Defective Regulation of the Phenylalanine Biosynthetic Operon in Mutants of the Phenylalanyl-tRNA Synthetase Operon

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Received 19 December 1985/Accepted 27 October 1986

Among mutants of Escherichia coli resistant to p-fluorophenylalanine (PFP) were some with constitutive expression of the phenylalanine biosynthetic operon (the pheA operon). This operon is expressed in the wild type by phenylalanine. The mutation in three of these mutants mapped in the aroH-aroD region of the E. coli chromosome at 37 min. A plasmid bearing wild-type DNA from this region restored p-fluorophenylalanine sensitivity and wild-type repression of the pheA operon. Analysis of subclones of this plasmid and comparison of its restriction map with published maps indicated that the mutations affecting regulation of the pheA operon lie in the structural genes for phenylalanyl-tRNA synthetase, pheST, probably in pheS. Thus, the pheST operon has a role in the regulation of phenylalanine biosynthesis, the most likely being that wild-type phenylalanyl-tRNA synthetase maintains a sufficient intracellular concentration of Phe-tRNA for attenuation of the pheA operon in the presence of phenylalanine. A revised gene order for the 37-min region of the chromosome is reported. Read clockwise, the order is aroD, aroH, pheT, and pheS.

The phenylalanine operon of Escherichia coli, located at 56 min on the genetic map, has a single gene, pheA, which encodes the bifunctional enzyme chorismate mutase-prephenate dehydratase (EC 4.2.1.51, EC 5.4.99.5) (1). This protein catalyzes conversions of chorismic acid to prephenic acid and prephenic acid to phenylpyruvic acid, the immediate precursor of phenylalanine (9).

Regulation of the pheA operon is not fully understood. Golub et al. (15) reported the pheR gene of Salmonella typhimurium, which maps in the 95- to 100-min region of the chromosome of that species, as the repressor gene for pheA. An E. coli gene which restores the wild phenotype in S. typhimurium pheR mutants is carried by the F-prime plasmid F116, derived from the comparable region of the E. coli chromosome, 59 to 65 min (15). Gowerishankar and Pittard (16) propose that this gene is nonfunctional in E. coli and that a second functional copy, which they named pheR, lies at 93 min on the E. coli chromosome. They propose that this latter gene encodes the repressor protein of the pheA operon. There is no evidence reported, however, that this protein is a repressor in the sense that it binds to an operator locus in the pheA operon, thus blocking transcription.

A cis-acting pheA-linked regulatory region (designated pheA0) has been described previously (16, 17). It is proposed that this is an operator, regulated by a phenylalanine-activated repressor encoded by pheR. Evidence has not yet been reported, however, that distinguishes between this possibility and the possibility that the pheR and pheA0 loci are elements of the phenylalanine-mediated attenuation system for the pheA operon described by Zurawski et al. (37). While it is our view that from the evidence available, no conclusion can be drawn about the existence of an operator-repressor system of regulation for the pheA operon, there is evidence from in vitro transcription and nucleotide sequence information (37) that the phe operon is regulated by an attenuation mechanism analogous to that of the tryptophan (20, 35) and histidine (18, 19) operons.

For both the tryptophan and histidine operons, it has been shown that the cognate aminoacyl-tRNA synthetases are involved in their regulation by attenuation (18, 25, 34-36).

In this paper, we report the isolation and characterization of mutants with elevated, constitutive expression of pheA. When plasmids containing the wild-type phenylalanyl-tRNA synthetase operon, pheST, were introduced into these strains, wild-type control of pheA was restored. Thus, we propose that pheST regulates the phenylalanine biosynthetic operon, adding further support for an attenuation mechanism for this operon.

MATERIALS AND METHODS

Strains. Strain KB1360 is F− araD proA argE hisX thi lacZ gal xyl mtl tsx shiA3 (5). Strain KB9195 was derived from KB1360 by mutation and P1 kc transduction; it is KB1360 aroD+ gal+ araF aroG aroH. Strains KB8021, KB8026, and KB8090 were derived from KB1360; they are KB1360 phe(ST)1, phe(ST)2, and phe(ST)3, respectively. Strain NP37 (11), obtained from B. Bachmann, is pheST relA1 fhuA22 ompF627 pit-10.

Isolation of mutants resistant to PFP. Colonies of KB1360 were grown in L broth (22) to 5 × 10⁹ cells per ml. Washed cells (10⁶) were spread on minimal agar containing non-aromatic growth factors plus shikimic acid (100 μg/ml) and p-fluoro-di-phenylalanine (PFP) (6 mM). After 48 h at 37°C, approximately 50 PFP-resistant colonies appeared per plate.

Media. Basal medium was that of Vogel and Bonner (33). It was supplemented when necessary with shikimic acid and amino acids at 100 μg/ml each and thiamine at 10 μg/ml. Complete medium was basal medium with L-tyrosine, L-phenylalanine, and L-tryptophan at 10⁻⁴ M each, L-proline, L-arginine, and L-histidine at 100 μg/ml each, thiamine at 10 μg/ml, shikimic acid at 1 μg/ml, and p-aminobenzoic acid, 2,3-dihydroxybenzoic acid, and p-hydroxybenzoic acid at 10⁻⁶ M each. Limiting phenylalanine was 8 × 10⁻⁶ M.

Transformations. The technique used was that of Cohen et al. (7), except for transformation of the temperature-sensi-

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Table 1. Specific activities of prephenate dehydratase in cell extracts of mutants and merodiploids

<table>
<thead>
<tr>
<th>Strain and plasmid</th>
<th>Sp act (mU/mg of protein)</th>
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<tbody>
<tr>
<td></td>
<td>Phe-rep&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>KB1360</td>
<td>6</td>
</tr>
<tr>
<td>KB8026</td>
<td>79</td>
</tr>
<tr>
<td>KB8026(F148)</td>
<td>6</td>
</tr>
<tr>
<td>KB8026(pAROH13)</td>
<td>12</td>
</tr>
<tr>
<td>KB8026(pBO7)</td>
<td>19</td>
</tr>
<tr>
<td>KB8026(pBO8)</td>
<td>85</td>
</tr>
<tr>
<td>KB8026(pBO78)</td>
<td>13</td>
</tr>
<tr>
<td>KB8026(pBO79)</td>
<td>8</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cells extracts were prepared and specific activities of prephenate dehydratase were estimated essentially as described previously (6).

<sup>b</sup> Cells were grown in complete aromatic medium and harvested in the exponential phase at 3 × 10⁸ cells/ml.

<sup>c</sup> Cells were grown in complete aromatic medium but with limiting phenylalanine (8 × 10⁻⁴ M) and harvested after 3 h in the stationary phase (5 × 10⁸ cells/ml).

<sup>d</sup> NT. Not tested.

Results

Isolation of mutants derepressed for chorismate mutase-prephenate dehydratase. PFP inhibits the growth of E. coli (26). Mutants resistant to PFP have been isolated with alterations in phenylalanyl-tRNA synthetase (13), general aromatic amino acid transport (4), feedback inhibition of the first enzyme of the aromatic pathway (3-deoxy-D-arabinoheptulosonic acid 7-phosphate [DAHP] synthetase) (15), and regulation of the phenylalanine operon (15, 17). We searched for mutants whose phenylalanine operon was constitutively derepressed by screening PFP-resistant mutants for high, constitutive activity of prephenate dehydratase. KB1360 was the parent in our mutant hunt, as it is aroD and is blocked in the third step (dehydroshikimate synthesis) of the aromatic pathway. KB1360 grows well on the intermediate shikimic acid, since it has the shiA3 allele for efficient shikimate uptake (5). We reasoned that in this strain, only regulatory mutations which cause overproduction of phenylalanine by interfering with regulation of the terminal phenylalanine pathway would be detected. Mutations affecting regulation of the aromatic pathway before aroD, e.g., at DAHP synthetase, would not be expressed.

We measured prephenate dehydratase in 50 independent PFP-resistant mutants of KB1360 grown under conditions of pheA repression (complete medium). Ten had high specific activities of prephenate dehydratase relative to the parent. The mutations which conferred PFP resistance and high, constitutive prephenate dehydratase synthesis in three of these mutants, KB8021, KB8026, and KB8090, mapped in the aroD-aroH region of the chromosome and are the subject of this paper. The specific activities of prephenate dehydratase in KB8021, KB8026, and KB8090 were, on average, 13.7 times higher than in KB1360 (Table 1; only KB8026 and KB1360 shown). When KB1360 was grown on limiting phenylalanine, there was ninefold derepression of prephenate dehydratase relative to cells grown on complete medium (Table 1). When the three phe regulatory mutants were grown under these conditions, there was approximately a twofold increase in prephenate dehydratase activity relative to complete medium-grown cells. Moreover, the derepressed activities were twice that in the parent KB1360 (Table 1). Thus, the three regulatory mutants with mutations of the pheA operon could be derepressed twofold by phenylalanine limitation but could not be repressed by phenylalanine below the level of the derepressed wild type.

Mapping the phe regulatory locus. When the F-prime plasmid F148 was introduced by conjugation into mutants KB8026, KB8021, and KB8090, the merodiploids were rendered PFP sensitive and prephenate dehydratase was repressed by phenylalanine (Table 1). Thus, the wild-type phenotype was restored by F148, which carries the 37- to 39- and 42- to 44-min regions of the chromosome (21).

P1 kc transduction analysis of the 37-min region indicated that the wild-type phe regulatory locus was transduced with aroD<sup>+</sup> into the three regulatory mutants at high frequency (69%) (Table 2; only KB8026 shown). To determine the side of aroD on which the phe regulatory locus lies, the inheritance of a third marker, aroH, was examined. The regulatory locus and aroH were transferred together as unselected markers at high frequency (91%) when aroD<sup>+</sup> transductants were selected (Table 2). Therefore, the regulatory locus and aroH lie close together on the same side of aroD. aroH was transduced with aroD at a frequency of 72%, indicating that aroH is closer to aroD than is the regulatory locus. No published information orienters aroH with respect to aroD. Its current reported position anticonwise to aroD at 37 min on the E. coli map (1) is arbitrary. If aroH is repositioned on the opposite side of aroD, then the position of our phe regulatory locus corresponds to the reported position of the phenylalanyl-tRNA synthetase operon (pheST) at 38 min (8).

We cloned the wild-type phe regulatory locus on plasmid pAROH13 and found it to be pheST (see below). Complementation of KB9195 and KB8026 showed that this plasmid contains aroH but not aroD. Therefore, the revised gene order in the 37-min region, reading clockwise, is aroD, aroH, pheT, and pheS.

Cloning the phe regulatory locus and identification as pheST. Plasmid pAROH13 (38) contains a 22.6-kilobase (kb) EcoRI fragment from F148 with the aroH region, inserted in vector pCR1 (10). When transformed with pAROH13, the three regulatory mutants were rendered PFP sensitive and wild-type repression of prephenate dehydratase was restored (Table 1). pAROH13 was mapped with several restriction enzymes (Fig. 1). The restriction map of the EcoRI insert in pAROH13 resembles that of the 22-kb insert in plasmid pID1, isolated by Elseviers et al. (12), while the restriction sites within the 9.8-kb EcoRI-HindIII region of

Table 2. P1 kc transduction of the 37- to 38-min region of the E. coli chromosome

<table>
<thead>
<tr>
<th>Genotype of aroD&lt;sup&gt;+&lt;/sup&gt; transductants&lt;sup&gt;a&lt;/sup&gt;</th>
<th>aroH&lt;sup&gt;b&lt;/sup&gt;</th>
<th>phe regulatory locus&lt;sup&gt;c&lt;/sup&gt;</th>
<th>% Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>6</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>−</td>
<td>22</td>
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<tr>
<td>+</td>
<td>−</td>
<td>+</td>
<td>63</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>−</td>
<td>9</td>
</tr>
</tbody>
</table>

<sup>a</sup> The P1 kc transduction method was described previously (3).

<sup>b</sup> Donor strain, KB9195; relevant genotype, aroD<sup>+</sup>, aroH<sup>−</sup>, wild-type phe regulatory locus. Recipient strain, KB8026. recipient genotype, aroH<sup>−</sup>, aroH<sup>+</sup>, mutant phe regulatory locus. Selected marker, aroD<sup>+</sup>. Number of transductants examined, 400.

<sup>c</sup> aroH mutant marker (−) was scored by the inability of aroD<sup>+</sup> transductants of KB8026 to grow on minimal medium plus phenylalanine and tyrosine (both 1 mM) following receipt of this unselected marker.

<sup>d</sup> The phe regulatory locus was scored by sensitivity (+) or resistance (−) to PFP.
the pAROH13 insert are similar to those of the 9.8-kb insert in plasmid pB1 isolated by Plumbridge et al. (29). Both pB1 and pD1 carry the pheST operon (12, 29). pAROH13 complemented the temperature-sensitive pheS mutation in strain NP37 (11). When cured of the plasmid, its temperature-sensitive phenotype returned. The pheST genes are 6 kb distant from arOH. The position of arOH in pAROH13 was determined by complementation of the arOH mutation in KB9195 by subclones of pAROH13 (data not shown) and from the previously reported nucleotide sequence (38).

A subclone of pAROH13, pB07 (Fig. 1 and 2), made by inserting the 10.1-kb SalI-BamHI fragment into pBR322, partially complemented the regulatory mutations in strains KB8021, KB8026, and KB8090 (as determined by repressibility of prephenate dehydratase [Table 1]). This plasmid contains 8.2 kb of the E. coli chromosome. A restriction map comparison between pB07 and the similar region in pB1 indicates that it carries the genes thrS, infC, rplT, and pheS and approximately the first third of pheT. That pB07 contains the pheS gene was confirmed by its ability to complement the temperature-sensitive pheS mutation in NP37. pB08, another subclone of pAROH13 which contains the distal portion of pheT, had no effect on the levels of prephenate dehydratase in the phe regulatory mutants (Table 1; Fig. 1 and 2).

To examine the effect of the entire pheST operon, pB07 and pB08 were cut with BamHI, ligated, and then transformed into KB8026. The fusion of pB07 and pB08 to reconstruct the pheST operon results in reformation of an intact tetracycline resistance gene from the two pBR322 vector fragments of the parents. Hence, cells bearing such a plasmid, designated pB078 (Fig. 1 and 2), could be selected for by resistance to tetracycline. Merodiploids of KB8026, KB8021, and KB8090 containing pB078 were PFP sensitive and had Phe-repressible prephenate dehydratase (Table 1).

To test whether our regulatory mutations occur in the pheST operon, we constructed plasmid pB079, which contains no chromosomal DNA other than structural genes pheST. pB079 was constructed by ligating the 7.4-kb SmaI-BamHI fragment of pB078 with the 0.5-kb EcoRV-SalI fragment of pBR322 (Fig. 2). Restriction mapping (28, 29) and DNA sequence analysis (14, 24) indicate that the pheST structural genes are contained within the 3.4-kb SmaI-HindIII region of pB079 (Fig. 1). pB078 and pB079 were shown to contain pheS by complementation of pheS in NP37. When the three phe regulatory mutants were transformed with pB079, the full wild-type phenotype was restored (Table 1). Thus we conclude that the regulatory mutations in KB8026, KB8021, and KB8090 lie in the pheST operon.

FIG. 1. Restriction maps of the 37-min region. (A) pAROH13 insert. (B) Comparison between pAROH13 DNA and DNA cloned from this region of the chromosome into plasmid pB1 by Plumbridge et al. (29). Symbols: A, AvoI; B, BamHI; G, BglII; E, EcoRI; H, HindIII; h, HpaI; K, KpnI; P, PstI; M, SmaI; S, SstI; X, XhoI. Arrows indicate transcription of pheST and arOH. There are four HpaI sites between the HpaI sites at 2.2 and 5.6 kb in pB078 and pB1. The order of these sites in pB078 was not determined, but fragments obtained by HpaI digestion of this region are similar in size to HpaI fragments from the same region of pB1 (28). Restrictions and gel electrophoresis were performed as described by Maniatis et al. (23).

DISCUSSION

We isolated PFP-resistant mutants with altered regulation of the phenylalanine operon. Complementation of these mutants by wild-type phenylalanyl-tRNA synthetase (pheST) genes restored wild-type repression of pheA. This indicates that wild-type phenylalanyl-tRNA synthetase is required for normal regulation of the phenylalanine operon.

Partial restoration of phe regulation in these phenylalanyl-tRNA synthetase mutants by pB07, which contains all of pheS but only part of pheT, suggests that the mutated gene in the three regulatory mutants is pheS (Fig. 2; Table 1). The incompleteness of restoration of wild-type phe regulation in pB07 merodiploids may be because pheS and pheT are cotranscribed on the chromosome (27) and hence are translated on the same mRNA. The mutant α subunit (the chromosomal pheS product) may be better able (owing to proximity) to compete for binding with the normal β subunit (the pheT product) than the wild-type α subunit, whose synthesis is directed by pB07. Hence, pheST regulatory mutants bearing pB07 would have a mixed population of phenylalanyl-tRNA synthetase molecules characteristic of wild-type and mutant genes. Alternative explanations are that (i) our mutants are pheS with a polar effect on pheT or that (ii) they are double pheST mutants.
phenylalanine starvation leads to higher pheA expression in our pheST mutants than in wild-type cells (Table 1). This may be explained if under conditions of Phe starvation in the wild-type strain (KB8526), there remains a low concentration of Phe-tRNA_phe that is able to cause some attenuation. In pheST mutants, the residual level of Phe-tRNA_phe may be further reduced in phenylalanine-starved cells owing to defective phenylalanyl-tRNA synthetase. Hence, the starved cells show greater transcriptional readthrough of pheA.

It has recently been shown that the pheST operon is regulated by an attenuation mechanism analogous to the attenuation mechanism postulated for the pheA operon (14, 31). It is likely that our pheST mutants are defective in this regulation as well as in pheA regulation, causing depression of the pheST operon itself and hence synthesizing less-active phenylalanyl tRNA synthetase. This possibility, however, has not been investigated.

An alternative explanation for the derepression of pheA in our pheST mutants is that phenylalanyl-tRNA synthetase is a repressor activated by phenylalanine to bind to a hypothetical operator locus of the pheA operon to block pheA transcription. An analogy occurs in the alaS operon (30), in which the operon product (alanyl-tRNA synthetase), in the presence of alanine, represses transcription of its own gene by binding specifically to a palindromic sequence centered at the transcription start site.

While our rationale for isolation of phe regulatory mutants by selecting for PFP resistance did yield such mutants, it does not follow that the overproduction of Phe causes the resistance. Possibly, the alteration of phenylalanyl-tRNA synthetase per se causes resistance, since thereby PFP may not be efficiently activated to give rise to nonfunctional proteins (a major cause of PFP toxicity [26]). Thus our mutants could have altered charging activities for both Phe and PFP. Fangman and Neidhardt (13) indeed showed that their PFP-resistant mutants had a reduced ability to activate PFP.

ACKNOWLEDGMENTS

We thank the Australian Research Grants Committee for financial support (project no. D72/15153).

We thank Leila Blackman for technical assistance.

LITERATURE CITED


