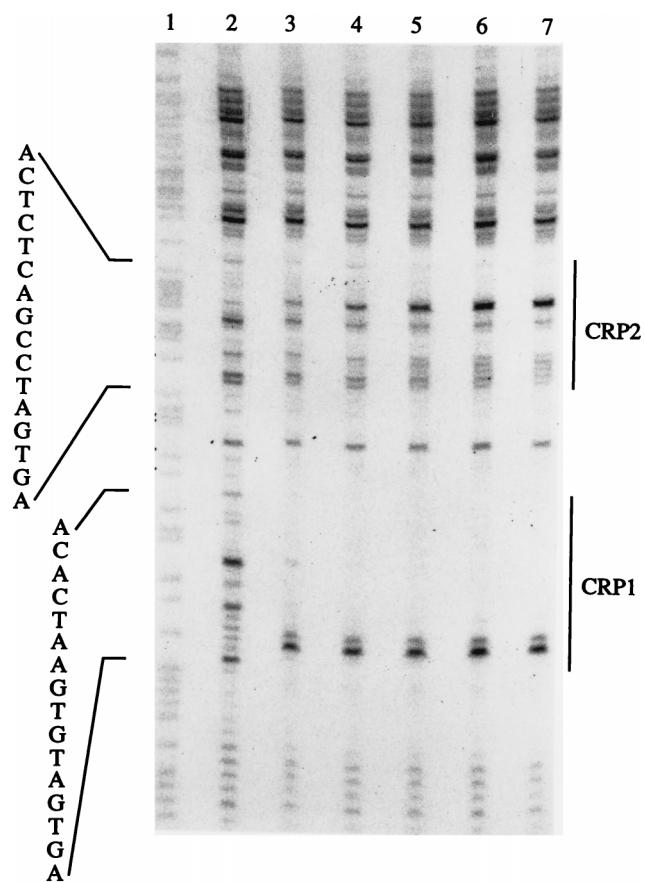


FIG. 3. DNase I footprints of CRP bound to the *tpl* promoter region. The DNA fragment used was generated by *EcoRI-PstI* digestion of pUC19-*tpl* and labeled with ^{32}P at the 5' end. The DNA was treated with DNase I in the presence (lanes 3 to 7) or absence (lane 2) of CRP and cAMP. The concentrations of DNA and cAMP were 1 nM and 100 μM , respectively. The concentrations of CRP were as follows: lane 3, 10 nM; lane 4, 25 nM; lane 5, 50 nM; lane 6, 75 nM; lane 7, 100 nM. Lane 1 contains the A+G sequence of the DNA. Segments protected by CRP are indicated to the right. The sequences read from the 5' end (bottom) toward the 3' end (top) as shown in Fig. 1.

unit of IHF, was knocked out in a pair of *tyrR*⁺ and $\Delta(\textit{tyrR})$ strains [SP1628 (*P_{tpl}-lacZ*⁺) and SP1629 (*P_{tpl}-lacZ*⁺)]. The ability of TyrR to activate the *tpl* promoter was severely affected in IHF-negative strains. An IHF-specific reduction of about 35-fold in reporter enzyme levels was detected (Table 2, compare lines 1 and 9). The utilization of the *tpl* promoter was also studied in strains with knockouts of both the *himD* and *crp* genes [SP1630 (*P_{tpl}-lacZ*⁺) and SP1631 (*P_{tpl}-lacZ*⁺)]. In glycerol-grown cultures, severe reductions in enzyme level were observed. Reporter enzyme levels were approximately 500-fold lower than in the *tyrR*⁺ reference strain SP1312 (*P_{tpl}-lacZ*⁺) grown in the same medium (Table 2, compare lines 1 and 12). On the other hand, about a 10-fold reduction below that of the $\Delta(\textit{tyrR})$ host SP1313 (*P_{tpl}-lacZ*⁺) was observed in glycerol-grown cells (compare Table 2, lines 2 and 13), indicating that IHF can slightly activate the *tpl* promoter in the absence of TyrR. This activation was strongly enhanced to about 150-fold when TyrR was present (Table 2, lines 4 and 13). Introduction of pHN $\beta_2\alpha$, which encodes two copies of the β subunit of IHF and one copy of the α subunit, fully restored the activation function of the TyrR protein (Table 2, lines 1, 8, and 14). The results indicated that IHF is not only involved in the regulation

of *tpl* but also plays a critical supporting role in enabling TyrR to function as an activator.

A 22-bp binding site for IHF, centered at coordinate -85 of the *tpl* promoter, was located by DNase I footprinting (Fig. 6). The importance of this segment in transcription from the *tpl* promoter was confirmed by site-directed mutagenesis. As described in Materials and Methods, the IHF binding site was changed from A₆CTTGTTGAATATGAAC to A₆CTTGTCGACTATGAAC. Reporter enzyme measurements from single-copy versions of this altered *tpl* promoter in *tyrR*⁺ cells showed that there had been an 8.8-fold reduction in promoter strength (compare lines 1 and 10 of Table 2). Basal expression from the *tpl* promoter, measured in $\Delta(\textit{tyrR})$ cells, was essentially unchanged when the IHF binding site was mutated (compare lines 2 and 11 of Table 2). This set of results strongly supports a role for IHF in the activation of the *tpl* promoter.

To address the possibility that IHF alters the affinity of the *tpl* promoter for TyrR, a DNase I footprinting experiment was performed in the presence of various combinations of cAMP-CRP, IHF, and TyrR. Since the protection by TyrR of boxes B and C is the most sensitive indicator of possible cooperativity in the occupancy of these target sites by the three transcription factors, attention was focused on this segment of the *tpl* pro-

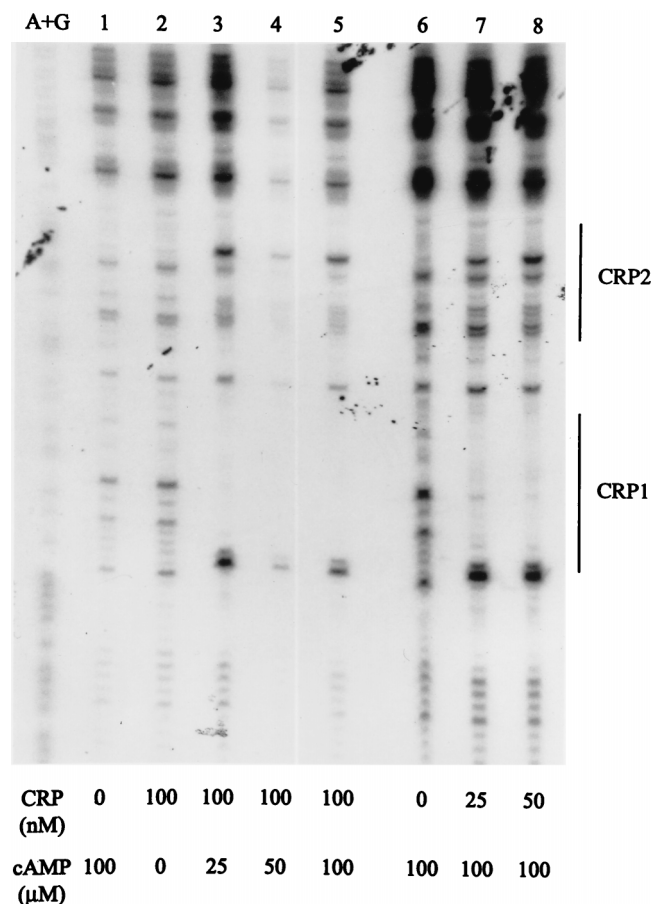


FIG. 4. Requirement for cAMP in DNase I footprinting of *tpl* promoter DNA by CRP. The DNA fragment used in lanes 1 to 5 was an *EcoRI-PstI* fragment from pUC19-*tpl*. The fragment used in lanes 6 to 8 was an *EcoRI-PstI* fragment from pUC19-*tplmut1* which contains a critical G-to-A change in box A. Each DNA fragment was labeled with ^{32}P at the 5' end. The concentrations of CRP and cAMP are shown at the bottom. The segments protected by CRP are indicated to the right. A+G standards are in the leftmost lane. TyrR (50 nM), L-tyrosine (0.2 mM), and ATP (0.2 mM) were present in each tube.

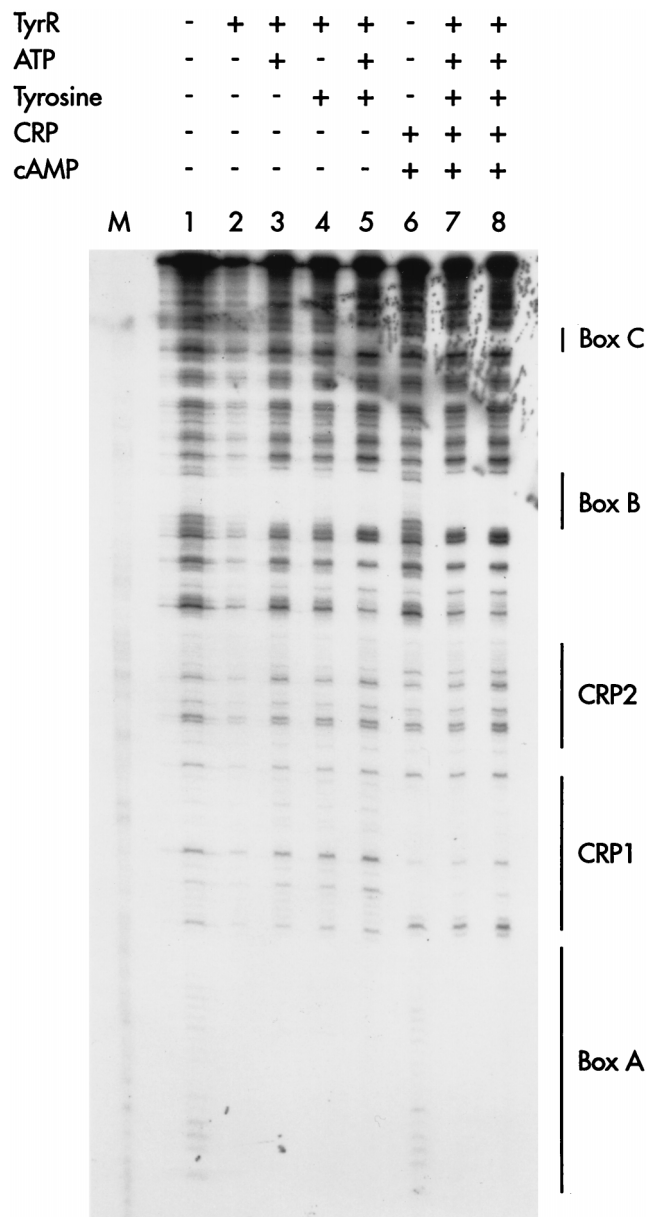


FIG. 5. Effects of ATP and tyrosine on DNase I footprinting by TyrR and cAMP-CRP. The DNA used in each case was 1 nmol of a ³²P-labeled *EcoRI-PstI* fragment from pUC19-*tpl*. The label was at the 5' end (see Materials and Methods). The presence (+) or absence (-) of other compounds added to each tube are shown at the top of the figure. When present, TyrR was included at a final concentration of 100 nM, except in lane 7, when the TyrR concentration was 50 nM. The concentrations of other additives were as follows: ATP, 0.2 mM; L-tyrosine, 0.2 mM; CRP, 50 nM; cAMP, 100 μM. The segments protected by TyrR (boxes A, B and C) and cAMP-CRP are indicated to the right. Lane M, A+G standards.

moter. To a first approximation, neither IHF nor cAMP-CRP altered the affinity of TyrR for either box B or box C (Fig. 7). The only IHF-specific effect that was noted was a slight reduction in the cleavability of phosphodiester bonds on either side of the IHF target site (compare lanes 2 and 3, 4 and 5, 6 and 7, etc.). However, this effect was not correlated with TyrR concentration. In agreement with a previous result (Fig. 4), cAMP-CRP had no effect on the binding of TyrR to its target sites.

Effects of mutations within the TyrR boxes of the *tpl* promoter. Previous comparisons (39) of TyrR binding sites have shown that there are only two absolutely conserved nucleotides (GN₁₄C) within this class of operators. Alteration of either residue severely reduced the TyrR-mediated regulation of promoter activity. To examine the roles of boxes A, B, and C in the regulation of the *tpl* promoter, G-to-A changes were introduced at the first invariant position in each of the TyrR boxes. When box A of the *tpl* promoter was mutated, the β-galactosidase activity of glucose-grown cells fell to a level characteristic of Δ(*tyrR*) host strains, i.e., there was a reduction of approximately 13-fold in reporter enzyme level from that in *tpl* promoter systems where box A was intact (compare line 1 of Table 3 to line 1 of Table 2). The effect of a G-to-A change in box B was not as severe as that in box A. In glucose-grown cells, a sevenfold reduction in reporter enzyme levels was observed (compare line 2 of Table 3 to line 1 of Table 2). Considering only glycerol-grown cells, where the cAMP-CRP effect is maximal, the β-galactosidase activities were reduced about 4.6-fold when either box A or box B was mutated (compare lines 1 and 2 of Table 3 to line 1 of Table 2). These data are consistent with the results of Table 2 (lines 1 and 4), where the contribution of cAMP-CRP to *tpl* promoter activity was found to be about threefold. There was no detectable TyrR or cAMP-CRP-mediated activation when box C was mutationally inactivated (Table 3, lines 3 and 4). This was unexpected, since box C showed little sequence homology to other TyrR boxes and fits the criteria for a weak box. Evidently cAMP-CRP can stimulate transcription from the *tpl* promoter when either box A or box B is disabled, but not when box C is incapable of engaging the TyrR protein.

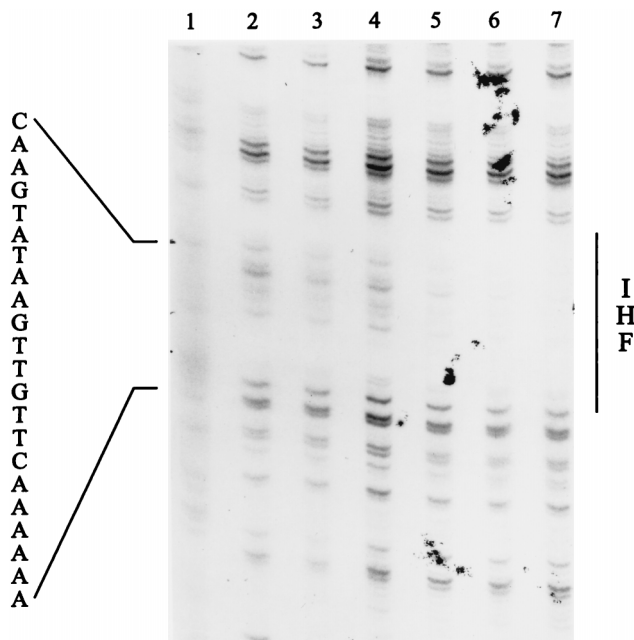


FIG. 6. DNase I footprint of the IHF binding site within the *tpl* promoter region. The *EcoRI-PstI* fragment from pUC19-*tpl* was labeled with ³²P at the 5' end as described in Materials and Methods. The DNA was treated with DNase I in the presence (lanes 3 to 7) or absence (lane 2) of IHF. The concentration of DNA was 0.7 nM in each reaction mixture. The concentrations of IHF were as follows: lane 3, 9 nM; lane 4, 18 nM; lane 5, 36 nM; lane 6, 72 nM; lane 7, 144 nM. Lane 1 contains the A+G sequence. The region protected by IHF is indicated to the right. The protected sequence reads from the 5' end (bottom) toward the 3' end (top) as shown in Fig. 1.

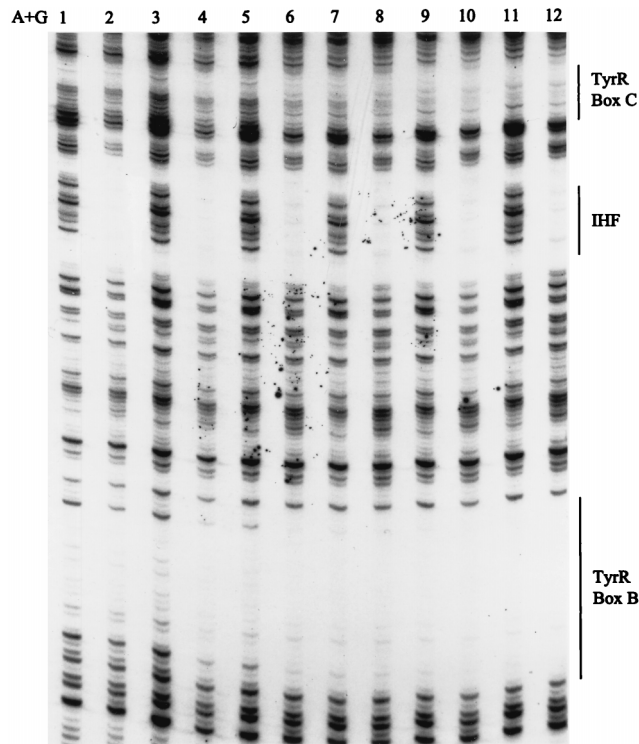


FIG. 7. Effects of IHF and cAMP-CRP on DNase I footprinting of boxes B and C. The DNA used was 0.7 nmol of an *EcoRI-PstI* fragment from pUC19-*tpl* that had been ^{32}P labeled at the 5' end (see Materials and Methods). The tubes alone were used (lane 1), or CRP (20 nM) and IHF (145 nM) (lanes 2, 4, 6, 8, 10, and 12) were added to the tubes. The TyrR concentrations were as follows: lanes 2 and 3, 10 nM; lanes 4 and 5, 20 nM; lanes 6 and 7, 40 nM; lanes 8 and 9, 60 nM; lanes 10 and 11, 80 nM; lane 12, 100 nM. Each reaction mixture contained ATP and tyrosine (0.2 mM each). Tubes containing CRP also contained cAMP (100 μM).

In previous work, it was shown that the *tpl* promoter became nonfunctional when upstream segments of DNA containing boxes A or A and B were deleted (50). The deletions in question shortened the *tpl* promoter by 64, 130, and 179 nucleotides

from the 5' upstream region (Fig. 1). This result was confirmed and extended in the present study. In no case was there significant β -galactosidase activity. In glycerol-grown cells, the $\Delta 130$ mutant with both CRP binding sites deleted showed much lower reporter enzyme levels (threefold drop) than the $\Delta 64$ mutant with intact CRP sites (Table 3, lines 5 to 8). This result is consistent with the general notion that cAMP-CRP stimulation functions largely in conjunction with the occupancy by TyrR of box C. If either target site is removed from the *tpl* promoter, reporter enzyme levels fall to basal values.

The DNase I footprinting result (Fig. 2) showed that the 3' end of box C either overlapped or was immediately adjacent to the -35 region of the *tpl* promoter. This suggested that box C plays a critical role in TyrR-mediated transcriptional activation. In order to address the functional importance of box C, we constructed a multiple mutant with a G-to-A change in box B in combination with two inactivating mutations in box C. The promoter activity of the resulting construct was totally abolished in both glucose- and glycerol-based media (Table 3, lines 9 and 10). Recall that it had previously been shown that a single mutational change in box B had only a slight effect. The possibility of cooperativity in the binding of TyrR to boxes A, B, and C was raised in the experiment of Fig. 2. If the role of cooperativity in the binding of TyrR to the *tpl* promoter were only to provide a local high concentration of activator near the RNA polymerase binding site, then converting box C to a strong box identical to box A (a high-affinity target for the TyrR protein) in promoters lacking box A or boxes A and B would be predicted to generate a TyrR-responsive *tpl* promoter. Surprisingly, only a fourfold TyrR-mediated stimulation of reporter enzyme was detected in a promoter containing a strong box at location C in combination with a deletion of boxes A and B [SP1312 (*Ptpl-lacZ*⁺) $\Delta 179\text{C}$] (Table 3, lines 13 and 14). A TyrR-mediated stimulation of about sixfold was detected in a promoter containing a strong box C together with a deletion of box A [SP1312 (*Ptpl-lacZ*⁺) $\Delta 64\text{C}$] (Table 3, lines 11 and 12). Introduction of pJC100, which enables cells to make TyrR protein at slightly higher than haploid levels, into SP1313 (*Ptpl-lacZ*⁺) $\Delta 64\text{C}$ significantly increased the level of reporter enzyme. A TyrR-specific induction of 26-fold was observed (Table 3, compare lines 12 and 15). A similar amplitude of change was obtained when pJC100 was introduced into

TABLE 3. Mutational analysis of transcriptional activation at the *tpl* promoter

Strain	Relevant genotype	β -Galactosidase activity ^a	
		Glucose	Glycerol
SP1312 (<i>Ptpl-lacZ</i> ⁺)mut1	<i>tyrR</i> ⁺ , G→A in box A	57.9	555
SP1312 (<i>Ptpl-lacZ</i> ⁺)mut2	<i>tyrR</i> ⁺ , G→A in box B	111.0	549
SP1312 (<i>Ptpl-lacZ</i> ⁺)mut3	<i>tyrR</i> ⁺ , G→A and C→T in box C	47.8	85.3
SP1313 (<i>Ptpl-lacZ</i> ⁺)mut3	Δ (<i>tyrR</i>), G→A and C→T in box C	22.0	22.6
SP1312 (<i>Ptpl-lacZ</i> ⁺) $\Delta 64$	<i>tyrR</i> ⁺ , 64-nucleotide deletion	41.2	278
SP1313 (<i>Ptpl-lacZ</i> ⁺) $\Delta 64$	Δ (<i>tyrR</i>), 64-nucleotide deletion	13.1	30.0
SP1312 (<i>Ptpl-lacZ</i> ⁺) $\Delta 130$	<i>tyrR</i> ⁺ , 130-nucleotide deletion	34.1	88.9
SP1313 (<i>Ptpl-lacZ</i> ⁺) $\Delta 130$	Δ (<i>tyrR</i>), 130-nucleotide deletion	17.1	55.2
SP1312 (<i>Ptpl-lacZ</i> ⁺)mut2,3	<i>tyrR</i> ⁺ , G→A and C→T in box C, G→A in box B	42.0	72.7
SP1313 (<i>Ptpl-lacZ</i> ⁺)mut2,3	Δ (<i>tyrR</i>), G→A and C→T in box C, G→A in box B	28.3	38.2
SP1312 (<i>Ptpl-lacZ</i> ⁺) $\Delta 64\text{C}$	<i>tyrR</i> ⁺ , 64-nucleotide deletion, box C is replaced by box A	93.6	170
SP1313 (<i>Ptpl-lacZ</i> ⁺) $\Delta 64\text{C}$	Δ (<i>tyrR</i>), 64-nucleotide deletion, box C is replaced by box A	12.1	28.7
SP1312 (<i>Ptpl-lacZ</i> ⁺) $\Delta 179\text{C}$	<i>tyrR</i> ⁺ , 179-nucleotide deletion, box C is replaced by box A	65.9	72.9
SP1313 (<i>Ptpl-lacZ</i> ⁺) $\Delta 179\text{C}$	Δ (<i>tyrR</i>), 179-nucleotide deletion, box C is replaced by box A	14.3	22.0
SP1313 (<i>Ptpl-lacZ</i> ⁺) $\Delta 64\text{C}$ /pJC100	Δ (<i>tyrR</i>) <i>ptyrR</i> ⁺ $\Delta 64\text{C}$	316	527
SP1313 (<i>Ptpl-lacZ</i> ⁺) $\Delta 179\text{C}$ /pJC100	Δ (<i>tyrR</i>) <i>ptyrR</i> ⁺ $\Delta 179\text{C}$	410	ND
SP1313 (<i>Ptpl-lacZ</i> ⁺) $\Delta 179\text{C}$ /pJC100/pJC136	Δ (<i>tyrR</i>) (<i>ptyrR</i> ⁺) $\Delta 179\text{C}$	614	ND

^a β -Galactosidase activity is expressed in Miller units. Cultures were grown in glucose or glycerol as indicated. ND, not determined.

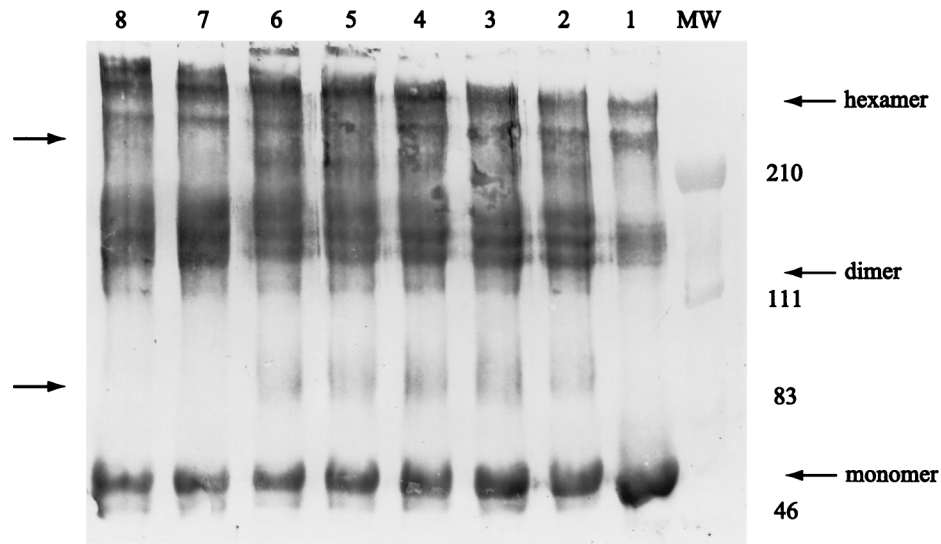


FIG. 8. DNA dependence of chemical cross-linking of TyrR demonstrated by immunoblotting. The cross-linking reagent was DMP. The reaction was carried out with 3.6 μ M TyrR, 1.2 μ M CRP, 100 μ M ATP, 50 μ M cAMP, and 200 μ M L-tyrosine in the presence (lanes 1 to 6) or absence (lanes 7 and 8) of pUC19-*tpl* DNA. Lane 1 is a control (no DMP). The concentration of DMP was 4 mg/ml. The incubation times for each reaction mixture were 10 min (lane 2), 20 min (lane 3), 40 min (lanes 4 and 7), 80 min (lanes 5 and 8), and 160 min (lane 6). The locations of molecular weight markers (in thousands) are indicated to the right. The positions of TyrR monomer, dimer, and hexamer are indicated with arrows to the right. The positions of presumptive TyrR-CRP complexes are indicated with arrows to the left. For details, see Materials and Methods.

SP1313 (*P_{tpl}-lacZ*⁺) Δ 179C (Table 3, compare lines 14 and 16). Further elevations in reporter enzyme levels were observed when TyrR levels were boosted via the introduction of a second compatible plasmid (pJC136) encoding TyrR (Table 3, line 17). These data suggest that elevated levels of TyrR facilitate transcriptional activation of the *tpl* promoter via box C. It was noted earlier that DNase I footprinting of box C also required high levels of TyrR (Fig. 2). We hypothesize that the TyrR operators in the *tpl* promoter facilitate the formation of a higher-order protein complex necessary for the productive interaction between TyrR and RNA polymerase.

Interaction between TyrR dimers at the *tpl* promoter. Mutational studies on DNA carrying TyrR boxes suggested that the DNA-mediated association of TyrR dimers plays a role in activating transcription at the *tpl* promoter. Earlier studies demonstrated that TyrR exists as a dimer in solution (9) and that dimers could interact to give rise to hexamers (59). To further investigate the range of aggregation states available to TyrR, a series of chemical cross-linking studies were conducted. Upon incubation with DMP, the subunits of TyrR protein were readily converted, in a time-dependent manner, to species with increasing multiples of the molecular weight of the monomer (Fig. 8). DMP mediates cross-linking via the ϵ amino groups of lysine residues, provided that these functional groups are separated by no more than 9.2 \AA . In the absence of DNA, complexes corresponding to the dimeric, tetrameric, hexameric, and octameric forms of TyrR were detected both on stained SDS-polyacrylamide gels or on Western blots developed with anti-TyrR antibodies (Fig. 8, lanes 7 and 8). When *tpl* DNA, bearing operator targets for TyrR, was present, the percentage of hexamer was dramatically increased, while the octamer form of TyrR became almost undetectable (data not shown). When cAMP-CRP was included in the cross-linking reaction mixtures with TyrR and DNA, two major complexes likely to contain TyrR-CRP cross-linked species were demonstrated by immunoblotting (Fig. 8). The molecular mass of each complex was estimated to be 83 to 95 kDa (broad) and 200 kDa. The 83- to 95-kDa complexes consist of

two species. One is likely to contain one subunit of TyrR and one subunit of CRP; the other is likely to contain one subunit of TyrR and two subunits of CRP. The 200-kDa protein complex is likely to have originated from cross-linking between one TyrR dimer and two CRP dimers. This is reasonable because there are two CRP binding sites near TyrR boxA (Fig. 1).

Attempts to verify the proposed composition of these species through the use of polyclonal goat anti-CRP immunoglobulin G (a gift from Sankar Adhya) were unsuccessful, presumably because the epitopes of CRP recognized by this reagent were unavailable by Western blotting. The inclusion of IHF in the cross-linking reactions produced no detectable changes from the patterns shown in Fig. 8 (data not shown). The apparent heterogeneity in molecular mass of the observed TyrR dimers, tetramers, and hexamers probably reflects cross-linking between different lysine residues of TyrR located at widely different positions within the TyrR monomers. These results suggest that a higher-order nucleoprotein complex containing TyrR, such as a hexamer, forms at the *tpl* promoter. This could be the species that interacts with RNA polymerase to activate *tpl* expression.

DISCUSSION

The TyrR protein of *E. coli* is known to regulate the expression of a number of genes involved in the biosynthesis and transport of aromatic amino acids (39). The present results, together with previous data (50), enlarge our understanding of TyrR-regulated systems. TPL, an enzyme of *C. freundii* that degrades tyrosine to phenol, pyruvate, and ammonia is clearly a member of the TyrR regulon. The regulation of *tpl* requires L-tyrosine as a cofactor. Like *mtr* and *tyrP* (18, 21, 46, 58), *tpl* is positively regulated by TyrR. In tester strains bearing a deletion of the *tyrR* gene, there was a reduction of at least 20-fold in the production of β -galactosidase from a single-copy *tpl-lacZ* reporter system from that of *tyrR*⁺ controls. Promoter function was completely restored when a plasmid expressing the *tyrR*⁺ gene was introduced into the Δ (*tyrR*) strain. As

revealed by DNase I footprinting experiments, TyrR binds to three operator targets, named A, B, and C, within the *tpl* promoter region. The binding sites are centered at positions -49.5 , -158.5 , and -272.5 relative to the transcriptional start point. Mutational alteration of any of the three operators abolished or severely reduced transcription.

Inspection of the sequence of each TyrR binding site (Fig. 1) reveals that boxes A and B are quite symmetrical and closely resemble the TyrR consensus sequence (TGTAAN₆TTT ACA). This was not the case for box C. Unliganded TyrR readily bound to the box A region of *tpl*. The DNase I footprint of Tyr bound to box A was unaffected by L-tyrosine and ATP. Box B was also protected by unliganded TyrR, but binding was enhanced when ATP was present. Under the same conditions, the box C region was protected by TyrR only when ATP and L-tyrosine were present (Fig. 5). Thus, box C conforms to the general criteria for a weak TyrR box first enunciated by Pittard and coworkers (39). The inability of TyrR to bind to box C in the absence of tyrosine and ATP probably reflects a poor fit between the operator recognition site of TyrR and the DNA of box C. TyrR binds to the three targets within the *tpl* promoter with progressively diminishing affinity, in the order box A > box B > box C. We assume that these different affinities for TyrR are functionally related to the regulatory response that is observed during the expression of *tpl*. It appears that transcription from the *tpl* promoter occurs only when TyrR occupies the lower-affinity site. Our data are consistent with the notion that TyrR binds cooperatively to the three boxes. When box A or boxes A and B were deleted, the utilization of the *tpl* promoter fell to a very low level. The interdependence of TyrR targets observed in vivo was supported by DNase I footprinting. The protection of box C was significantly diminished when box A or boxes A and B were deleted. In the $\Delta 64$ version of the *tpl* promoter (box A deleted), the DNase I protection of box C required >80 nM TyrR compared to a requirement of 80 nM TyrR for wild-type *tpl* DNA. With the $\Delta 179$ version of the *tpl* promoter (boxes A and B deleted), the protection of box C required >120 nM TyrR compared to a requirement of 80 nM for wild-type *tpl* DNA (data not shown). This apparently cooperative binding of the TyrR protein to its multiple binding sites is likely to offer a physiological advantage by increasing the overall effectiveness and specificity of occupancy by TyrR of binding sites within the *tpl* promoter. Similar situations exist for other systems, including the Lrp-regulated *ilvIH* promoter (57), the simian virus 40 early promoter (5), *Drosophila* heat shock promoters (62), and yeast promoters that are subject to general amino acid control (4, 20).

In contrast to the situation that prevails in several other TyrR-regulated promoters, two of the operator targets (boxes A and B) of the *tpl* promoter lie far upstream of the transcriptional start point, while box C is immediately adjacent to the RNA polymerase-binding site (Fig. 1). The three targets are separated from each other by 10 helical turns of B-form DNA. In the *mtr* and *tyrP* promoters, there are only two TyrR binding sites within each promoter whose centers are separated by no more than 30 bp. Neither *mtr* nor *tyrP* has TyrR target sites further upstream than coordinate -66 (21). The importance to transcriptional activation of the spacing between TyrR boxes has been studied in the *tyrP* system. Activation of *tyrP* can be detected if the boxes are separated by one turn of the helix but not if the separation involves three turns of the helix (2). In contrast, we found that even TyrR binding sites situated far upstream from the promoter were essential for the regulation of the *tpl* promoter. It is uncommon to find such remote regulatory elements in association with σ^{70} promoters, where activators tend to bind predominantly to targets between coor-

dinate -80 and -30 (16). The σ^{70} *tpl* promoter is an exception (50). The regulatory function of boxes A and B appears to be obligatorily linked with box C, located near the -35 recognition element of the *tpl* promoter. Inactivation of box C essentially eliminates the function of the other two sites, even when boxes A and B are fully intact. Since boxes A and B are far from the *tpl* promoter region, only looping of DNA would allow interactions between TyrR proteins, thus generating higher-order multimers of TyrR. Our cross-linking study of TyrR in the presence of *tpl* promoter DNA (Fig. 6) provides supporting evidence for multimerization of the TyrR protein.

Effects of global transcriptional regulatory proteins. Two DNA-binding proteins, IHF and CRP, were shown to participate in the regulation of *tpl* expression. Although IHF and CRP are generally considered to be global regulators, the involvement of both factors in the activation of a single promoter has been reported only for the *tdc* promoter (60). Here we explored a transcriptional regulatory system in which IHF and CRP are both required for full TyrR-mediated activation of *tpl*. Initially, this was studied with appropriately constructed background strains carrying a *tpl* reporter system. When a Δ (*himD*) mutation was introduced into host strains that contained a *tpl-lacZ* reporter system, the transcriptional activity of the *tpl* promoter became virtually undetectable, even when TyrR was available. On the other hand, a Δ (*crp*) mutation lowered the expression of *tpl* only about threefold. Although both IHF and CRP were shown to interact directly with the *tpl* promoter region, the IHF appears to be more critical in *tpl* regulation than CRP. In the absence of TyrR, IHF showed about a 10-fold effect on induction of the *tpl* promoter. This induction was dramatically increased when TyrR was present, indicating that IHF acts as a coactivator of the *tpl* system. Given what is known about the interaction of IHF with DNA (1), a plausible role for IHF is to bend *tpl* DNA in the region between boxes B and C. The bend angle, predicted to be 140° or greater, would alter the shape of DNA from approximately a straight line to something resembling a hairpin (42). IHF is involved in numerous processes in *E. coli* and some of its bacteriophages and plasmids, including site-specific recombination, DNA replication, and gene expression (12, 15). Most IHF-specific transcriptional regulatory events involve σ^{54} -dependent promoters, which become fully functional only when activator proteins bind to remote upstream sites. In NtrC-responsive σ^{54} promoters such as *nifA*, IHF binds to a site midway between an upstream activation site and the promoter, where it mediates the formation of a DNA loop that brings these elements into close proximity (19, 27). In contrast, σ^{70} -dependent promoters are rarely regulated via remote upstream activator binding sites.

How IHF is involved in many regulatory systems is a question that has been pursued for many years. The focus of interest has been on systems where removal of IHF causes qualitative changes. However, many of the reported effects on transcription of mutations in IHF are modest (two- to fivefold). Large effects attributable to IHF have been reported in only three cases: (i) induction by the NifA regulator of the *nifHDK* operon (19, 36); (ii) induction by the NarL regulator of the gene encoding nitrate reductase (41, 47); (iii) induction by the TdcR regulator of the gene encoding threonine dehydratase (60). We have demonstrated a large effect of IHF on the TyrR-mediated regulation of the *tpl* promoter. It has been reported that the capacities of NifA, NarL, and TdcR to produce a marked increase in enzyme levels are virtually abolished by mutations in the genes encoding IHF subunits and/or the target sites for IHF that lie upstream of the relevant promoters. Remarkably, all of these cases, including the TyrR-regu-

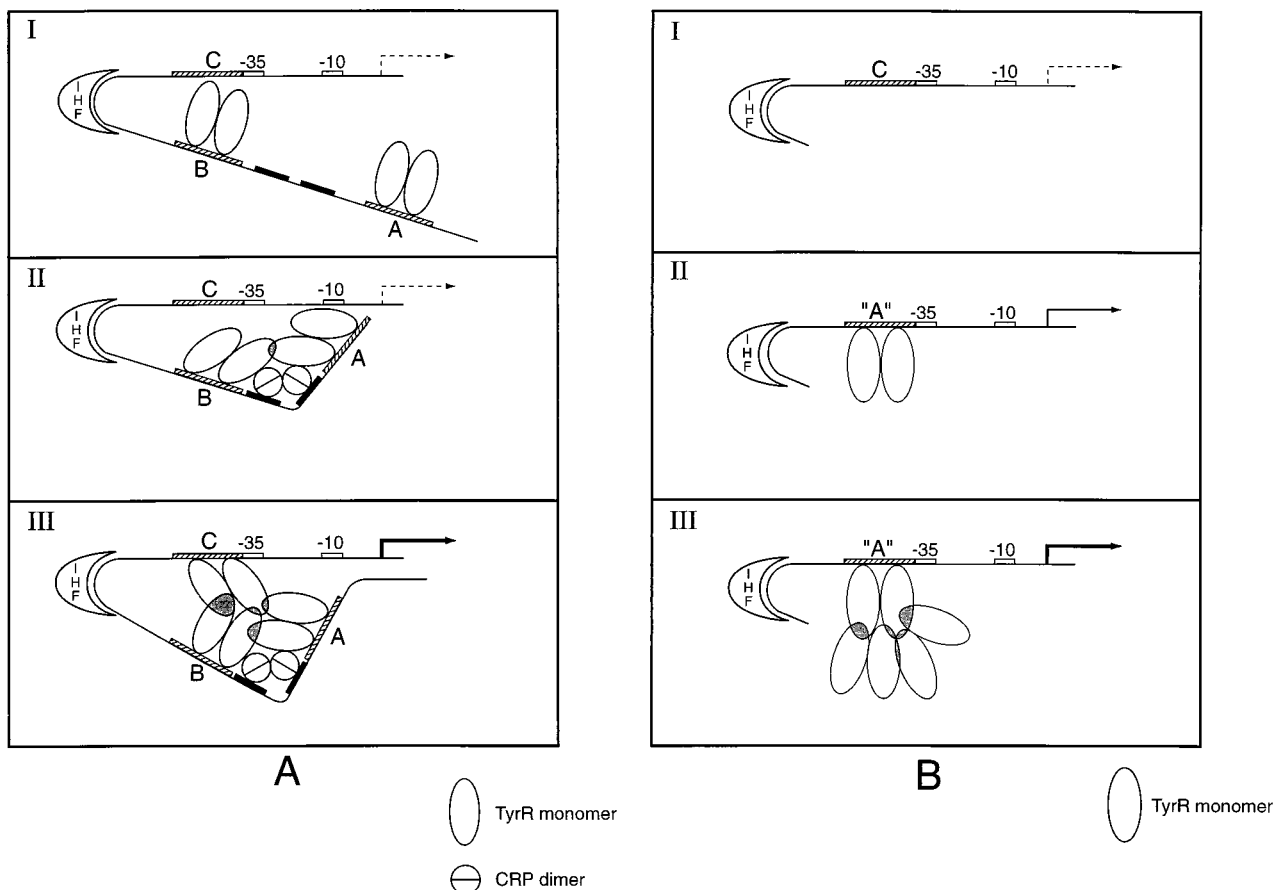


FIG. 9. Model for involvement of IHF and CRP in TyrR-mediated activation of *tpl*. TyrR boxes A, B, and C are shown as hatched bars. Two CRP binding sites are shown as black bars. The σ^{70} promoter region is indicated by white bars. See Fig. 1 for the sequences. (A) Activation of the wild-type *tpl* promoter. (I) Resting state. At low tyrosine concentrations, TyrR dimers occupy strong boxes A and B, while weak box C is unoccupied. The DNA between boxes B and C is bent by IHF. (II) Catabolite repression lifted. cAMP-CRP binds to target sites, introducing a bend between boxes A and B. Interaction between TyrR dimers bound to boxes A and B, induced by DNA bending, stabilizes DNA-TyrR interaction. In states I and II, the transcriptional activity, induced by IHF, is at a very low level (indicated by a broken arrow). The mechanism of activation by IHF is not clear. (III) Tyrosine induction. At increased concentrations of tyrosine, TyrR dimers are able to occupy weak box C. Interactions between TyrR dimers bound to boxes A, B, and C become possible. The interactions between TyrR dimers are indicated by shaded regions. This gives rise to a very stable TyrR-hexamer DNA structure. This higher-order nucleoprotein complex is proposed to fully activate the *tpl* promoter either by inducing a conformational change within promoter DNA or by interacting directly with σ^{70} RNA polymerase. Activation of the *tpl* promoter is indicated by a heavy black arrow. (B) Model for activation of $\Delta 179$ mutant *tpl* promoter. A DNA segment of 179 nucleotides that included boxes A and B was deleted from the region upstream of the *tpl* promoter region. In a separate operation, weak box C, centered at coordinate -49.5 , was replaced by a strong box essentially identical to box A (see Materials and Methods). This construct is designated box A. (I) Promoter inactivity. The $\Delta 179$ *tpl* promoter cannot be utilized when wild-type box C is the only TyrR target, probably because the affinity of TyrR for box C is too low to enable higher-order structures required for promoter activity to form. (II) Weak promoter activity. When box C is replaced by strong box A, TyrR dimers can attach to this region of the promoter. Occasional formation of higher-order structures would allow σ^{70} RNA polymerase to launch transcription. A low level of promoter activity is observed (indicated by a solid arrow). (III) Promoter activation. When the cellular content of TyrR dimers is elevated, there is an increased chance for the formation of complexes between TyrR dimers bound at box A and free TyrR dimers in solution. The formation of such higher-order complexes between TyrR dimers permits launching of σ^{70} RNA polymerase. Activation of the *tpl* promoter is indicated by a heavy black arrow.

lated *tpl* promoter, lead to the formation of ammonia from alternate sources (nitrogen, nitrate, threonine, and tyrosine). It has been suggested (37) that IHF may be essential for a range of cellular responses to nitrogen limitation, especially during simultaneous oxygen deprivation. Our data support a role for IHF as an enhancer of the *tpl* regulatory system and also provide evidence for a larger role for IHF in gene regulation.

It is not surprising that the cAMP-CRP complex is a transcriptional activator of the *tpl* promoter, given the fact that CRP is a generally recognized global regulator of gene expression (7a). In many cases, CRP acts by binding to a single site, located slightly upstream of the RNA polymerase binding site. In the absence of CRP, the promoter often displays a low affinity for σ^{70} RNA polymerase ($E\sigma^{70}$), while CRP and $E\sigma^{70}$ bind cooperatively to the promoter in the presence of CRP (11). In a few other cases, several adjacent CRP binding sites

have been observed, but the nature of their involvement in promoter activation is not well understood (8, 29, 38, 49, 55). A recent study of an artificial promoter having two CRP sites whose positions were systematically varied has clarified the functions of dual CRP targets (5a).

Our data suggest that CRP binds to two adjacent sites located far upstream of the RNA polymerase binding site of the *tpl* promoter and that this binding leads to full TyrR-mediated activation. In the presence of the TyrR protein, *tpl* promoter activity was increased by a factor of three by cAMP-CRP (Table 2). Two cAMP-CRP binding sites, situated between TyrR boxes A and B, were identified by DNase I footprinting (Fig. 3). The possibility of direct interaction between cAMP-CRP and the TyrR protein was addressed in chemical cross-linking experiments. Although this approach gave evidence consistent with TyrR-CRP proximity, DNase I footprinting analysis was

inconclusive in showing any effects of TyrR on the binding of cAMP-CRP or vice versa.

What might be the role of CRP in the *tpl* system? Two properties of CRP that were observed in studies of other promoters may be relevant to this question. First, the results of biochemical and genetic experiments have suggested that other proteins are capable of binding to CRP (11). Second, the binding of CRP to its target induces a 90° bend in the DNA (48). Thus, the role of CRP in the *tpl* regulatory system may be to contribute to the assembly of a TyrR-containing nucleoprotein complex. This could be accomplished either by specific interactions of CRP with TyrR or via cAMP-CRP-mediated bending of the DNA between boxes A and B that might favor interactions between TyrR dimers bound at these sites (1).

Model for activation of transcription from the *tpl* promoter.

The *in vivo* and *in vitro* results presented herein, viewed within the framework of the established properties of the IHF and CRP proteins, suggest a model for the regulation of *tpl* expression (Fig. 9). This model is based on the hypothesis that the occupancy of the promoter-proximal TyrR binding site (box C) is a prerequisite for transcription. Box C binds TyrR weakly. The cooperative binding of TyrR to the *tpl* promoter via boxes A and B, with the assistance of IHF and cAMP-CRP, is proposed to facilitate the binding of TyrR to box C. At low tyrosine concentrations, it is proposed that TyrR, bound to box A, can become positioned near box B of the *tpl* promoter region, provided that there is sufficient cAMP-CRP available to bend *tpl* DNA between boxes A and B. This would happen readily in glycerol-grown cells but would not occur if the production of cAMP or CRP were blocked by mutation. A TyrR dimer bound at box B is presumed to interact with TyrR at box A, forming a stable complex. When the concentration of tyrosine increases, TyrR acquires the ability to bind to box C; meanwhile, the tetrameric TyrR-box A-box B complex could approach the *tpl* promoter near box C by virtue of the DNA bending activity of IHF. It is proposed that this gives rise to a very stable TyrR hexamer-DNA structure that can interact with RNA polymerase to initiate transcription. The nonavailability of any of the three proteins or relevant target sites would impair transcription.

It is not clear whether TyrR, IHF, and CRP must physically interact to achieve optimal promoter expression or if simultaneous occupancy of binding sites in DNA is the critical feature. In DNase I footprinting experiments in the presence of all three proteins (TyrR, IHF, and CRP), there were no indications of binding cooperativity (Fig. 7). For none of the other genes regulated by IHF has physical contact between IHF and an upstream regulator or RNA polymerase been demonstrated. Thus, it is unlikely that IHF interacts directly with TyrR and/or RNA polymerase. The presumed role of IHF in *tpl* expression is to bend *tpl* promoter DNA to enhance the interaction between TyrR dimers.

Our model is consistent with the work of Wilson et al. (59), who demonstrated that TyrR undergoes ligand-induced hexamerization. By sedimentation equilibrium methods, these workers found that TyrR dimers undergo reversible association to form hexamers in the presence of tyrosine and ATP. In the present study (Fig. 8), we used chemical cross-linking to demonstrate the oligomerization of TyrR in the presence of *tpl* promoter DNA. Mutational alterations within *tpl*-specific TyrR targets proved that three sites must be functional in order for full activation of the *tpl* promoter to proceed.

The available information is also compatible with an alternative model where the formation of a higher-order complex containing three or more dimers of TyrR is the key feature in the transcriptional activation of *tpl*. Such a model has been

proposed by Porter et al. (40) and Wyman et al. (61), who reported that NtrC-P from *Salmonella typhimurium* must self-assemble into oligomers in order to activate transcription. It has long been known that the central region of TyrR bears significant homology to the central region of NtrC. The central domains of proteins in the NtrC superfamily are thought to interact directly with σ^{54} RNA polymerase and to mediate protein dimerization. It is reasonable to hypothesize that TyrR and NtrC utilize similar mechanisms of gene regulation. The physiological advantage of oligomerization was suggested (40, 61) to be as follows: DNA target sites, by virtue of the inability to bind dimeric transcription factors, facilitate interactions between regulatory proteins. Activation therefore occurs only at the correct locations on the chromosome. This is important because other activators that are homologs of TyrR or NtrC could activate transcription in response to a variety of physiological signals that are unrelated to the specific regulons to which they belong (40).

Distinctions in mode of action of TyrR at *tpl*, *mtr*, and *tyrP*.

It is likely that the mechanism of TyrR-regulated *tpl* expression is quite different from the role of TyrR as a positive regulator of *mtr* and *tyrP*. In the case of *tyrP*, there is dual regulation, namely, tyrosine-mediated repression or phenylalanine-mediated activation, at the same promoter. Mutational studies (2) of two *tyrP* targets (boxes 1 and 2) showed that both TyrR boxes were required for repression but that only the upstream box (box 2) was required for activation. The degree of activation of the *tyrP* promoter was critically related to the location of box 2. Maximal activation was observed when box 2 was moved 3 or 12 to 14 residues upstream, while no activation was seen at intermediate positions such as +7 and -4 (2). This positional restriction of box 2 in *tyrP* was attributed to the dual role of box 2 in both repression and activation. We predict that the relative positions of the TyrR targets (boxes A and B) in the *tpl* promoter will be less critical owing to their remote distance from the promoter. In *mtr*, mutational experiments were used to demonstrate that the strong box plays an important role in activation by phenylalanine and tyrosine. Mutations in the weak box had no effect on activation by phenylalanine but decreased activation by tyrosine (45). Comparing these results with our data obtained from mutational studies of the *tpl* promoter, one is forced to conclude that phenylalanine- and tyrosine-mediated TyrR activation involves distinct regulatory mechanisms. Whether a multimeric TyrR complex is involved in tyrosine-mediated regulation of *mtr* remains to be determined. Further studies will be necessary to establish the precise mechanistic roles of IHF and CRP in *tpl* expression and how the formation of hexameric TyrR is regulated by tyrosine.

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