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Mutational Analysis of Repression and Activation of the *tyrP* Gene in *Escherichia coli*

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In a previous report it had been suggested that the *tyrP* gene of *Escherichia coli* may be expressed from two separate promoters. We have endeavored to confirm this suggestion by primer extension studies and the separate subcloning of each of these promoters. In these studies, we found a single promoter whose expression was repressed by TyrR protein in the presence of tyrosine and activated by TyrR protein in the presence of phenylalanine. Two adjacent TYR R boxes, with the downstream one overlapping the *tyrP* promoter, are the likely targets for the action of TyrR protein. Mutational analysis showed that both TYR R boxes were required for tyrosine-mediated repression but that only the upstream box was required for phenylalanine-mediated activation. In vitro DNase protection studies established that whereas in the absence of tyrosine TyrR protein protected the region of DNA represented by the upstream box, at low TyrR protein concentrations both tyrosine and ATP were required to protect the region of DNA involving the downstream box and overlapping the RNA polymerase binding site.

The *tyrP* gene encodes a cytoplasmic membrane protein which represents the tyrosine-specific transport system of *Escherichia coli* (28, 30). Its expression is repressed when cells are grown in medium supplemented with tyrosine but is activated when phenylalanine but not tyrosine is present in the medium (13, 29). When both tyrosine and phenylalanine are present, repression is dominant. When cells are grown in minimal medium, expression of *tyrP* is partially repressed. Both repression and activation require functional TyrR protein, making *tyrP* a member of the TyrR regulon (14, 29).

The *tyrP* gene and its upstream region have been sequenced, and two adjacent TYR R boxes, one of which overlaps the −35 region of a likely promoter, have been identified (13). TYR R boxes are related to the palindromic sequence TGTAAA-N_6-TTTACA and, in every case in the TyrR regulon in which repression is mediated by TyrR protein and tyrosine, two adjacent boxes have been found to occur (5, 6, 9, 11, 13, 22, 31).

Two mutants in which a base change in the right-hand box of *tyrP* has resulted in a decrease in tyrosine repression but not in phenylalanine activation have been described (13). In these studies, in which S1 nuclease was used to identify the transcription start site in *tyrP*, the presence of two promoters was reported. One of these was positioned so that its −35 region overlapped the right-hand TYR R box, whereas the other was located some 40 bases further upstream. This latter transcription start site seemed to be specifically activated by growth in Casamino Acids and to a lesser extent by growth in the presence of phenylalanine. Other experiments showed that the Casamino Acids effect was independent of TyrR.

In this paper, we report new experiments that establish that there is in fact only one promoter for *tyrP*, confirm its position overlapping the right-hand TYR R box, and offer an explanation for the apparent presence of a second promoter in previous results. In addition, we describe base substitution mutations in both the left- and right-hand boxes and discuss the role that the different boxes play in repression and activation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. The bacterial strains used in this study were all derivatives of *E. coli* K-12, and their relevant characteristics are shown in Table 1. The plasmids used are also shown in Table 1. Bacteriophages M13tg130 and M13tg131 have been described elsewhere (15).

Media and chemicals. The minimal medium used was prepared from the 56/2 buffer of Monod et al. (20) and supplemented with appropriate growth requirements. To study regulation, we added tyrosine and/or phenylalanine to the minimal medium at a final concentration of 1 mM. Trimethoprim was used in nutrient and minimal media 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. The chemicals used were all obtained commercially and not purified further. [α-^{35}S]dATP (1,200 Ci/mmol; 10 mCi/ml) was obtained from Amersham International.

Recombinant DNA techniques. Standard techniques were used essentially as described previously (16). DNA sequencing of operator mutants involved cloning the respective promoter-operator regions into the M13tg130 and M13tg131 vectors (15) and sequencing by the chain termination method of Sanger et al. (23) with modified T7 polymerase (27).

Primer extension. Two single-stranded DNA primers carrying the sense strand of *tyrP* were isolated from an M13tg131 derivative carrying the 320-bp *Sph*I promoter fragment. The primers were produced by extension of the single-stranded template with the M13 17-base sequencing primer and [α-^{35}S]dATP as a label. Following de novo synthesis, the DNA was digested with either *Sau*3A or *Rsa*I and the probes were isolated on a 6% sequencing gel. *Sau*3A cuts at position 235 and *Rsa*I cuts at position 151 (see Fig. 1), producing probes of 123 and 211 bases, respectively, including the M13 polylinker region and the primer. These probes

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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*	13
JP4822	JP3561 *tyrR366*	P1 transduction
JP4855	JP3561/pMU360	This work
Plasmids		
pBR328	Ap^r Tc^r Cm^r	26
pKO-1	Ap^r *galK*$^+$	18
pMC1403	Ap^r *lac'ZYA'*	4
pMU360	Ap^r pBR322 *tyrR*$^+$	7
pMU530	Tp^r *trp'BA'lac'ZYA*; low-copy-number promoter cloning vector	13
pMU562	Tp^r; 470-bp *tyrP* fragment in pMU530	13
pMU574	Tp^r *galK'lac'ZYA*; low-copy-number promoter cloning vector	This work
pMU575	Tp^r *galK'lac'ZYA*; low-copy-number promoter cloning vector	This work
pMU2003	Tp^r; 320-bp *tyrP* fragment in pMU575	
pMU2007	Tp^r; 152-bp *tyrP* fragment in pMU575	This work
pMU2015	pMU2003 derivative with a C-to-T mutation at position +8 in the left-hand TYR R box	This work
pMU2063	Tp^r; 181-bp *tyrP* fragment in pMU575	This work
pMU2064	pMU2003 derivative with a C-to-A change at position −7, a G-to-T change at position +6, and a G-to-A change at position +9 in the right-hand TYR R box	This work
pMU2065	pMU2003 derivative with a C-to-T change at position +8 in the left-hand TYR R box and a C-to-A change at position −7, a G-to-T change at position +6, and a G-to-A change at position +9 in the right-hand TYR R box	This work
pMU2066[b]	pMU2003 derivative; C→A, −5, left	This work
pMU2070[b]	pMU2003 derivative; G→A, −8, left	This work
pMU2071[b]	pMU2003 derivative; G→A, −8, left; C→T, +8, left	This work
pMU2072[b]	pMU2003 derivative; A→G, +7, left	This work
pMU2073[b]	pMU2003 derivative; T→A, +4, left	This work
pMU2074[b]	pMU2003 derivative; T→G, +4, left	This work
pMU2075[b]	pMU2003 derivative; T→C, +4, left	This work
pMU2076[b]	pMU2003 derivative; T→G, −9, right	This work
pMU2077[b]	pMU2003 derivative; T→C, −9, right	This work
pMU2078[b]	pMU2003 derivative; T→C, −7, left	This work
pMU2093[b]	pMU548 (12) derivative; A→G, −6, right	This work
pMU2094[b]	pMU532 (12) derivative; G→C, −8, right	This work
pMU2322	pMU2065 derivative; deletion of left-hand box	This work
pREG151	Tp^r; low-copy-number plasmid	S. Falkow

[a] The nomenclature for genetic symbols follows that described by Bachmann (2). Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; Cm^r, chloramphenicol resistance; Tp^r, trimethoprim resistance.

[b] Changes are shown, e.g., as follows: C→A, −5, left represents a C-to-A change at position −5 in the left-hand box.

were used to determine the 5′ ends of *tyrP* in vivo transcripts by the primer extension technique described by Hudson and Davidson (12).

The 123-bp probe was expected to hybridize to a region downstream of both putative transcription start sites; however the 5′ end of any transcript arising from the putative upstream promoter would require an extension of up to 160 bp with this probe, so the second probe, of 211 bp, requiring a much shorter extension, was used to examine the possible presence of such a transcript.

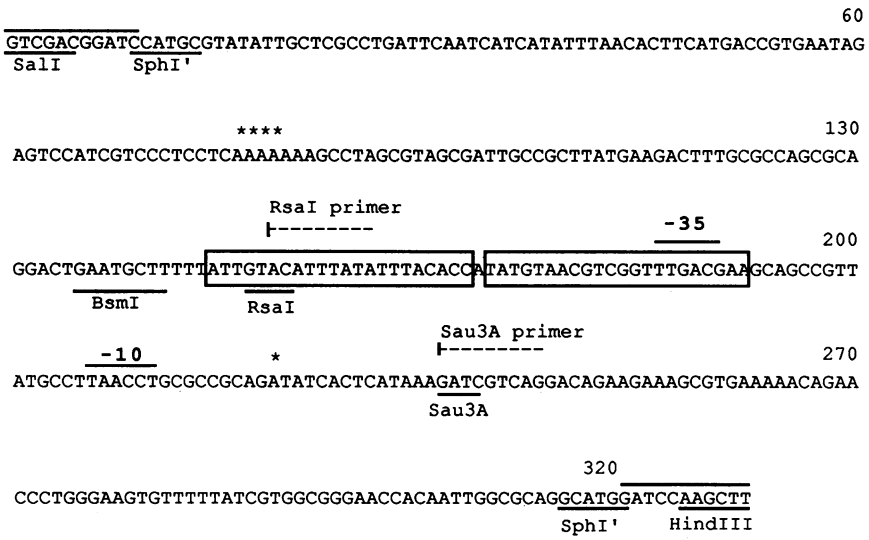
Sodium bisulfite mutagenesis. Sodium bisulfite mutagenesis within single-stranded loops of DNA heteroduplexes was performed by the methods of Peden and Nathans (21) and Shortle and Nathans (25) and involved pMU2003 and pMU575. A multicopy *tyrR*$^+$ plasmid was included in the recipient strain to maximize the color difference between repressed and derepressed colonies.

Oligonucleotide-directed mutagenesis. Oligonucleotides were synthesized on either an Applied Biosystems 381A DNA synthesizer or a Pharmacia GeneAssembler Plus. Mutagenesis was performed on M13tg131 derivatives containing the *tyrP* promoter-operator region from pMU2003 with an oligonucleotide-directed in vitro mutagenesis system kit from Amersham. Following screening and isolation of desired mutants, the entire *tyrP* fragment was sequenced to ensure that no base changes other than that planned had occurred. The *tyrP* fragments were cloned into pMU575, the resulting plasmids were transformed into JP3561, JP4822, and JP4855, and β-galactosidase assays were performed.

β-Galactosidase assay. β-Galactosidase activity was assayed as described by Miller (19). Repression ratios are expressed as the level of β-galactosidase expression in a *tyrR366* strain over the level in *tyrR*$^+$ cells grown in minimal medium plus 1 mM tyrosine. Activation ratios are expressed as the level in *tyrR*$^+$ cells grown in minimal medium plus 1 mM phenylalanine over the level in a *tyrR366* strain.

DNase I footprinting of the *tyrP* operator with purified TyrR protein. A strain in which TyrR protein is overproduced was constructed in our laboratory (30a). Purified repressor was produced by a method based on that developed by Argyropoulos et al. (1b). Footprinting of the *tyrP* operator was performed with 336-bp *Sal*I-*Hin*dIII fragments from pBR328 derivatives of pMU2003 and the various mutants. Plasmid DNA was digested with *Hin*dIII and labelled with the

A



B

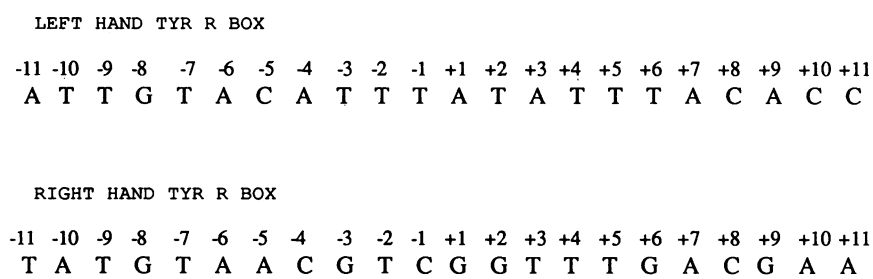


FIG. 1. (A) Nucleotide sequence of the 320-bp *Sph*I fragment carrying the *tyrP* promoter-operator region. This fragment was cloned into pMU575 to produce pMU2003, with the nucleotide numbers shown referring to the *tyrP* antisense sequence. The overlined flanking sequences are those found in the pMU575 polylinker and show the *Sal*I and *Hin*dIII sites used in DNase I protection experiments. The original *Sph*I sites were destroyed during cloning and are indicated as *Sph*I'. The *Bsm*I and *Rsa*I sites used to construct the *tyrP* promoter subclones are marked. The two putative TYR R box sequences are boxed, and the transcription start sites determined by S1 nuclease mapping are marked by asterisks. The −35 and −10 regions of the promoter initiating the downstream transcript are shown. The 5′ ends of the two primers used in primer extension analysis are indicated by the broken lines. (B) Numbering system used to describe base positions within the two *tyrP* TYR R boxes. The number assigned to each base relative to the axis of symmetry is shown above the box sequences.

Klenow fragment in the presence of [α-^{32}P]dATP and [α-^{32}P] dCTP. Following phenol-chloroform extraction and ethanol precipitation, the DNA was digested with *Sal*I and the resultant fragment was isolated on a 5% polyacrylamide gel. The DNA was eluted by the method of Maxam and Gilbert (17), phenol-chloroform extracted, and ethanol precipitated.

TyrR protein was added at increasing concentrations to the labelled operator fragment (sufficient to read 50 cps on a hand-held monitor) in footprinting buffer (15 mM Tris [pH 7.6], 80 mM KCl, 8 mM $MgCl_2$, 4% glycerol, 1 mM dithiothreitol). ATP, tyrosine, and phenylalanine were added when required at final concentrations of 0.2, 1, and 1 mM, respectively. TyrR protein was allowed to bind to the DNA for 15 min at 37°C before the addition of 1 U of DNase I. The reaction was stopped after 30 s with an equal volume of phenol-chloroform, and the solution was quickly vortexed. The aqueous layer was removed following centrifugation and ethanol precipitated. Samples were resuspended in formamide dye mix, denatured, and loaded onto a 6% sequencing gel.

Footprinting samples were electrophoresed next to a sequencing ladder produced by G+A chemical cleavage of the corresponding labelled operator fragment (17).

RESULTS

How many promoters exist for *tyrP*? The nucleotide sequence of the upstream region of *tyrP* is shown in Fig. 1 and includes the two putative transcription start sites that had been previously identified (13). A *Sph*I-*Rsa*I fragment including nucleotides 1 to 151 and a *Bsm*I-*Sph*I fragment including nucleotides 141 to 320 were separately cloned into expression vector pMU575 to yield plasmids pMU2007 and pMU2063, respectively. The *Sph*I fragment of 320 nucleotides which was expected to contain both putative promoters was also cloned into pMU575 to yield plasmid pMU2003.

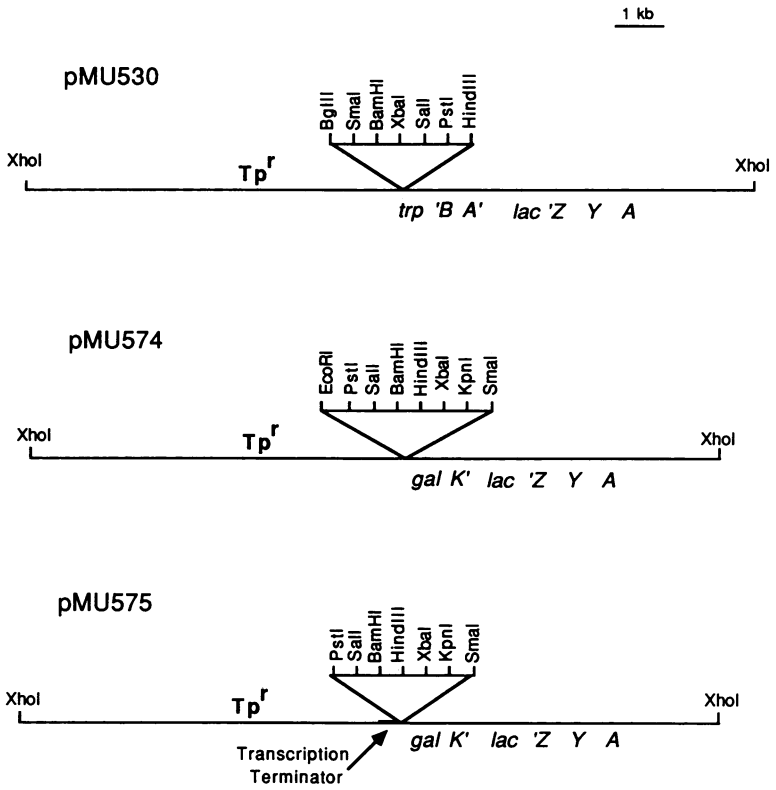


FIG. 2. Diagramatic representation of the three expression vectors relevant to this work. All three were derived from the low-copy-number trimethoprim resistance plasmid pREG151 (S. Falkow) and contain the *lac'ZYA* genes from pMC1403 (4). pMU530 has been described previously (13). In both pMU574 and pMU575, the *trp'BA'* region of pMU530 has been replaced by a 342-bp fragment of the *galK* gene from pKO-1 (18). This fragment contains the *galK* translational signals minus the promoter and the first 55 amino acids, which in pMU574 and pMU575 have been fused in phase with the eighth codon of *lacZ*. The polylinker was derived from M13tg131 (15). In pMU575, a transcription terminator has been placed immediately upstream of this polylinker.

Expression vector pMU575 which, along with pMU530 and pMU574 is depicted in Fig. 2, has numerous advantages over vector pMU530, which had been used in previous work. In pMU575, the *lacZ* coding sequence is joined in phase at the eighth codon with the proximal portion of *galK*, avoiding the problems of translational coupling observed with pMU530, in which *lacZ* is fused to the *trpB* and *trpA* genes, so that the translation efficiency of *trpA* and thus *lacZ* is governed by initiation events occurring at *trpB* (1c). Furthermore, pMU575 has a transcription terminator immediately upstream of the polylinker cloning site, preventing any readthrough transcription from vector promoters. It also has termination codons in all three reading frames in front of the *galK* initiation codon to prevent any inadvertent translational readthrough. Plasmid pMU575 is based on the same plasmid as pMU530 (pREG151) and, as such, is a low-copy-number plasmid.

Plasmids pMU2003, pMU2007, and pMU2063 were each transformed into the *tyrR*$^+$ strain JP3561 and the almost isogenic *tyrR366* strain JP4822. Transformants were assayed for β-galactosidase activity after growth in various media; the results are shown in Table 2. The results obtained with transformants containing pMU2003 and pMU2063 were virtually identical. Both strains exhibited good promoter activity and TyrR-mediated tyrosine repression and phenylalanine activation. In addition, slight (1.5- to 2-fold) TyrR-independent activation was seen when the strains were grown in the presence of Casamino Acids. Transformants with pMU2007, on the other hand, had virtually no detectable levels of β-galactosidase and hence exhibited no promoter activity under any of these growth conditions.

Primer extension. Since subcloning failed to reveal the putative second upstream promoter, primer extension studies were carried out to try to confirm the results of previous experiments with S1 nuclease. RNA was isolated from two

TABLE 2. β-galactosidase assays of strains containing various *tyrP-lac* plasmids

Strain	Plasmid[a]	β-Galactosidase activity[b] of cells grown in[c]:			
		MM	MMT	MMP	MMCAA
JP3561 (*tyrR*$^+$)	pMU2003 (1–320)	146	76	625	173
JP4822 (*tyrR366*)		260	265	234	347
JP3561	pMU2007 (1–151)	2	2	1	1
JP3561	pMU2063 (141–320)	106	55	540	152
JP4822		286	257	222	466

[a] Number in parentheses indicate the region introduced into pMU575.
[b] As defined by Miller (19).
[c] MM, minimal medium; MMT, minimal medium containing 1 mM tyrosine; MMP, minimal medium containing 1 mM phenylalanine; MMCAA, minimal medium plus 0.2% Casamino Acids.
[d] NT, not tested.

strains, one of which carried the new pMU575 expression vector with the 320-bp *Sph*I fragment (pMU2003) and the other of which carried the pMU530 expression vector with the 470-bp fragment (pMU562) which had been used in the S1 nuclease experiments (13). Strains with parental plasmids pMU575 and pMU530 were used as controls. Two primers (Fig. 1), one obtained from a *Sau*3A digest and extending to position 235 and the other obtained from an *Rsa*I digest and extending to position 151, were used. A description of their preparation is included in Materials and Methods. The primers were hybridized with mRNA isolated from cells grown in minimal medium, minimal medium supplemented with phenylalanine, and minimal medium supplemented with Casamino Acids. Following hybridization, the primers were extended with reverse transcriptase and the products of extension were electrophoresed next to the same clones as those used to generate the primers; the results are shown in Fig. 3. The primer extending to position 235 was extended to yield a single transcription start site at about positions 220 to 224 with mRNA derived from strains with pMU2003 (lanes 5, 6, 7, and 8) or pMU562 (lane 10). The multiple extension bands appearing in some of the lanes resulted from the multiple bands produced in the *Sau*3A digest and do not represent different start sites, as shown in lane 5, in which the top band of the *Sau*3A digest was purified and found to extend to a single band. Transcription from this start site was clearly stimulated by phenylalanine.

The *Rsa*I primer, which extended to position 151, was used to detect any transcripts originating at a start site in the vicinity of position 80 (Fig. 1). No such transcripts were detected with mRNA from strains with plasmid pMU2003, but such a transcript can be seen in Fig. 3, lane 12, in which mRNA from a strain with plasmid pMU562 was used. To confirm that the difference in these results was attributable to the presence or absence of a transcription terminator preventing readthrough transcription from a vector promoter, we constructed a plasmid in which the 320-bp *Sph*I fragment was introduced into expression vector pMU574; this vector is the precursor of pMU575 and lacks the transcription terminator. When mRNA from strains with this new plasmid, pMU2008, was used, the transcript appearing to originate at position 80 was again apparent (data not shown).

The failure to subclone the putative second promoter coupled with the absence of any relevant transcript in strains in which the 320-bp *Sph*I fragment was positioned downstream of a transcription terminator led us to the conclusion that *tyrP* has a single promoter with transcription starting at about position 220 (Fig. 1). Previous results indicating the presence of a second promoter were artifacts, almost certainly resulting from transcription readthrough from an unknown vector promoter outside the cloned fragment. For this latter hypothesis to be correct, some processing of this transcript must occur to produce an apparent start site at position 80. Although analysis of possible secondary structures in this transcript by the programs of Zuker and Stiegler (32) revealed possible cleavage points in this region, we have not pursued this analysis any further. From all of these results, it now seems clear that *tyrP* has a single transcription start site and that phenylalanine-mediated activation influences transcription initiation at that site.

Analysis of mutants with base substitutions. Previous results (13) had shown that two base substitutions mutations at positions −6 and −8 of the right-hand TYR R box (the bases are numbered from the axis of symmetry in the center of each TYR R box −1 to −11 in the left-hand direction and +1 to +11 in the right-hand direction, as shown in Fig. 1) were no longer repressible by tyrosine but still retained the ability to be activated by phenylalanine. Sodium bisulfite mutagenesis and screening on X-Gal plates supplemented with phenylalanine or tyrosine (see Materials and Methods) were used to search for a mutant which had lost the capacity to be activated by phenylalanine. One such mutant, which had lost the capacities for both repression and activation, was obtained. This mutation was sequenced and shown to have a C-to-T change at the +8 position of the left-hand TYR R

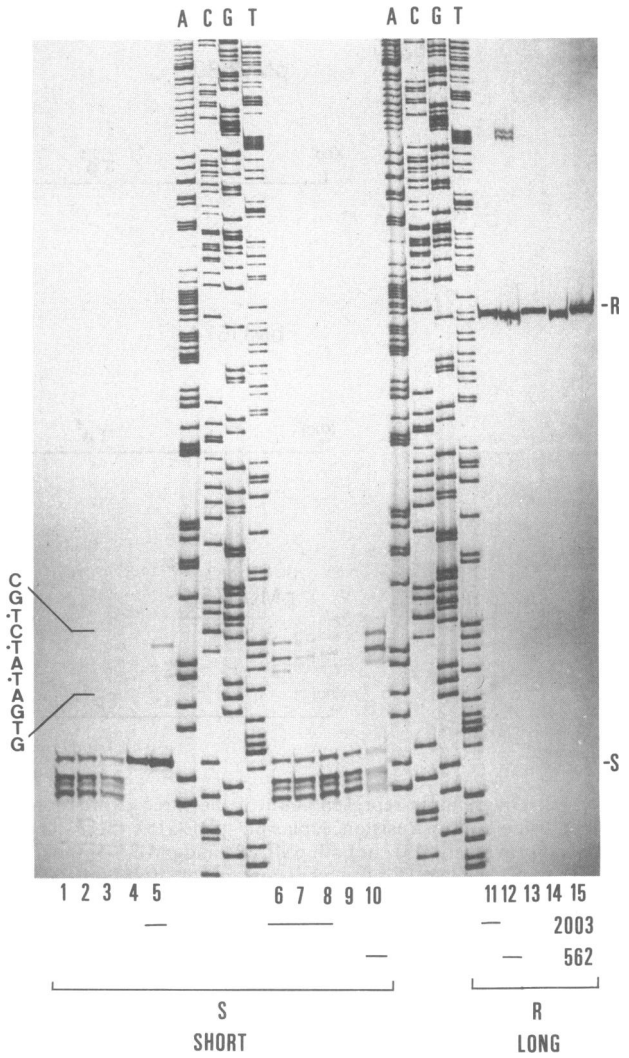


FIG. 3. Primer extension analysis to determine the transcription initiation sites in *tyrP*. Two probes (see Materials and Methods), S (lanes 1 to 10), a 123-bp *Sau*3A-derived probe, and R (lanes 11 to 15), a 211-bp *Rsa*I-derived probe, were hybridized to RNA prepared from strains carrying various plasmids and grown in minimal medium (lane 7), minimal medium plus 1 mM phenylalanine (lane 6), or minimal medium plus 0.2% Casamino Acids (remaining lanes). RNA from strains carrying pMU2003 was used in lanes 5 to 8 and lane 11, and RNA from strains carrying pMU562 was used in lanes 10 and 12. Controls included primers alone (lanes 1, 4, 9, and 13) or primers extended with RNA from strains carrying the parental plasmids pMU530 (lanes 3 and 15) and pMU575 (lanes 2 and 14). The sequence corresponding to the region of extension with the 123-bp *Sau*3A probe is given, with the final extension points marked by asterisks.

TABLE 3. Repression and activation ratios in strains with mutations in the left-hand box

Plasmid	Mutation[a]	β-Galactosidase levels in *tyr*R strains (U)	Tyrosine-mediated repression ratio in the following strains:		Phenylalanine-mediated activation ratio in the following strains:	
			tyrR$^+$	*tyrR*$^+$ (multicopy)	*tyrR*$^+$	*tyrR*$^+$ (multicopy)
pMU2003	None (wild type)	306	6.1	18	2.0	0.7
pMU2070	G→A, −8	376	1.1	3.7	1.0	0.5
pMU2078	T→C, −7	360	2.9	9.7	1.8	0.9
pMU2066	C→A, −5	296	2.9	19.7	3.0	2.2
pMU2073	T→A, +4	319	4.0	7.3	1.5	1.3
pMU2074	T→G, +4	263	4.2	29.2	1.7	0.8
pMU2075	T→C, +4	295	3.7	29.5	1.9	0.7
pMU2072	A→G, +7	297	2.2	19.8	1.6	0.6
pMU2015	C→T, +8	329	0.7	2.4	1.4	0.7
pMU2071	G→A, −8; C→T, +8	353	1.0	0.9	1.0	0.8

[a] See Table 1, footnote *b*.

box. The plasmid with this mutation was called pMU2015. The DNA fragments containing the two mutations previously reported by Kasian and Pittard (14) were also introduced into pMU575 to produce plasmids pMU2093 and pMU2094.

Additional mutations with specific changes in either the left-hand or the right-hand box were made by oligonucleotide-directed mutagenesis. Each new mutation was sequenced to confirm its identity, and the plasmid containing it was transformed into three strains: JP3561, which is haploid *tyrR*$^+$; JP4822, which is a *tyrR366* derivative; and JP4855, which is a derivative of JP3561 that contains the multicopy *tyrR*$^+$ plasmid pMU360. The *tyrR366* mutation is a frameshift mutation that causes translation to stop 134 residues before the carboxyl terminus. The truncated protein so formed does not contain the DNA binding domain and, as *tyrR366* does not behave as a negative dominant mutation, we assume that the shortened protein is unable to dimerize. *tyrR366* is therefore regarded as a null mutation (24). Strains were grown in minimal medium supplemented with phenylalanine and minimal medium supplemented with tyrosine and assayed for β-galactosidase activity as described in Materials and Methods. Values for *tyrR* mutant derivatives were the same for cells grown in minimal medium with tyrosine or phenylalanine.

Mutations in the left-hand box. Table 3 presents the results obtained with the left-hand box mutations, expressed as repression and activation ratios (see Materials and Methods). The β-galactosidase levels in *tyrR* mutant derivatives are also shown; for these strains, both activation and repression ratios were 1.0. Base substitutions at positions −8 (pMU2070), +8 (pMU2015), and −8 and +8 together (pMU2071) all resulted in the loss or significant diminution of both repression and activation. All of these base substitutions were chosen to lessen agreement with the consensus sequence TGTAAA-N_6-TTTACA.

It should be noted that the increased levels of TyrR protein in strains with multicopy *tyrR*$^+$ plasmids were nevertheless able to cause significant repression in all of these strains, with the exception of the strain with pMU2071, which had two base changes, at positions +8 and −8. This was the only strain with repression and activation ratios approaching unity under all conditions, as might be expected if operator function were completely destroyed. It should also be noted that phenylalanine-mediated activation was not seen in strains with multicopy *tyrR*$^+$ plasmids, with the exception of the strains with pMU2066, in which a C-to-A change at position −5 increased the agreement with the consensus sequence of the left-hand box, and with pMU2073, in which the T at position +4 was changed to an A. The implications of this result will be discussed below (see Discussion). Mutations at positions +4, +7 and −7, which decreased agreement with the consensus sequence, reduced repression and activation but did so to a lesser extent than the previously mentioned mutations at +8 and −8. These results led to the conclusion that an intact left-hand box is a necessary requirement for both tyrosine-mediated repression and phenylalanine-mediated activation of *tyrP*.

Mutations in the right-hand box. Table 4 presents the results obtained with the right-hand box mutations, also expressed as repression and activation ratios. The levels of β-galactosidase in *tyrR* mutant derivatives are also shown. Base substitutions at positions −9, −6, and −8 all resulted in a significant loss of repression, while phenylalanine-mediated activation appeared to be enhanced in both haploid and multicopy *tyrR*$^+$ strains.

Taken together, the results obtained with these various mutations indicate that base substitutions which lessen agreement with the consensus sequence in either the left-hand or the right-hand box can cause a decrease in repression, whereas phenylalanine-mediated activation is only reduced by mutations in the left-hand box.

Creation of a "strong" right-hand box. In the case of *tyrP*, the TYR R box overlapping the −35 region of the promoter differs from the consensus sequence TGTAAA-N_6-TTTACA at three positions, whereas the left-hand box differs at only one position. Using oligonucleotides, we created two new box sequences. The first plasmid, pMU2064, had an unaltered left-hand box but had three changes in the right-hand box (C to A −4; G to T +6; G to A +9), so that this box now contained the sequence TGTAAA-N_6-TTTACA. In the second plasmid, pMU2065, this strong right-hand box was linked to a left-hand box in which the invariant C at position +8 had been changed to a T. In the wild type, such a change had a severe effect on both repression and activation (Table 3). The results with these two strains are also shown in Table 4. These changes decreased promoter activity, as indicated by the β-galactosidase levels in the *tyrR* mutant strains; however, there were still marked effects on repression. The strain with pMU2064 seemed to have an increased sensitivity to repression in the presence of multiple copies of *tyrR*$^+$,

TABLE 4. Repression and activation ratios in strains with mutations in the right-hand box

Plasmid	Mutation[a]	β-Galactosidase levels in *tyrR* strains (U)	Tyrosine-mediated repression ratio in the following strains:		Phenylalanine-mediated activation ratio (repression ratio) in the following strains:	
			tyrR$^+$	*tyrR*$^+$ (multicopy)	*tyrR*$^+$	*tyrR*$^+$ (multicopy)
pMU2003	None (wild type)	306	6.1	18	2.0	0.7
pMU2076	T→G, −9	366	0.6	2.6	4.8	4.3
pMU2077	T→C, −9	420	2.0	10.8	3.7	2.0
pMU2094	G→C, −8	585	0.6	0.4	5.1	3.9
pMU2093	A→G, −6	359	0.6	0.7	6.2	5.4
pMU2064	C→A, −4; G→T, +6; G→A, +9	145	4.7	72.5	0.3 (3.0)	0.1 (16.1)
pMU2065	C→T, +8[b]; C→A, −4; G→T, +6; G→A, +9	196	2.3	19.6	0.8 (1.3)	0.1 (12.3)
pMU2322	Deleted left-hand box; C→A, −4; G→T, +6; G→A, +9	78	0.9	1.3	1.3 (0.8)	0.2 (5.2)

[a] See Table 1, footnote *b*.
[b] This change is at position +8 on the *tyrP* left-hand box.

and activation was abolished. Activation is therefore reduced by mutations which decrease affinity between the TyrR protein and the left-hand box or which increase affinity between the TyrR protein and the right-hand box. Moreover, in both haploid and multicopy *tyrR*$^+$ strains with pMU2064, phenylalanine had a repressing effect. This behavior was also evident with pMU2065, where, in the presence of multiple copies of *tyrR*$^+$, phenylalanine caused significant repression. Although tyrosine-mediated repression was somewhat reduced when this plasmid was present in a strain with a single copy of *tyrR*$^+$, it was strong in a strain with multiple copies of *tyrR*$^+$. This result is in contrast to those obtained with pMU2015, in which a mutation at +8 in the left-hand box in conjunction with a wild-type right-hand box resulted in a total loss of repression in a haploid *tyrR*$^+$ strain.

This difference was examined further by construction of pMU2322, in which the left-hand TYR R box of pMU2065 was deleted, thus leaving a single strong box overlapping the −35 region of the promoter. Both haploid and multicopy *tyrR*$^+$ strains carrying pMU2322 were found to have lost tyrosine-mediated repression (Table 4); however, the repression mediated by phenylalanine in the presence of multiple copies of *tyrR*$^+$ was still observed.

DNase I protection studies. Using purified TyrR protein and purified templates (see Materials and Methods), we performed DNase I footprinting. In addition to TyrR protein, phenylalanine, tyrosine, and ATP were used as effectors. TyrR protein contains an ATP binding site, and mutants with substitutions at this site (glycine to aspartate at position 239) cannot repress any of the genes of the TyrR regulon (10a). ATP has also been shown to be involved in the tyrosine-mediated binding of TyrR protein to a number of the operators of the TyrR regulon (1a, 30a).

Figure 4 shows the results of DNase I protection studies involving the wild-type *tyrP* operator and its mutant derivatives.

With the wild type (Fig. 4A and B), in the presence of both tyrosine and ATP, both boxes were protected when TyrR protein was added at concentrations of 5 to 10 nM or higher. When ATP was omitted, protection was not seen with 10 nM TyrR protein but was seen when higher concentrations, 35 nM or higher, were used. In the absence of tyrosine, the left-hand box was protected by TyrR protein at concentrations of 35 nM or higher. The addition of phenylalanine or ATP appeared to have no effect on the latter result. No protection of the right-hand box was seen, except when tyrosine was present. Figure 4C shows a footprint involving a template from pMU2094, in which the invariant G at position −8 of the right-hand box was changed to a C. In this case, protection of the right-hand box was abolished, except for very weak protection at a high TyrR protein concentration (175 nM) in the presence of ATP and tyrosine. The left-hand box, however, was still protected. Figure 4D shows the footprint from pMU2015, in which the invariant C at position +8 of the left-hand box was changed to a T. With this template, no protection of either box was seen at TyrR protein concentrations lower than 35 nM. In the presence of tyrosine and ATP, both boxes were protected by TyrR protein at concentrations of 35 nM or higher. In the absence of tyrosine, weak protection was seen with the highest concentration of TyrR protein used (175 nM) in the presence of ATP and phenylalanine, but at lower concentrations, no protection was seen. In this case, repression was abolished in haploid *tyrR*$^+$ strains and reduced to about 20% of normal in multicopy *tyrR*$^+$ strains.

Figure 4E shows the results obtained with a template from pMU2064, which contained a strong right-hand box. In this case, tyrosine-mediated repression was increased in multicopy *tyrR*$^+$ strains and phenylalanine repressed rather than activated. In the footprint, in the presence of tyrosine and ATP, TyrR protein protected both boxes at very low concentrations (5 nM). At concentrations of 70 nM or higher and in the presence of phenylalanine and ATP, both boxes were also protected. Figure 4F shows the results obtained with a template from pMU2065, which combined a strong right-hand box with a mutated left-hand box. In the presence of tyrosine and ATP, both boxes were protected when the TyrR protein concentration was 35 nM or higher. At these concentrations and in the presence of ATP and phenylalanine, protection of the strong right-hand box was also apparent. Under these conditions, no protection of the mutated left-hand box was observed. As can be seen in Table 4, repression by phenylalanine was observed in strains with multiple copies of *tyrR*$^+$, confirming the role of the right-hand box in repression.

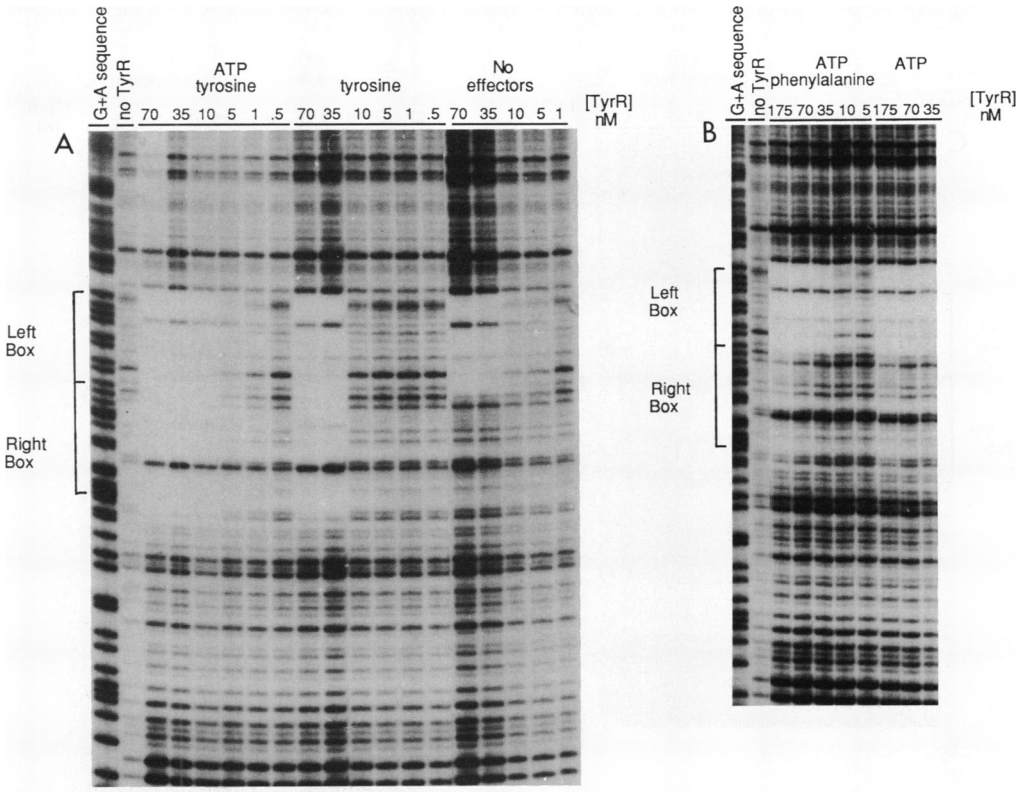


FIG. 4. DNase I footprinting of the antisense strand of *tyrP* wild-type and mutant operators. A 328-bp DNA fragment containing the *tyrP* regulatory region was ^{32}P labelled at the 3′ end of the antisense strand and subjected to partial DNase I digestion (see Materials and Methods) in the presence of purified TyrR protein. Tyrosine, phenylalanine, and ATP were added at final concentrations of 1, 1, and 0.2 mM, respectively, to the preincubation mixture. The concentrations of TyrR protein are shown above the gels. The G+A Maxam-Gilbert sequence of the operator fragments is shown, with the regions corresponding to the left-hand and right-hand TYR R boxes marked. (A and B) Wild-type operator. (C) Operator from pMU2094, in which the invariant G at position −8 in the right-hand TYR R box has been changed to a C. (D) Operator from pMU2015, in which the invariant C at position +8 in the left-hand TYR R box has been changed to a T. (E) Footprint of the operator from pMU2064, containing the strong right-hand box. (F) Footprint of the operator from pMU2065, containing the combination of the strong right-hand box and the C-to-T change at position +8 of the left-hand box.

DISCUSSION

Although some genes, such as *glnA*, which are subject to both activation and repression utilize two separate promoters, one subject to repression and the other subject to activation (10), the work reported in this paper establishes that *tyrP* has a single promoter. Activation and repression both affect transcription initiation from this one promoter. The previously reported upstream transcription initiation site has been found to be an artifact dependent on readthrough from an unidentified upstream vector promoter.

Mutations within each of the TYR R boxes have major effects on the expression of *tyrP*. Changes in either of the invariant G-N_{14}-C bases in the two *tyrP* TYR R boxes have major effects on the ability of the altered box to function. Similar findings have been reported for a number of genes in the TyrR regulon (3, 5, 6, 11, 13, 31). Changes in other bases that are part of the consensus sequence TGTAAA-N_6-TTTACA, such as the T at position −7 and the T at position +4 in the left-hand box, have less severe effects on repression.

Changes away from the consensus sequence in the left-hand or promoter-distal TYR R box of *tyrP* affect both tyrosine-mediated repression and phenylalanine-mediated activation. Similar mutations in the right-hand box, however, only affect repression. As mentioned previously, changes in the right-hand box which bring it closer to the consensus sequence increase repression and at the same time abolish activation. TyrR protein bound to the left-hand box would be in a position to interact with RNA polymerase without blocking its binding to the promoter sequence. On the other hand, occupancy of the right-hand box by TyrR protein would prevent effective binding of RNA polymerase. DNase I footprinting experiments confirm that in the presence of tyrosine, TyrR protein protects both the left-hand and the right-hand boxes. At low TyrR protein concentrations, this protection requires both ATP and tyrosine. At the same concentrations in the presence of phenylalanine and ATP, only the left-hand box is protected. In the presence of phenylalanine, protection of the right-hand box is only seen at the highest concentration of TyrR protein used. The dominance of tyrosine-mediated repression over phenylalanine-mediated activation which is seen when cells are grown in a mixture of phenylalanine and tyrosine (14) implies that the affinity of TyrR-tyrosine for both boxes is greater than the affinity of TyrR-phenylalanine for the left-hand box.

One of the consequences of a mutation in the right-hand box which abolishes repression is that the level of expression observed in cells grown in the presence of phenylalanine increases about fourfold over wild-type levels. This result implies that when wild-type cells are grown in minimal

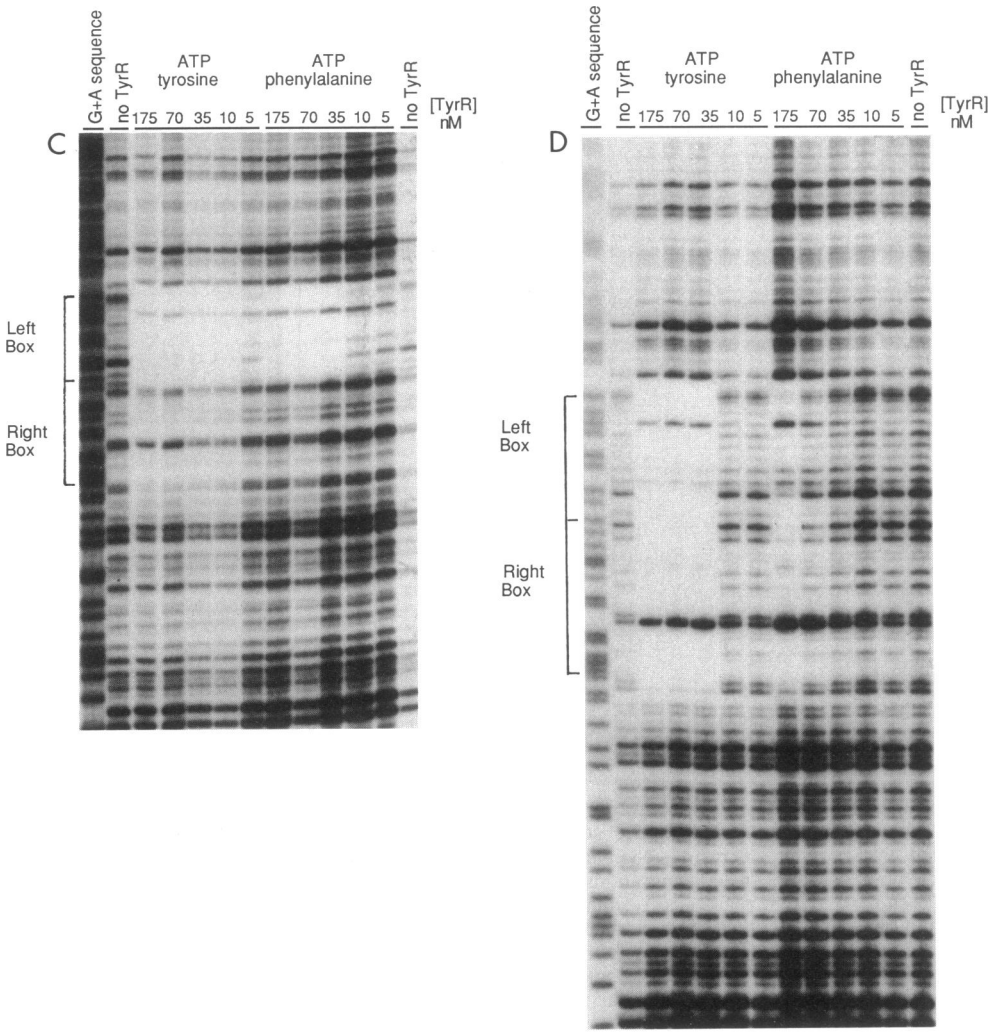


FIG. 4—*Continued.*

medium supplemented with phenylalanine, endogenous tyrosine pools are high enough to ensure that any three of four operators are occupied by TyrR-tyrosine rather than TyrR-phenylalanine.

A number of mutants in which the TYR R box sequences were changed to make them closer to the consensus box sequence were derived. Changing the C at position −5 to an A in the left-hand box of *tyrP* results in phenylalanine-mediated activation occurring in both haploid and multicopy *tyrR*$^+$ strains. Phenylalanine activation is not seen in wild-type cells with a multicopy *tyrR*$^+$ plasmid, presumably because the increased levels of TyrR protein result in increased occupancy of the boxes by TyrR-tyrosine, preventing activation, or because at the higher concentrations of TyrR protein, phenylalanine causes some binding to the right-hand box. The C-to-A change may increase the affinity between TyrR-phenylalanine and the left-hand box, allowing binding and exclusion of TyrR-tyrosine.

The gene *aroG* has a single TyrR box, which overlaps the −35 region of the promoter (3). In wild-type cells, *aroG* expression is repressed by phenylalanine and not by tyrosine (3). When the *aroG* promoter region is cloned into a low-copy-number vector and expression is measured in cells grown in the presence and absence of phenylalanine, only twofold repression is seen (8). On the other hand, increasing the level of TyrR protein results in about eightfold repression (3). When the sequence of the right-hand box of *tyrP* is changed to TGTAAA-N_6-TTTACA, cells grown in the presence of either tyrosine or phenylalanine become more sensitive to repression. In the strain with pMU2065, in which, in addition to this improved right-hand box, the left-hand box is disabled by a C-to-T change at position +8, this phenylalanine-mediated repression is only evident in the presence of multiple copies of *tyrR*$^+$. With this multicopy *tyrR*$^+$ strain, growth with phenylalanine results in more than 10-fold repression. Similarly, multicopy *tyrR*$^+$ strains carrying pMU2322, in which the left-hand box is deleted, leaving a single strong box overlapping the promoter, also exhibit significant phenylalanine-mediated repression. This phenylalanine effect is particularly interesting, as it mimics the situation found normally with the *aroG* gene. Protection studies show that the right-hand box of pMU2065 is protected by TyrR protein (35 nM) in the presence of phenylalanine and ATP.

Tyrosine repression is observed in strains carrying pMU2065 but is lost in strains carrying pMU2322, implying

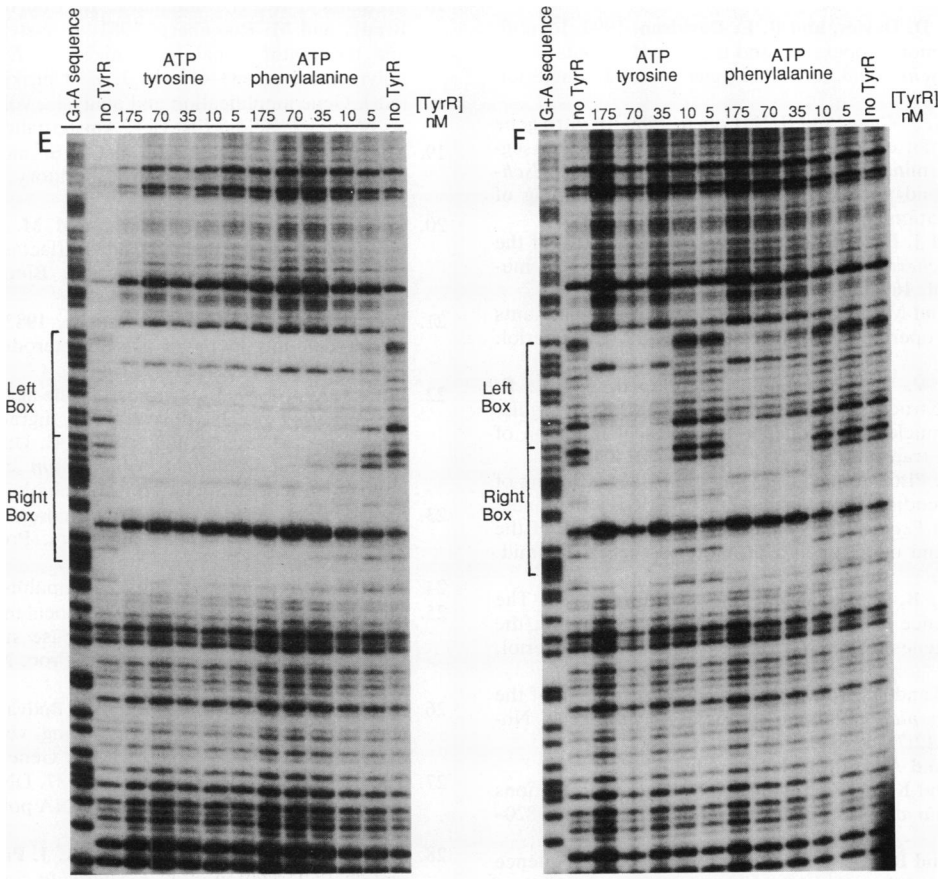


FIG. 4—*Continued.*

that there is a strict requirement for two boxes in tyrosine-mediated repression. In general, genes of the TyrR regulon whose expression is repressed by tyrosine have adjacent TYR R boxes (22). With the exception of *aroP*, in which the boxes are so far downstream that they cannot interfere with RNA polymerase binding, the adjacent boxes are arranged so that the one closest to the promoter has the least resemblance to the palindromic sequence TGTAAA-N_6-TTTACA. DNase I protection studies of *tyrP*, *aroF*, *tyrB*, and *aroL* (1a, 30a; this paper; unpublished results) reveal that the promoter-distal box is the one to which TyrR protein preferentially binds in the absence of tyrosine. It seems likely that in the presence of tyrosine, cooperative binding to the second box occurs. This cooperativity would be essential for repression to occur and would be the key to the specificity of tyrosine-mediated repression. In the case of pMU2065, it is possible that in the presence of tyrosine and ATP, the binding of TyrR protein to the right-hand box acts to facilitate the binding of a second molecule to the weakened left-hand box.

The significance of the relative positions of these TYR R boxes for both repression and activation is addressed in the accompanying paper (1).

The observation that in vitro TyrR protein requires only tyrosine and ATP to specifically bind to both TYR R boxes of *tyrP* suggests that the level of *tyrP* expression is determined by the intracellular level of tyrosine, and there seems to be no need to postulate a second protein component in the control system. Phenylalanine-mediated activation cannot be inferred from DNA binding studies and may require in vitro transcription studies for further elucidation. Both the gene for the tyrosine-specific system (*tyrP*) and the gene for the tryptophan-specific transport system (*mtr*) are activated by phenylalanine in $tyrR^+$ cells (29).

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