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著者	Yang Ji, Ogawa Yoshito, Camakaris Helen, Shimada Tomohiro, Ishihama Akira, Pittard A. J.
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folA, a New Member of the TyrR Regulon in *Escherichia coli* K-12[▽]

Ji Yang,[1] Yoshito Ogawa,[1,2] Helen Camakaris,[1] Tomohiro Shimada,[2] Akira Ishihama,[2] and A. J. Pittard[1]*

Department of Microbiology and Immunology, The University of Melbourne, Victoria 3010, Australia,[1] and Department of Frontier Bioscience and Micro-Nano Technology Research Center, Hosei University, Koganei, Tokyo 184-8584, Japan[2]

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The *folA* gene was identified as a new member of the TyrR regulon by genomic SELEX. Binding of TyrR to two sites in *folA* activated its transcription. Mutations in the N-terminal or central domain of TyrR, the α subunit of RNA polymerase, or integration host factor all abolished activation of the *folA* promoter.

The TyrR regulon of *Escherichia coli* K-12 comprises at least eight separate transcription units (6). These various transcription units have been identified over time by observing fluctuations in the levels of particular proteins caused by the presence or absence of the amino acid phenylalanine or tyrosine in the medium or as a result of the introduction of various mutations into the regulator gene *tyrR*. In addition to the *tyrR* gene itself, all of the known genes of the regulon encode proteins which have a role in either the biosynthesis or transport of the aromatic amino acids. Flanking or overlapping the promoters of each of these transcription units are 18-bp sequences to which the TyrR protein selectively binds and which are referred to as TyrR boxes (6). These sequences are related to the palindrome TGTAAAN$_6$TTTACA and vary from one another in both their location and their agreement with the ideal consensus. The only absolutely invariant feature of these boxes is the G, the C, and the spacing of 14 bp between them.

In solution, TyrR protein is a dimer, but in the presence of tyrosine and ATP, it self-associates to form a hexamer (11). Although phenylalanine is reported not to facilitate hexamerization at physiological concentrations, it does facilitate the binding of TyrR dimers to multiple binding sites (12).

In the case of activation, TyrR protein is bound to a TyrR box upstream of the promoter. The addition of one of the aromatic amino acids induces a change in the protein, facilitating an interaction between the N-terminal domain of TyrR and the α subunit of RNA polymerase, resulting in enhanced transcription.

Using the method of genomic SELEX (9) with purified TyrR protein to identify TyrR-specific DNA binding sites, we identified seven of the eight known members of the TyrR regulon and five other possible new members. *lacZ* transcriptional fusions were constructed to test whether the transcription of any of these five genes exhibited TyrR-mediated responses in vivo. Expression of only one of these, namely, *folA*, encoding the enzyme dihydrofolate reductase, showed clear and unambiguous TyrR-mediated effects. This paper reports a genetic analysis of the interactions between TyrR protein and this new member of the TyrR regulon.

Selection and identification of TyrR-bound fragments by SELEX. A plasmid library carrying random *E. coli* K-12 fragments of 100 to 300 bp was constructed as described previously (9). Using this library as templates, linear genomic DNA fragments were amplified by PCR, and the resulting PCR fragments were used in SELEX. The binding assay to detect fragments with TyrR binding sites used a purified TyrR protein carrying six histidine residues at its amino terminal end (H_6-TyrR). The selection was carried out under three different conditions: first, with H_6-TyrR alone; second, with H_6-TyrR and ATP (ATP increases the affinity between TyrR and TyrR boxes [6]); and third, with H_6-TyrR, ATP, and tyrosine (ATP and tyrosine facilitate TyrR hexamer formation [11]). Following incubation at 37°C for 30 min, the H_6-TyrR–DNA complexes were purified by chromatography on Ni-nitrilotriacetic acid columns. The H_6-TyrR-bound DNA fragments were then cloned into vector pT7Blue (Novagen). A total of 250 clones from the three experiments were sequenced. Following an analysis of the sequences, those fragments which covered the promoter region of a coding sequence and contained at least one recognizable TyrR box were identified.

The results in Table 1 are the accumulated results from the three experiments. Except for *aroG*, which possesses a single TyrR box, all of the known transcription units of the TyrR regulon were identified. The least effective screen was H_6-TyrR alone, which yielded only fragments with *aroF* and *tyrP* promoters. In the presence of ATP (100 μM), a much wider range of fragments were selected, in both the absence and presence of tyrosine (100 μM). Two members of the regulon, *tyrB* and *aroP*, were obtained only with the screen including ATP and tyrosine. The most frequent isolates overall (*aroF* and *aroL*) contain multiple boxes with strong resemblances to the ideal consensus. In addition to the known members of the TyrR regulon, we identified five new targets, *cusC*, *cyaA*, *proP*, *folA*, and *holE*, whose expression could theoretically be affected by TyrR. These genes encode a number of unrelated functions. The gene *cusC* encodes a component of a copper transport system, *cyaA* encodes adenyl cyclase, *proP* encodes a proline specific permease, *folA* encodes dihydrofolate reductase, and *holE* encodes a component of DNA polymerase III. Furthermore, *cyaA*, *folA*, and *holE* have also been identified by algorithm as possible members of the TyrR regulon (4, 8).

* Corresponding author. Mailing address: Department of Microbiology and Immunology, The University of Melbourne, Victoria 3010, Australia. Phone: 03-8344-5696. Fax: 03-9347-1540. E-mail: aj.pittard@microbiology.unimelb.edu.au.

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TABLE 1. TyrR-bound DNA fragments isolated by SELEX

Promoter region covered by fragment	No. of clones isolated by SELEX	Identification by algorithm	Regulation experimentally confirmed[a]
aroF	62	+	+
aroL	48	+	+
tyrP	25	+	+
mtr	8	+	+
tyrB	6	−	+
tyrR	6	−	+
aroP	1	+	+
aroG	0	+	+
cusC	19	−	−
cyaA	13	+	−
proP	3	−	−
folA	3	+	+
holE	1	+	−

[a] Confirmed by in vivo studies with promoter-*lacZ* transcriptional fusions.

TABLE 2. TyrR-mediated activation of *folA-lacZ* fusion

Phenotype of host strain	TyrR phenotype of plasmid[a]	β-Galactosidase sp act (fold activation) expressed from *folA-lacZ* fusion in the indicated medium[b]		
		MM	Tyr	Phe
$TyrR^-$	NA[c]	108	110 (1)	102 (1)
$TyrR^+$	NA	103	271 (2.6)	127 (1.2)
$TyrR^-$	$TyrR^+$	101	458 (4.5)	267 (2.7)
$TyrR^-$	$TyrR_{RQ10}$	105	155 (1.5)	96 (1)
$TyrR^-$	$TyrR_{DA103}$	100	190 (1.9)	150 (1.5)
$TyrR^-$	$TyrR_{\Delta 226–419}$	110	188 (1.7)	231 (2.1)
$TyrR^-$	$TyrR_{EQ274}$	95	139 (1.5)	201 (2.1)
$HimA^-$ $HimD^-$	$TyrR^+$	36	54 (1.5)	46 (1.3)
$RpoA_{DN250}$	$TyrR^+$	40	45 (1.1)	60 (1.5)

[a] *tyrR*$^+$ was carried on plasmid pACYC177, and mutant *tyrR* alleles were carried on pACYC177 derivative pSU39.

[b] Units are those defined by Miller (5). The values of β-galactosidase activities are averages from three independent assays, with the standard deviation below 15%. MM, minimal medium; Tyr, MM containing 1 mM tyrosine; Phe, MM containing 1 mM phenylalanine. Fold activation is the specific activity of β-galactosidase obtained in the presence of tyrosine or phenylalanine divided by that obtained in MM.

[c] NA, no *tyrR* gene is present on the plasmid.

In order to determine the consequences of TyrR binding for gene expression, we constructed promoter/operator-*lacZ* fusions for each of the genes under consideration using the single-copy vector pMU2385 (7). Each of the promoter/operator-*lacZ* fusions was introduced into strains that were *tyrR* (*tyrR*366), haploid *tyrR*$^+$, or multicopy *tyrR*$^+$ with the *tyrR*$^+$ gene present on plasmid pACYC177. Cultures were grown in minimal medium and in minimal medium supplemented with phenylalanine or with tyrosine. The only gene to show significant modulation of expression in a haploid *tyrR*$^+$ strain and enhanced activation in a multicopy *tyrR*$^+$ background was *folA*. The data for the other four genes whose expression was not regulated by TyrR are not shown. The data for *folA* are shown in Table 2, along with results obtained using various *tyrR* mutants and strains with mutations affecting integration host factor (IHF) production and the α subunit of RNA polymerase. The mutant *tyrR* alleles, each of which has been previously described (6), were cloned on plasmid pSU39, a pACYC177 derivative, and introduced into strain JP8042, which has a *tyrR* null phenotype (*tyrR366*) and also carries the plasmid with the *folA-lacZ* fusion (single copy).

The $tyrR_{RQ10}$ allele specifies a protein with an arginine-to-glutamine substitution at position 10 of the TyrR N-terminal domain which inactivates a putative aromatic amino acid binding site involved in activation. The aspartate-to-alanine change at position 103 ($tyrR_{DA103}$) affects a putative interaction site between the N-terminal domain and the α subunit of RNA polymerase. Both mutants have been shown to have a greatly reduced ability to activate expression of *tyrP*, *aroP*, and *mtr* (6). The host strain with a mutant *rpoA* allele, $rpoA_{DN250}$, has been shown also to be unable to activate expression of *tyrP* or *mtr* in *tyrR*$^+$ strains (6).

The deletion of residues 226 to 410 in the central domain of TyrR ($TyrR_{D226-410}$) destroys the ability of TyrR protein to hexamerize and to repress a number of genes specifically repressed by tyrosine (6). The more specific change in the central domain, $tyrR_{EQ274}$, has a similar effect on tyrosine-mediated regulation (3).

Because of certain similarities between the regulation of *folA* and the TyrR-mediated regulation of the gene *tpl* in *Citrobacter freundii* (1), we also used a *himA himD* double mutant to investigate a possible role for IHF in TyrR-mediated regulation of *folA*.

Overall, the results in Table 2 clearly show that tyrosine-mediated activation of *folA* requires first a fully functional TyrR N-terminal domain to carry out aromatic amino acid binding and interaction with the α subunit of RNA polymerase, as is the case for *tyrP*, *aroP*, and *mtr*. Unlike the situation with these genes, activation of *folA* also requires a fully functional TyrR central domain, which is necessary for the binding of a second molecule of tyrosine and the subsequent self-association to form a hexamer. The failure to observe significant activation in the IHF-negative strain indicates that in this case, IHF presumably is required to facilitate bending of the DNA to bring the TyrR protein close enough to the promoter to allow interactions with the α subunit of RNA polymerase. The weaker phenylalanine-mediated activation shows the same absolute requirement for a fully functional N-terminal domain, but the requirement for a fully functional central domain is less marked.

Specific interaction of the TyrR protein with the *folA* regulatory region. Inspection of the *folA* regulatory region revealed the presence of three putative TyrR boxes which are centered at positions −106.5 (TyrR box 1), −145.5 (TyrR box 2), and −186.5 (box 3) (Fig. 1). DNase I footprinting was carried out to characterize the interaction of the TyrR protein with the *folA* regulatory region. The ^{32}P-labeled *folA* fragment spanning the region between positions −254 and +1 relative to the start site of transcription was generated by PCR. The ^{32}P-labeled *folA* fragment was incubated with various amounts of native TyrR protein (11) in the presence of ATP (100 μM) and phenylalanine (100 μM) or tyrosine (100 μM). Binding reactions were carried out for 15 min at 37°C in a total volume of 25 μl of transcription buffer (50 mM Tris-HCl [pH 7.8], 50 mM NaCl, 3 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 25 μg/ml bovine serum albumin). The samples were then treated with 0.02 unit of DNase I (Boehringer Mannheim) to allow partial digestion of the DNA. After incu-

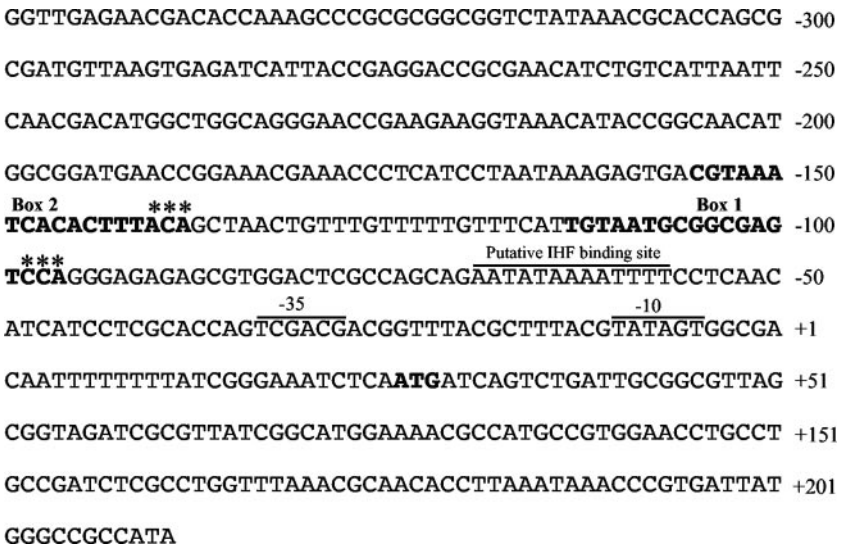

FIG. 1. Nucleotide sequence of the *folA* regulatory region. The numbering of the sequence is relative to the start site of transcription. The −35 and −10 regions of the *folA* promoter (10), the two TyrR boxes, and the putative IHF binding site are indicated. In the TyrR boxes 1 and 2, the positions where mutations were introduced are marked with asterisks above the bases. To inactivate box 1, the three nucleotides CCA were changed to GAT, and to inactivate box 2, the three nucleotides ACA were also changed to GAT.

bation for 45 s at room temperature, the reaction was terminated by phenol extraction. The resulting DNA fragments were analyzed on a 6% sequencing gel (Fig. 2). In the presence of either tyrosine or phenylalanine, full protection corresponding to TyrR box 2 (the strong box) was seen at all TyrR concentrations used (from 12.5 to 250 nM). However, protection of box 1 (the weak box) occurred in the presence of tyrosine only at concentrations of TyrR protein of 50 nM or above, and in the presence of phenylalanine, box 1 was only weakly protected at concentrations of 100 nM or above. In the presence of tyrosine and high concentrations of TyrR, there was also evidence of some protection of the region between boxes 1 and 2. The third putative box centered at −186.5 failed to show any protection, and subsequent mutation of this box failed to affect expression (data not shown). Consequently, this third box has been excluded from the discussion.

To investigate the role of each of the two TyrR boxes in regulation, mutations affecting one of the invariant GC pairs were introduced separately into each one (Fig. 1). As can be seen in Table 3, inactivation of either box destroys TyrR-mediated activation of *folA*.

Discussion. The gene *folA* encodes the enzyme dihydrofolate reductase, which carries out the reversible reaction dihydrofolate plus NADPH = tetrahydrofolate plus $NADP^+$. Tetrahydrofolate plays a central role in one-carbon metabolism in the synthesis of glycine, methionine, and purines. The substrate of this reaction, dihydrofolate, is a product of a pathway leading from chorismic acid via *para*-aminobenzoic acid. Little is known about the regulation of this pathway or the way in which the various pathways to aromatic vitamins compete with the pathways for phenylalanine, tyrosine, and tryptophan biosynthesis for the important initial substrate chorismic acid. It can be argued, however, that of the three aromatic amino acids, tyrosine has the most significant effect on chorismic acid biosynthesis. The TyrR-mediated repression of *aroF* (a gene which encodes a major isoenzyme for the first reaction of the pathway) and *aroL* (a gene encoding an enzyme for the fifth reaction of the pathway), coupled with the feedback inhibition by tyrosine of the *aroF*-encoded DAHP synthase, significantly affects the level of synthesis of chorismic acid. Although tyrosine also reduces the conversion of chorismic acid to tyrosine by causing TyrR to repress both *tyrA* and *tyrB* and by inhibiting prephenate dehydrogenase, it is possible that the net effect of these interactions is a reduced availability of chorismate for folic acid biosynthesis. If that is so, it may be that TyrR-tyrosine activation of *folA* expression in some way balances this effect on chorismate supply. Alternately, it is possible that under conditions in which the cell normally encounters high levels of tyrosine, there is an increased demand for tetrahydrofolate and one-carbon transfers. Further work will be required to explain the physiological significance of this complex system of TyrR-tyrosine mediated activation of *folA* expression. It is worth noting that no other gene of this chorismate-to-folate pathway has been selected by TyrR protein in genomic SELEX or has been revealed in bioinformatic searches (4, 8).

Although there are unresolved questions relating to the physiology, the genetics of the system is fairly clear. TyrR-mediated activation does not occur if the *tyrR* gene has mutations affecting the N-terminal domain's interaction with the aromatic amino acids or with RNA polymerase. Similarly, a mutation in *rpoA* which affects activation of *tyrP*, *aroP*, and *mtr* also affects *folA* activation. Unlike the situation with these three genes but similar to what is probably the case for the *tpl* gene of *Citrobacter freundii* (1), activation of *folA* also requires a fully functional central domain. Activation of *folA* also requires IHF, which is not required for the simpler activation of genes such as *mtr* (data not shown). A putative IHF binding site was found between the TyrR boxes and the *folA* promoter (Fig. 1). Furthermore, mutational studies have established that both of the identified TyrR boxes play an important role in activation. As in the case of *tpl*, the box with the least agree-

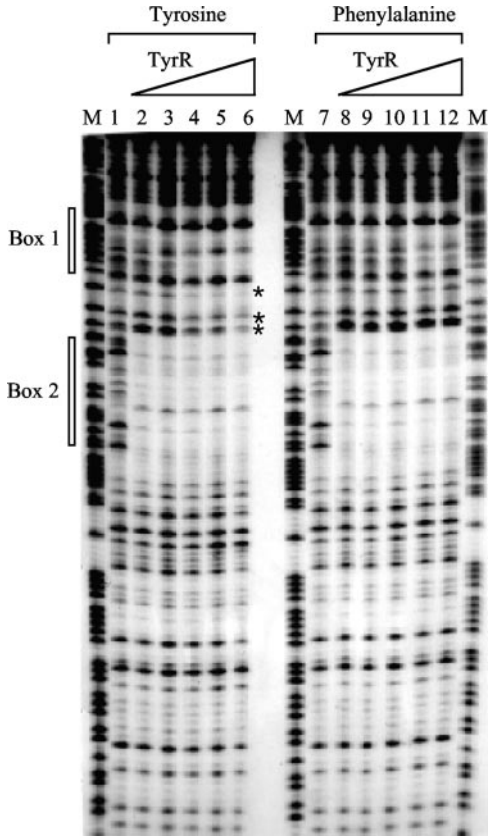

FIG. 2. DNase I footprinting of the *folA* promoter region. The DNase I protection assay was carried out in the presence of either tyrosine (lanes 1 to 6) or phenylalanine (lanes 7 to 12). The protected regions corresponding to TyrR boxes 1 and 2 are indicated with open bars. The base positions in the intervening region between the two TyrR boxes, which were protected by TyrR only in the presence of tyrosine, are marked with asterisks. The final concentrations of TyrR protein used in each reaction are as follows: lanes 1 and 7, no TyrR; lanes 2 and 8, 12.5 nM; lanes 3 and 9, 25 nM; lanes 4 and 10, 50 nM; lanes 5 and 11, 100 nM, and lanes 6 and 12, 250 nM. Lanes M, GA DNA sequence ladders. The protein used was native TyrR protein purified as described by Wilson et al. (11). The method for DNase I footprinting has been described previously (12).

ment to the consensus is the one closest to the promoter. This box, box 1, has only 8 of the 12 bases in the arms of the palindrome which agree with the consensus and has a central region comprised exclusively of GC pairs. It has all the characteristics of a low-affinity binding site, which would require an adjacent stronger binding site for effective binding to occur. Box 2, with 11 of its 12 bases in the palindromic arms agreeing

TABLE 3. Effect of TyrR box mutations on *folA* activation

folA-lacZ fusion	β-Galactosidase sp act in the indicated medium[a]		
	MM	Tyr	Phe
Wild type	101	458	267
TyrR box 1 mutation	102	80	83
TyrR box 2 mutation	80	107	119

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

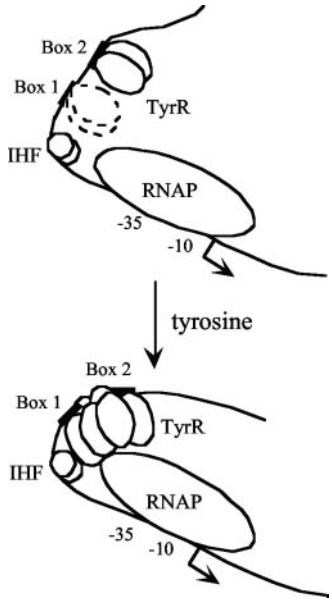

FIG. 3. A hypothetical model for tyrosine-dependent activation of the *folA* promoter by the TyrR protein. In vivo, in the absence of tyrosine, TyrR exists as a dimer, which binds strongly to TyrR box 2 and weakly to TyrR box 1 (the TyrR dimer is shown by dotted lines). The presence of tyrosine induces TyrR to form a hexamer, which strengthens the binding of TyrR to box 1. The TyrR molecule bound to box 1 interacts directly with the α subunit of RNA polymerase, leading to activation of transcriptional initiation of the *folA* promoter. The binding of IHF to the *folA* promoter is presumably required for creating an ideal DNA conformation, which facilitates TyrR-RNA polymerase interaction.

with the consensus and with three AT pairs in the central region, appears to play this role. We would hypothesize that the TyrR hexamer binds across both boxes and, with the assistance of IHF, produces a complex in which the N-terminal domain of one or more of the TyrR molecules is able to interact with the α subunit of RNA polymerase and activate transcription (Fig. 3). The DNase I protection experiment showed that, in addition to the protection of boxes 1 and 2, the intervening region between the two boxes was also partially protected by TyrR-tyrosine (Fig. 2). This may reflect the formation of a complex structure in this region. In the presence of phenylalanine, the weaker box 1 is protected only at higher TyrR concentrations (100 nM or above), which would explain why phenylalanine-mediated activation as shown in Table 2 is observed only in the presence of enhanced TyrR protein levels (i.e., with multicopy *tyrR*$^+$).

This complex system is designed to respond primarily to increased levels of tyrosine by activating expression of *folA*, in contrast to the well-studied situation in *tyrP*, where tyrosine-induced hexamerization causes repression. It is a good example of the selective application of the same mechanism to produce opposite outcomes.

The usefulness of the genomic SELEX approach is supported by the identification of seven of the eight known members of the TyrR regulon. The one which was not detected, *aroG*, is the only member of the regulon with a single TyrR box, and in this case it is possible that the GC-rich central region of this box generally associated with those called weak

TyrR boxes (6) may cause it to be a less effective binding site. Baseggio et al. (2) have shown that the affinity of TyrR protein for this single *aroG* box is about 1/10 of its affinity in the presence of tyrosine for the double box found in genes such as *tyrP*. The expression of *aroG* does not appear to be affected by the presence or absence of the aromatic amino acids, and its place in the TyrR regulon is dependent on observations of enhanced expression in *tyrR* mutants and decreased expression in strains with an elevated level of TyrR protein. The single TyrR box of *aroG* overlaps the −35 region of the promoter, and regulation appears to involve competition between RNA polymerase and TyrR protein in binding to the same region of DNA. Under these circumstances, it is not surprising to find that a binding site with a relatively low affinity for TyrR has been selected for *aroG* during evolution.

The finding that four of the five new genes with TyrR binding sites adjacent to their promoters showed no signs of TyrR-mediated regulation in vivo cautions against a too-ready acceptance of the conclusion that the existence of binding sites for a regulator protein near a promoter automatically implies effective regulation of that promoter by that protein. On the other hand, if such binding occurs in vivo, it will influence the overall concentration of nonbound TyrR protein available to interact with other binding sites associated with known members of the TyrR regulon. The finding of only one new member of the TyrR regulon, as a result of this latest search, may indicate that the TyrR system of regulation is fairly pathway specific and is unlikely to be involved in more global interactions within the cell.

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