Autoregulation of *Escherichia coli* purR Requires Two Control Sites
Downstream of the Promoter†

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The expression of *Escherichia coli* purR, which encodes the pur regulon repressor protein, is autoregulated. Autoregulation at the level of transcription requires two operator sites, designated purR₁ and purR₂ (O₁ and O₂). Operator O₁ is in the region of DNA between the transcription start site and the site for translation initiation, and O₂ is in the protein-coding region. The repressor protein binds noncooperatively to O₁ with a sixfold-higher affinity than to O₂, and saturation of O₁ by the repressor precedes saturation of O₂. Both O₁ and O₂ function in the two- to threefold autoregulation in vivo, as determined by measurement of β-galactosidase and mRNA from purR-lacZ translational fusions. Of all the genes thus far known to be regulated by the Pur repressor, only purR employs a two-operator mechanism.

In *Escherichia coli*, the genes encoding the enzymes for the de novo synthesis of IMP are arranged as individual loci and small polycistronic operons. The gene organization and map locations (in minutes) are as follows (2, 33): purR, 25.2; purHD, 90.3; purEK, 12.2; cyp purF dedF (11, 37), 50.0; purMN, 53.5; purC, 53.3; purL, 55.2. In addition, guaBA at min 53.9 is required for the two-step conversion of IMP to GMP, and purA at min 95.0 and purB are required for conversion of IMP to AMP. The addition of exogenous purines to the growth medium cause repression of all genes in the pathway (15, 36). However, the AMP and GMP branches appear to be under separate regulation from the pathway leading to IMP (15, 54). Genes for the pathway to IMP, except for purB (23), are coregulated by the purR-encoded repressor (23, 34) and a corepressor that is a small molecule (25). These genes constitute the *E. coli* pur regulon. Gene purR encoding the pur regulon repressor has been cloned (42) and sequenced, and operator binding sites have been identified (29, 42). Each of the coregulated pur genes has a 16-base-pair (bp) conserved operator sequence that is located in the promoter region (1, 11a, 32, 43, 45, 47, 47a, 48, 51; K. A. Flannigan, S. H. Hennigan, H. H. Vogelbacker, J. S. Gots, and J. M. Smith, Mol. Microbiol., in press; A. A. Tiedeman, D. J. DeMarini, J. Parker, and J. M. Smith, submitted for publication). Mutational analysis (41) and DNase I footprinting (23, 42) have established that the Pur repressor binds to these operator sequences for the negative regulation of pur regulon gene expression (23, 34).

Gene purR contains two operatorlike sequences located downstream of the promoter (42). In this report, we show that purR is autoregulated and that the operatorlike sequences purR₁ and purR₂ (O₁ and O₂) are authentic control sites. The mechanism of autoregulation involves the independent binding of Pur repressor to O₁ and O₂. Operator site O₂, located within the purR coding sequence, binds the repressor in vitro with a sixfold lower affinity than O₁ and yet makes an important contribution to in vivo autoregulation under the conditions studied. During the preparation of this manuscript, Meng et al. (34) reported the autoregulation of *E. coli* purR. Our work supports and extends their analyses and gives a more complete picture of the autoregulation of purR.

MATERIALS AND METHODS

**Bacterial strains and plasmids.** Strains and plasmids are described in Table 1.

**Media.** Minimal growth medium contained salts (50), 0.5% glucose, 0.2% casamino acids, and 0.2 μg of thiamine per ml. Adenine (100 μg/ml) was added to repress purine nucleotide synthesis. Extracts containing Pur repressor were prepared from cells grown in rich medium containing 2% tryptone, 1% yeast extract, and 0.5% NaCl. L agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside was used to visualize β-galactosidase activity from purR-lacZ fusions in cells on plates. For plasmid selection, antibiotics were added to the following concentrations: ampicillin, 100 μg/ml; spectinomycin, 50 μg/ml.

**Plasmid constructions.** A series of plasmids (Table 1) was produced in the construction of pPR1006, the source of fragments for binding assays. A 3.2-kilobase *Bal*I-*PstI* fragment (Fig. 1A) from purR⁺ plasmid pPR1003 was subcloned into the *HincII* and *PstI* sites of pUC119, yielding plasmid pPR1004. The 3’ noncoding sequences were removed by deleting sequences between the *PstI* site and the *HpaI* site located at position 1266 relative to the transcription start site of purR. The resulting plasmid, pPR1004-2, contained the entire coding sequence of purR and 136 of 155 nucleotides of the 5’ transcribed but untranslated sequence cloned in the opposite direction to the *lac* promoter. An *NdeI* site was constructed at the initiating ATG codon in plasmid pPR1004-2 by oligonucleotide-directed mutagenesis (31) to produce pPR1005. Plasmid pPR1006 is a derivative of pPR1005 in which most of the coding sequence was removed by deleting from an internal *HincII* site at nucleotide 321 to the downstream polylinker *SphI* site.

A series of plasmids was produced in the construction of purR-lacZ fusions and the mutagenesis of O₁ and O₂. Plasmid pPR2000 contains the promoter region and was constructed by cloning the *XhoI* (position −245)-to-*BglII* (position 258) fragment (Fig. 1A) from pPR1002 into the *HincII* site of pUC119. A purR-lacZ fusion was constructed in two steps by subcloning purR⁺ DNA from pPR2000 into the low-copy-number vector pGB2 by using EcoRI and *HindIII*.

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TABLE 1. E. coli strains and plasmids

| Strain or plasmid | Description | Reference or source |
|-------------------|-------------|---------------------|
| E. coli MC4100    | Δ(argF-lac)169 | 4                   |
| R320              | MC4100 purR300 | 41                  |
| CJ236             | dut-l ung-1   | 31                  |
| Plasmids          |             |                     |
| pPR1002           | 3.8-kilobase purR+ PstI fragment in pMS421 | 42 |
| pPR1003           | Opposite orientation of PstI fragment in pPR1002 | 42 |
| pPR1004           | purR Ball-to-PstI fragment in pUC119 | This work |
| pPR1004-2         | purR Ball-to-HpaI fragment in pUC119 | This work |
| pPR1005           | pPR1004-2 with NdeI mutation | This work |
| pPR1006           | purR' Ball-to-HincII fragment in pUC119 | This work |
| pPR2000           | purR' Xhol-to-BglII fragment in pUC119 | This work |
| pPR2000-1         | pPR2000 with O1- | This work |
| pPR2000-2         | pPR2000 with O2- | This work |
| pPR2000-3         | pPR2000-2 with O1- | This work |
| pPR2002           | Wild-type purR-lacZ fusion (O1O2) | This work |
| pPR2004           | purR-lacZ (O1- O2-) | This work |
| pPR2005           | purR-lacZ (O1 O2-) | This work |
| pPR2006           | purR-lacZ (O1- O2-) | This work |
| pGB2              | Low-copy-number vector, Spc' | 6 |
| pMS421            | lac' gene in pGB2, Spc' | 42, M. Suskind\(^a\) |
| pUC119            | High-copy-number phagemid, Amp' | 49 |
| pMC1871           | lac Z cassette plasmid | 46 |

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polylinker sites. A PstI fragment containing lac'Z from pMC1871 was ligated downstream of the purR sequences in the PstI site. The fusion gene created at this step (Fig. 1B) contained 35 codons of purR and 13 codons from a polylinker joined to codon 8 of lacZ. This purR-lacZ translational fusion was under the same controls as the purR gene. The copy number of this plasmid is expected to be maintained between four and six copies per cell (21).

Substitutions were made in the operators O1 and O2 by oligonucleotide-directed mutagenesis with pPR2000 as the source of single-stranded DNA. The purR-lacZ fusions were created by using the same cloning scheme described for the wild-type fusion. The plasmid designations for the purR-lacZ containing O1- O2-, O1- O2-, and O1- O2- are pPR2004, pPR2005, and pPR2006, respectively.

Oligonucleotide-directed mutagenesis. Mutagenesis was performed by the method of Kunkel (31) with the Mutagene Phagemid in vitro mutagenesis kit from Bio-Rad Laboratories. An NdeI site was created in pPR1004-2 by using the oligonucleotide 5'-TGGAGTGAATCATATGGCAGAACAATTAAAG-3' (mismatches are underlined). The operator mutations were made in pPR2000 by using the oligonucleotides 5'-GAAACGCCACACTACCTTGCGATTGTTG-3' and 5'-GAAACGCCACACTACCTTGCGATTGTTG-3' to mutate O1 and O2, respectively (Fig. 1). All mutations were verified by dideoxy sequence analysis (44).

Assay of β-galactosidase. Cells were grown to the midlog phase in minimal medium with and without adenine. The β-galactosidase activity of purR-lacZ fusions was determined by the Miller assay (35). Since purR and purR' strains were each grown with and without added purine, it was possible to calculate repression by two methods (Table 2). In every case, the values calculated by the two methods agreed closely and supported the reliability of these measurements of repression.

Repressor-operator binding. Protein extracts were prepared as described previously (42) from 20-ml cell cultures

FIG. 1. (A) Schematic representation of the purR gene and surrounding region. The drawing is not to scale so that details will show. The positions of several restriction sites with respect to the transcription start site (arrow at +1) are shown in parentheses. The two purR operators, O1 and O2, are indicated by the black boxes; the purR coding region is the open box. (B) Nucleotide and protein sequence of the purR-lacZ translational fusion junction. The sequence shown begins with the final two purR codons for Asn-34 and Ala-35 and ends with the first two lacZ codons for Pro-8 and Val-9. The purR-lacZ junction contains codons from a polylinker region that arose during the cloning. (C) Sequence of the two purR operators and the sites of mutation within each. Conserved positions are highlighted in reverse type.
TABLE 2. Regulation of purR-lacZ

| Plasmid (purR operator) | purR genotype<sup>a</sup> | β-Galactosidase<sup>b</sup> | Repression<sup>c</sup> (fold) | mRNA<sup>d</sup> (cpm) | Repression<sup>c</sup> (fold) |
|-------------------------|--------------------------|-----------------|-----------------|-----------------|-----------------|
|                         |                          | + Adenine     | - Adenine       | + Adenine       | - Adenine       |
| pPR2002 (O₁O₂<sub>2</sub>) | purR<sup>*</sup>         | 150 ± 8.4    | 380 ± 12       | 2.5–2.7<sup>f</sup> | 2,200     | 4,800  | 1.6–2.2 |
|                         | purR<sup>+</sup>         | 380 ± 16    | 420 ± 16       | 1.1             | 3,900     | 3,100  | 0.8   |
| pPR2005 (O₁O₂<sub>−</sub>) | purR<sup>+</sup>         | 220 ± 13    | 440 ± 17       | 1.9–2.0<sup>f</sup> | 2,400     | 3,700  | 1.5–1.6 |
|                         | purR<sup>−</sup>         | 400 ± 9.8   | 425 ± 22       | 1.1             | 3,900     | 4,000  | 1.0   |
| pPR2004 (O₁O₂<sub>−</sub>) | purR<sup>+</sup>         | 260 ± 23    | 420 ± 48       | 1.5–1.6<sup>f</sup> | 2,200     | 2,700  | 1.2–1.5 |
|                         | purR<sup>−</sup>         | 370 ± 35    | 410 ± 56       | 1.1             | 3,200     | 3,600  | 1.0   |
| pPR2006 (O₁O₂<sub>−</sub>) | purR<sup>+</sup>         | 200 ± 15    | 240 ± 21       | 1.1–1.2         | 6,400     | 7,500  | 1.1–1.2 |
|                         | purR<sup>−</sup>         | 210 ± 17    | 230 ± 25       | 1.1             | 6,300     | 8,400  | 1.3   |

<sup>a</sup> Strains MC4100 (purR<sup>−</sup>) and R320 (purR<sup>+</sup>).

<sup>b</sup> β-Galactosidase values are expressed in Miller units as the average of 4 to 13 experiments ± standard error. Cells were grown as described in Materials and Methods.

<sup>c</sup> Fold repression was calculated two ways and expressed as a range: (i) comparison of with and without adenine in purR<sup>+</sup>; (ii) values for with and without adenine in purR were averaged and divided by the value for purR<sup>+</sup> with adenine.

<sup>d</sup> Pulse-labeled RNA was quantitated as described in Materials and Methods. The values are from single experiments (pPR2002, pPR2005) or the average of two experiments (pPR2004, pPR2006).

<sup>e</sup> Calculated as for the β-galactosidase assays.

<sup>f</sup> Significant at greater than 97% confidence level with Student's t test.

<sup>g</sup> Strains carrying this construct consistently had low enzyme values (five experiments) and high RNA levels (two experiments). We were unable to determine the cause of this effect.

grown in rich medium. Fragments used in gel retardation assays were from plasmid pPR1006: a 324-bp EcoRI-HindIII fragment containing O₁ and O₂, a 153-bp EcoRI-Ndel fragment containing O₁, and a 173-bp Ndel-HindIII fragment containing O₂. These fragments are shown schematically in Fig. 2. Fragments were labeled with T4 polynucleotide kinase and [γ-<sup>32</sup>P]ATP and were isolated by electrophoresis from 5% polyacrylamide gels. Gel retardation experiments were performed as previously described (42), with the following changes: 10 fmol of each DNA fragment was used for binding; extract was added containing 0, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, and 20.0 μg of protein; N-2-hydroxyethylpyrano-rine-N'-2-ethanesulfonic acid (pH 8.0) and glycerol were added to 10 mM and 10%, respectively. Samples were resolved by electrophoresis at 100 V for 1.5 h.

For DNase I footprinting, plasmid pPR1006 was digested with either EcoRI or HindIII, end labeled with T4 polynucleotide kinase and [γ-<sup>32</sup>P]ATP, and then treated with the other restriction enzyme to cut on the opposite side of the polynucleotidyl. Fragments were electrophoresed from 5% polyacrylamide gel slices. For DNase I footprinting, repressor-operator complexes were prepared as described above by using 0, 1, 2.5, 5, 10, and 15 μg of protein. After complex formation, 2.5 mM MgCl₂ and 0.2 mg of DNase I per ml were added. Digestion was stopped after 1.5 min by the addition of 5 mM EDTA and placing samples on ice. Samples were extracted with phenol-chloroform (1:1), 20 μg of glycerol was added, and DNA was ethanol precipitated. Pellets were suspended in 2.5 μl of water and 2.5 μl of formamide dye (80% formamide, 0.1% EDTA, 0.1% each bromphenol blue and xylene cyanol). Half of this sample was loaded onto an 8% polyacrylamide–urea sequencing gel for electrophoretic resolution.

RNA analyses. Cells were grown to the midlog phase in minimal medium with or without adenine, and 10 mM of [5,6-<sup>3</sup>H]uridine (specific activity, 38 Ci/mmol) was added for 1.5 min. Cells were harvested by filtration through Whatman type GF/C glass fiber filters for 20 to 30 s. RNA was isolated as described previously (10). Concentrations of RNA samples were determined by A<sub>260</sub>. RNA from the purR-lacZ fusion was quantitated by RNA-DNA hybridization (14). Hybridizations were in vials containing one 13-mm nitrocellulose filter with 2 μg of immobilized DNA, 300 μl of 1× SSC (0.15 M NaCl, 0.015 M sodium citrate), and 50 μg of RNA diluted to 200 μl in TE (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA) containing 0.5 M KCl. RNA-DNA hybridizations were incubated for 16 h at 66°C. Nonspecific RNA was removed by washing at room temperature as follows: two washes in 2× SSC–10 μg of RNase A per ml for 30 min; two washes in 2× SSC for 30 min. Filters were dried for 10 min on each side under a heat lamp, placed in scintillation vials, and counted after complete dissolution in toluene-based scintillation fluid. All hybridizations were in duplicate to linearized pMC1871, which contains lac'Z in pBR322, and to linearized pBR322 as a control.

RESULTS

Autoregulation of purR. Two pur regulon operatorlike sequences flank the initiating methionine codon in the purR gene (42). Operator O₁ is located between positions 96 and 111 relative to the transcription initiation site, and operator O₂ is in the coding sequence at positions 184 to 199 (Fig. 1A). To determine whether the Pur repressor regulates expression of the purR gene, a purR-lacZ translational fusion was constructed in a low-copy-number vector and named pPR2002. Plasmid pPR2002 was transformed into purR<sup>−</sup> and purR<sup>+</sup> strains, and β-galactosidase activities were measured from cells grown in the presence and absence of adenine (Table 2). The data demonstrate a 2.5- to 2.7-fold repression of purR-lacZ in the purR<sup>−</sup> strain. Essentially no regulation was obtained in the purR strain. Thus, purR expression is autoregulated.

Repressor-operator interaction. Gel retardation assays were performed to determine whether the Pur repressor can interact with either one or both of the operatorlike sequences. A Ball-to-HindIII fragment containing purR DNA from nucleotides 20 to 321 was cloned into the Smal site of the pUC119 polylinker and used for binding experiments. This DNA fragment was excised as a 324-bp EcoRI-to-HindIII segment, end labeled, and incubated with extract prepared from a purR<sup>−</sup> strain. The results of repressor binding to the labeled DNA are shown in Fig. 2A. After electrophoresis, DNA was found in three positions: the band with the fastest mobility, which was free DNA, plus two
positions with protein-DNA complexes B1 and B2. With increasing protein there was initial formation of band B1 and subsequent formation of band B2 with a slower mobility than B1. The binding curves in Fig. 3 show the concentration dependence for conversion of free DNA to complexes B1 and B2. The half-maximal formation of B1 required 1.1 μg of protein, whereas the half-maximal formation of B2 required approximately 6.9 μg of protein. No binding was obtained with extract from a purR mutant (data not shown). The results in Fig. 2A suggest that binding of the repressor to one operator site results in a complex that migrates as band B1, whereas binding of repressors to O1 and O2 forms a protein-DNA complex that migrates as band B2.

To determine whether there are different affinities of the repressor for O1 and O2, and to determine whether the repressor initially interacts with O1 or with O2, binding assays were conducted with "half" fragments containing only one operator. Fragment 1 contained O1 (Fig. 2B), and fragment 2 contained O2 (Fig. 2C). Saturation of O1 and O2 with repressor is shown in Fig. 2B and C and is quantitated in Fig. 3. The saturation curves in Fig. 3 show that the concentration dependence for binding of the repressor to operator O1 was similar to the concentration dependence for band B1 and identifies band B1 as the repressor-O1 complex in the DNA fragment containing O1 and O2. The concentration dependence for saturation of O2 in fragment 2 was similar to the formation of B2 in DNA containing O1 and O2. Thus, the Pur repressor initially saturates O1, followed by saturation of O2. In this analysis there is no evidence for cooperativity for binding of the repressor to sites O1 and O2.

The gel retardation assays presented in Fig. 2B and C show decreased electrophoretic mobility for fragments 1 and 2, respectively, incubated with 10 or 20 μg of protein. It is likely that this decreased mobility is due to protein-protein or nonspecific protein-DNA interactions, since no additional protected regions appeared in a DNase I footprint (see below).

Binding of the repressor to the purF operator was also determined. Saturation curves for binding of the repressor to a DNA fragment containing the purF operator and to the fragment containing O1O2 were superimposable, as measured by the disappearance of free DNA (data not shown). Saturation of fragment 1 (95% binding) containing O1 required 10 μg of repressor compared with 5 μg of repressor required for binding to O1O2 or PurFp. Thus two purR operators were required to obtain repressor binding that was identical to that of the purF operator. As shown below, this requirement for both purR operators is not due to cooperative binding but reflects the availability of two binding sites for formation of a mixed population of complexes: repressor-O1, repressor-O2, (repressor)-O1O2.

**Protection of operator DNA by Pur repressor.** DNase I footprinting assays were performed to verify that binding of the repressor is specific for operator sites O1 and O2 and to search for evidence for interaction between operator sites. Sites O1 and O2 were saturated with increasing concentrations of repressor before treatment with DNase I. Figure 4A shows the protection of the noncoding (upper) strand, and Fig. 4B shows the protection of the coding (lower) strand. These results demonstrate clearly that the repressor initially binds to O1 and at higher concentrations saturates O2. The boundaries for DNase I protection extend 3 to 5 nucleotides beyond the conserved operator sites and are indicated by brackets under the sequence in Fig. 4C. One boundary was difficult to define due to poor digestion by DNase I on both strands. This boundary is indicated by a dashed bracket in Fig. 4C. These results demonstrate that the repressor binds to O1 and O2. We could detect no other sites of specific interaction of the Pur repressor with the DNA fragment.

There is no evidence in the DNA footprint for DNA binding that might result from interaction between repressor molecules bound at O1 and O2. Interaction between sites due to loop formation has been recognized by patterns of alternating DNase I-hypersensitive and -insensitive regions with a periodicity of about 10 bases reflecting the helical rotation (24). Loops are generally formed when the binding sites are separated by an integral number of helical turns (9, 30). The separation of the two purR operators is 88 nucleotides from dyad center to dyad center. This converts
FIG. 3. Concentration dependence of Pur repressor binding to operator DNA. This is a composite of two experiments. From one experiment with O₁O₂, DNA (Fig. 2A), the percentage of radioactivity recovered in each of the species is plotted: ○, free DNA; △, B₁; □, B₂. From a second experiment (Fig. 2C and D), the percentages of the two half-fragments found in the DNA-protein complex are plotted: ▲, EcoRI-NdeI fragment; ■, NdeI-HindIII fragment.

Effect of operator mutations on autoregulation of purR. The purR-lacZ fusion in pPR2002 was recreated in plasmids pPR2004, pPR2005, and pPR2006 with mutations in O₁, O₂, and O₁O₂, respectively. These plasmids were transformed into purR⁺ and purR strains, and β-galactosidase levels were measured from cells grown in the presence and absence of a repressing level of adenine (Table 2). Cells carrying pPR2005, O₁O₂⁻, showed somewhat less repression than that of the wild-type strain (1.9- to 2.0-fold versus 2.5- to 2.7-fold). Repression was further decreased in the strain carrying pPR2004, O₁⁻O₂. However, the 1.5- to 1.6-fold repression of purR-lacZ in this strain demonstrated the in vivo function of operator site O₂. The double operator mutant pPR2006 showed little or no repression. In each case, repression of purR-lacZ was essentially abolished in a purR strain, indicating that it is the Pur repressor that is mediating the response to adenine.

mRNA levels. To investigate whether repression of purR expression was by a transcriptional or posttranscriptional mechanism, we determined purR-lacZ mRNA levels in purR⁺ and purR strains. RNA was pulse-labeled with [³H]uridine, and purR-lacZ mRNA was determined by DNA-RNA hybridization. Values for mRNA synthesis, uncorrected for decay, are summarized in Table 2. The range of values for repression of mRNA synthesis was calculated by the method used for enzymes. These results demonstrate that repression of mRNA synthesis by the Pur repressor generally paralleled the repression of enzyme formation. Maximal repression of 1.6- to 2.2-fold was obtained in the wild type (O₁O₂), with marginally less repression from O₁O₂⁻ and O₁⁻O₂ purR-lacZ genes. These data demonstrate transcriptional regulation of purR by binding of the Pur repressor at operators O₁ and O₂. Although these results do not exclude low-level translational control, transcriptional regulation is sufficient to account for the modulation of enzyme production.

to 8.5 helical turns with a periodicity of 10.4 bp per turn. According to this analysis, repressors bound at operators O₁ and O₂ act independently of each other.

Operator mutations. The protein-DNA binding experiments indicate that the Pur repressor binds with a higher affinity to O₁ than to O₂, yet the position of O₂ within the coding sequence suggests that it has an in vivo role. To dissect the effects of each operator on in vivo regulation, mutations were made within each operator sequence. A comparison of pur regulon operator sequences from the genes purF, purMN, purL, purC, purEK, purHD, and purR (Fig. 5) revealed a number of invariant positions and highly conserved positions. Mutations were made in O₁ and O₂ to abolish repressor binding. The wild-type and mutant operators are shown in Fig. 1. Plasmid pPR2004 contains a three-base substitution in positions 9 through 11 of O₂: G104A, T105C, and T106A. This mutation changes one invariant position, G-9, and the two conserved T residues at positions 10 and 11 (operator numbering is from 1 to 16 from left to right). The T105C and T106A replacements mimic a purF operator-constitutive mutation (41). Plasmid pPR2005 contains a four-base mutation in positions 11 through 14 of O₂: T194C, T195A, C196G, and C197T. This change was designed to maintain the purR coding sequence at these positions yet alter two invariant positions: T-12 and C-14. Finally, a double mutant was constructed that contains replacements in both operators. Repressor binding assays with DNA fragments that contained single operator mutations demonstrated gel retardation to only a single position (data not shown). DNA fragments carrying mutations in either of the two operator sequences bound the repressor with identical affinity to the half-fragments reported above (data not shown). These results verify that each of the mutations eliminated the ability of the repressor to bind to mutant operator.
The results presented here demonstrate that the Pur repressor autogenously regulates expression of purR. Meng et al. (34) recently reported similar findings. This work supports their conclusions and extends our understanding of this autoregulation from studies of the isolated operators. Two species of DNA-protein complexes were detected in each study. We have identified the components of each of the complexes. At low repressor levels, the primary interaction with operator DNA occurs at O1 and causes a mobility shift of free DNA to B1. Thus, B1 is not a mixture of repressor-O1 and repressor-O2 complexes; B1 is largely repressor-O1 complex. Binding of additional repressor to B1 saturates O2 and results in a shift to B2. Our work demon-
FIG. 5. Comparison of the sequences from the pur regulon genes. In the consensus pur operator, uppercase letters refer to positions that are conserved in the operators with at least six of eight matches; lowercase letters refer to four or five of eight matches. n, Not conserved.

strates that interaction of the repressor with O₁ and O₂ is noncooperative. Binding of the repressor to either O₁ or O₂ in vitro is independent of the state of the second operator and is only dependent on protein concentration. In some cases, such as with lac O₁/O₂, loop formation only occurs in supercoiled DNA (see reference 16 for a review). Although we have not excluded the possibility that purR O₁/O₂ loops may form in vivo, the oligomeric state of the Pur repressor does not favor DNA looping. The Pur repressor has a molecular weight intermediate between the monomer and the dimer, suggestive of a monomer-dimer equilibrium (42a), in contrast to tetrameric lac repressor. Meng et al. (34) detected a third repressor-operator species by using a gel retardation assay. We did not identify a third species of reduced mobility, but we obtained smearing at high protein concentrations that was ascribed to protein-protein interaction or nonspecific protein-DNA binding. The footprint analysis (Fig. 4A and B) clearly shows that the Pur repressor preferentially saturates O₁ before O₂. These results extend our understanding of repressor-operator complex formation by showing different operator affinities for the repressor and demonstrate the noncooperative nature of in vitro binding.

The mutational analysis of operator function indicates an in vivo role for each operator. The full level of repression attained in the wild-type purR-lacZ fusion relies on both operators, not just O₁. This is in spite of the closer position of O₁ to the promoter and its greater affinity for the Pur repressor. The unique position of operator sequences relative to the promoter suggested the possibility of translational regulation. However, the close correspondence between the response of mRNA and enzyme to repression by purR+ and excess adenine provides evidence for transcriptional regulation. Although values for the lower repression of mRNA relative to enzyme (Table 2) leave open the possibility for some translational regulation, for the following reasons we favor the conclusion that most or all regulation is transcriptional. (i) Translational regulation should require some type of interaction between repressors bound to O₁ and O₂ or should require at least operator O₂, which is located in the coding sequence. However, we can find no evidence for an O₁-O₂ interaction in vitro or in vivo, nor can we find an absolute requirement for O₂. Substantial repression of purR-lacZ occurred in each of the mutants possessing only one operator. (ii) Small differences in RNA levels are difficult to quantitate. For this reason the somewhat lower values for repression of mRNA relative to enzyme are unlikely to be significant. The work of Meng et al. (34) was confined to autoregulation of wild-type purR in a lacZ transcriptional fusion and did not include measurements of mRNA.

The expression and regulation of the purR gene appear unique in two respects. First, the 5' end of the mRNA has been mapped and corresponds to a thymidine residue 155 nucleotides upstream of the ATG initiation site (42). Most E. coli transcripts begin with a purine nucleotide, usually an adenine (22). Sequences upstream of the mapped mRNA 5' end show poor homology with σ70 promoter consensus elements (22). Although there is a very good promoter consensus sequence located between nucleotides −50 and −85, corresponding mRNA 5' ends were not detected. Second, the location of the two purR operators is unusual. Both purR operators lie downstream of the promoter. Repressors that bind within the promoter are thought to function by preventing formation of closed or open transcription complexes (39). We propose that the large difference in repression of purF and purR by the Pur repressor reflects the location of operator sites. The Pur repressor regulates the expression of a purF-lacZ fusion by approximately 28-fold (41), compared with 2- to 3-fold for repression of purR-lacZ, notwithstanding that in vitro binding of the repressor to the two control regions is indistinguishable. We conclude that the mechanism for repression of purR expression is much less efficient than that for purF. Repression of purR may occur by inhibition of transcription elongation rather than at the step of transcription initiation as in purF. The advantages to the cell of this mechanism of repression of purR are not presently understood.

There are several examples for multiple operator sites in gene regulation (for reviews, see references 16 and 38). There are two operators in both the lac and gal operons. In these cases one operator site is upstream of (8) or overlapping (13) the site for transcription initiation, and the second is in the protein-coding region (12, 27, 40); repressor binding is cooperative, and DNA loops are formed (18, 26, 30). Gralla (reviewed in reference 16) has proposed two roles for the lac O₂ operator element within the lacZ gene. It contributes to repression of transcription initiation by enhancing the interaction of the repressor bound to O₂ via loop formation and also is thought to block elongation of lac transcription that has escaped repression at O₁. Alternative loop formation also plays a role in the regulation of araBAD and araC expression by the AraC regulatory protein (9, 20). AraC protein bound to araO₂ operator located within araC can interact with AraC-bound either at araO₁ or araO (20). Both of these latter operators are located in or near the promoters P₅ and P₁₆. Single and double loop formation has also been suggested to explain the regulation of the deo operon by DeoR (7, 19). In these examples, repressors bind to an operator site in or near the promoter, and binding to a second operator is stabilized through loop formation (16). Of the four bacterial repressors that utilize two operator sites, only araC is autoregulated; galR, lacI, and deoR are not. Gene trpR is autoregulated by the Trp repressor, which binds to an operator site overlapping the −10 and +1 regions of the promoter (3, 17, 28). The regulation of purR by the Pur repressor is distinct from these examples in both operator location and in lack of loop formation. The regulation of purR expression appears to be the first example of autoregulation that occurs by binding to two operator sequences, both downstream of the promoter.

The Pur repressor is now known to regulate a number of genes or operons involved in de novo synthesis of purine and pyrimidine nucleotides. These include purF, purMN, purL, purC, purHD, purEK, guaBA, pyrC, and pyrD (5, 23, 34, 52, 53). In each case, except for guaBA and pyrD, the Pur repressor has been shown to interact with a single operator.
site located in the promoter-transcription initiation region. Binding of the Pur repressor to guaBA and purD control sites has not been reported. Of all the genes thus far known to be regulated by the Pur repressor, only purR employs a two-operator mechanism.

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