Double Negative and Positive Control of tsx Expression in Escherichia coli

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The Escherichia coli tsx gene encodes an outer membrane protein that is involved in nucleoside uptake and serves as the receptor protein for colicin K and several bacteriophages. Regulation of its expression was studied by using tsx-lacZ protein and operon fusion strains carrying mutations in deoR, cytR, and crp. The cyrR-encoded repressor had a stronger influence on tsx transcription than the DeoR repressor did, and the level of tsx expression in a deoR cytR double mutant was approximately the sum of those found in the single deoR and cytR strains. This double negative control of Tsx synthesis was superceded by a positive control mechanism mediated by the cyclic AMP-catabolite activator protein (cAMP-CAP) complex. Our results suggest that tsx expression is controlled at two separate and differently regulated promoters: the weaker promoter (P_r) is repressible by DeoR, while the stronger promoter (P_s) is subject to negative and positive control by the CytR repressor and the cAMP-CAP complex, respectively. A mutant that showed unaltered tsx regulation by DeoR and the cAMP-CAP complex but strongly reduced repression by CytR. This tsx operator mutant was used to obtain a suppressor mutation located on a plasmid carrying the cloned cytR gene that restored CytR control of tsx expression. The direction of tsx transcription was determined and found to be counterclockwise on the E. coli chromosome.

The Tsx protein is a minor component of the outer membrane of Escherichia coli, with an apparent molecular weight of 28,000 (22). It acts as the receptor protein for colicin K and several bacteriophages, including phage T6 (23, 38). The Tsx protein is involved in the permeation of nucleosides across the outer membrane. This was first demonstrated by Hantke (16), who found that strains lacking Tsx were impaired in nucleoside uptake. Further analysis of this phenomenon revealed a remarkable specificity in the Tsx-mediated permeation of nucleosides across the outer membrane (19, 20, 27). The rate of uptake of adenosine and thymidine is strongly reduced in the absence of Tsx, whereas that of cytidine remains unchanged. The importance of the Tsx protein for nucleoside uptake becomes apparent only at low (<1 µM) substrate concentration or under conditions of fast cell growth (19, 27). At higher concentration of substrate, the Tsx protein becomes dispensable and the nucleosides permeate the outer membrane by diffusion through the non-specific porins OmpC and OmpF (28, 37, 39). These and other studies (18) led to the suggestion that Tsx is a pore-forming protein with some specificity for nucleosides and deoxynucleosides. Recently, we reconstituted purified Tsx protein in vitro into black lipid membranes (C. Maier, E. Bremer, A. Schmid, and R. Benz, J. Biol. Chem., in press). These experiments demonstrated that Tsx can form a pore in vitro. They also provided strong evidence that this protein contains a nucleoside-specific binding site that has a high affinity for adenosine and a low affinity for cytidine. Thus, the Tsx protein appears to be functionally comparable to another pore-forming outer membrane protein, LamB, which specifically facilitates the permeation of maltose and maltodextrins and contains a substrate-binding site (5, 30).

The involvement of Tsx in nucleoside uptake is reflected in the regulation of its structural gene, tsx, which is located at 9 min on the E. coli linkage map (2). As first demonstrated by Krieger-Brauer and Braun (19), expression of the tsx gene is under double negative control of the DeoR and CytR proteins, which are known to regulate the synthesis of two nucleoside transport systems (NupC and NupG) and several nucleoside-catabolizing enzymes (for reviews, see references 14 and 26). In addition, the cyclic AMP-catabolite activator protein (cAMP-CAP) complex (11) has been shown to positively affect the transcription of the tsx gene (1, 20, 24). However, the interrelationship of the double negative (DeoR; CytR) and positive (cAMP-CAP) control in tsx expression has not been analyzed to any great extent. Furthermore, it has been difficult to quantitate the effects of these regulatory proteins on Tsx synthesis.

To gain a deeper insight into the complex regulation of the tsx gene, we have studied Tsx synthesis in an isogenic set of strains with mutations in deoR, cytR, and crp. We have monitored and quantitated tsx expression by using tsx-lacZ protein and operon fusions and have isolated a strain with a mutation in the tsx regulatory region that reduces the binding of the CytR repressor.

MATERIALS AND METHODS

Media and chemicals. Bacteria were grown aerobically in a shaking incubator (28 or 37°C, as indicated) in LB medium (10 g of tryptone [Difco Laboratories] per liter, 5 g of yeast extract [Difco] per liter, 5 g of NaCl per liter) or in minimal medium A (MMA; 10.5 g of K_2HPO_4 per liter, 4.5 g of KH_2PO_4 per liter, 1 g of (NH_4)_2SO_4 per liter, 0.5 g of hydrated sodium citrate per liter, 0.1 g of MgSO_4·7H_2O per liter) supplemented with a carbon source (glucose or glycerol) at 0.4%. The medium was supplemented with a solution of sodium valine, and isoleucine to a final concentration of 0.02% as required. MacConkey and tetrazolium media were prepared as previously described (25, 29). Agar plates spread with 0.1 ml of a 10-mg/ml solution of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in dimethylformamide were used to distinguish between LacZ− and LacZ+ phenotypes. The indicator dye 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.
phosphate (XP) was used in a similar manner to detect colonies synthesizing high levels of alkaline phosphatase.

Strains, phages, and genetic procedures. The bacteria and phages used are described in Table 1. All strains were E. coli K-12 derivatives. Standard techniques were used for the growth and titration of bacteria and bacteriophages and for generalized transduction with phage P1 (25, 29). Lysoys of phage Mu were obtained by host induction of a Mu cI857 lysogen (29), and immunity to superinfecting Mu phages was tested as described previously (7). The procedure for the induction of a λ placMu prophage by UV irradiation has been reported previously (29).

Construction of bacterial strains. The Δ(arg-F-lac) U169 mutation was transduced with phage P1 into strains SO656 (deoR* cytR*), SO664 (deoR* cytR9), SO665 (deoR8 cytR*), and SO774 (deoR8 cytR9) by using a phage lysate prepared on strain ECB457 [proC::Tn10 Δ(arg-F-lac)-U169]. One tetracycline-resistant LacZ− colony from each group of transductants was purified on LB agar containing tetracycline (20 μg/ml), and the proC::Tn10 insertion was subsequently removed from these strains by P1 transduction with a P1 lysate containing the proC::Δ(arg-F-lac)-U169 strain MC4100. Strains SO656, SO664, SO665, and SO744 (15) were found to be resistant to λ vir. This phenotype must result from a lamB mutation, because we found that these strains are Mal+. To remove this lamB mutation from the Δlac derivatives, the malF::Tn10 insertion from strain TST7 (λamB+) was first introduced with phage P1. Then the malF::Tn10 insertion was removed from λ vir* (λamB+) colonies by transduction to Mal− with a P1 phage lysate prepared on strain MC4100. These steps yielded strains BRE2047 (deoR* cytR*), BRE2048 (deoR* cytR9), BRE2049 (deoR8 cytR*), and BRE2050 (deoR8 cytR9). The presence of the deoR and cytR mutations was verified by streaking these strains on minimal plates with inosine or uridine as the sole carbon source (14). The Δ(crp)96 mutation present in strain BD18 (Table 1) was transduced with phage P1 into various strains by selecting for the closely linked zhd-732::Tn10 insertion and then testing the tetracycline-resistant transductants for a Mal− phenotype by streaking on maltose MacConkey plates.

Isolation of the tsx-lacZ fusion. We used the transposable λ placMu phages (8) to construct tsx-lacZ protein and operon fusions. Strains were infected with λ placMu9 (lacZ protein fusions) and λ placMu55 (lacZ operon fusions) in the presence of the λ pmu07 helper phage, and kanamycin-resistant colonies were selected as previously described (8). Approximately 10,000 colonies each were pooled, washed three times with 40 ml of LB medium containing 40 mM sodium citrate, and suspended in 5 ml of LB medium. To select lacZ fusions to the tsx gene, we prepared serial dilutions from the pooled kanamycin-resistant cells (10−1 to 10−8) in LB medium and plated them onto lawns of phage T6 (0.2 ml of a 1011 PFU/ml lysate) on LB agar containing kanamycin and X-Gal. Nonmuroid LacZ− colonies were purified twice and restested for their resistance to phage T6. To confirm that the LacZ+ and T6+/ phenotype were due to λ placMu insertion into the tsx gene, we prepared a P1 lysate on such strains. These lysates were used to transduce strain BRE2047 to kanamycin resistance, and the resulting Lac− transductants were tested for T6 resistance. We retained one tsx-lacZ prophage and one tsx-lacZ operon fusion strain. The λ placMu9 and λ placMu55 insertions were originally isolated in strains MC4100 and BRE2047, respectively.

β-Galactosidase assay. Specific β-galactosidase activity was assayed as described previously (25) and expressed as micromoles of substrate cleaved per minute per milligram of protein. Protein concentration was estimated from the optical density at 600 nm of the culture, assuming that an optical density at 600 nm of 1.4 corresponds to approximately 150 μg of protein per ml of culture (25).

Preparation of proteins samples, gel electrophoresis, and immunological detection of a Tsx-β-galactosidase hybrid protein. Cell envelopes were prepared from 40-ml overnight cultures grown in LB medium as described by Henning et al. (17). The membrane proteins were solubilized in sample buffer containing 2% sodium dodecyl sulfate (SDS) by boiling for 10 min, and proteins were electrophoresed on an SDS-polyacrylamide gel (12% polyacrylamide) by the method of Lugtenberg et al. (21). Protein bands were visualized by staining with Coomassie brilliant blue. To detect the hybrid protein synthesized in the Φtsx-lacZ/Hyb

### Table 1. Bacterial strains, bacteriophages, and plasmids

| Strain, phage, or plasmid | Relevant genotype* | Source or reference |
|--------------------------|-------------------|---------------------|
| Strains                 |                   |                     |
| MC4100                  | F' araD19 Δ(arg-F-lac) U169 | 10                  |
|                         | rpsL30 relA deoC1 psl25 |                     |
|                         | rbsR ffbB5301      |                     |
| ECB457                  | MC4100 proC::Tn10  | S. Benson           |
| TST7                    | MC4100 malF::Tn10  | T. I. Silhavy       |
| K797                    | phoR::Tn10         | M. Villarejo        |
| RM1036                  | F' traD36 proAB lacI Δlac M15(Δlac-pro) thi rpsL supE endA sbcB r m" matD5 zaf-13::Tn10 | R. Maurer through P. Göt
| BD18                    | HfrH rpsL thi Δcya-851 Δcrp-96 zhd-732::Tn10 | 4                  |
| SO656                   | F' metB ilv rpsL lamB | 15                   |
| SO664                   | F' metB ilv rpsL lamB cytR9 | 15               |
| SO665                   | F' metB ilv rpsL lamB deoR8 | 15     |
| SO744                   | F' metB ilv rpsL lamB cytR9 deoR8 | 15 |
| BRE2047                 | SO656 lamB+ Δ(arg-F-lac) U169 | This study          |
| BRE2048                 | SO664 lamB+ Δ(arg-F-lac) U169 | This study          |
| BRE2049                 | SO665 lamB+ Δ(arg-F-lac) U169 | This study          |
| BRE2050                 | SO744 lamB+ Δ(arg-F-lac) U169 | This study          |
| BRE2055                 | BRE2047 Δ(crp)96 zhd-732::Tn10 | This study          |
| BRE2056                 | BRE2048 Δ(crp)96 zhd-732::Tn10 | This study          |
| BRE2057                 | BRE2049 Δ(crp)96 zhd-732::Tn10 | This study          |
| BRE2058                 | BRE2050 Δ(crp)96 zhd-732::Tn10 | This study          |
| BRE2101                 | BRE2047 phoR::Tn10 | This study          |
| Bacteriophages          |                   |                     |
| λ placMu9               | Mu cI562 ner+ A' ara' Mu S' lacZ lacI- Y lacA+ kan Imm | 8                  |
| λ placMu55             | Mu cI562 ner+ A' amI103 E. bremer 'uvrD' Mu S' trip lacZ lacI- Y lacA+ kan Imm | 15     |
| λ pmu07                 | Mu cI562 ner+ A' B' dts857 | 8                  |
| Plasmids                |                   |                     |
| pBR322                  |                   |                     |
| pCB008                  |                   |                     |
| pGP 1'                  |                   |                     |

* Genes or sequences marked with a prime are incomplete. The genetic nomenclature is that of to Bachmann (2).  
* This phage is a derivative of λ placMu53 (8). It carries the Mu Aaml103 mutation, which prevents secondary transposition.  
* This plasmid is a derivative of pCB008 obtained after mutD mutagenesis.
fusid strain GP4, we prepared total cellular proteins (29) from a 5-ml overnight culture grown in LB medium. These were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (12% acrylamide) and stained with Coomassie brilliant blue. A high-molecular-weight calibration kit (Sigma Chemical Co.) was used to estimate the molecular weight of the hybrid protein. For the immunological detection of the Tsx–β-galactosidase hybrid protein, we transferred the separated cell proteins electrophoretically onto a sheet of nitrocellulose and probed the bound proteins with a rabbit antibody raised against β-galactosidase. The formed antigen-antibody complex was visualized with a second goat anti-rabbit immunoglobulin G peroxidase coupled antibody (33).

RESULTS

Negative and positive regulation of tsx expression. To study the expression of the tsx gene of E. coli, we used an isogenic set of strains (kindly provided by A. Munch-Petersen) carrying mutations in the deoR and cytR regulatory genes. These strains were made lamR+ and Δlac (see Materials and Methods) (Table 1), so that they could also be used for isolating tsx-lacZ protein and operon fusions with the λ placMu system (8). Figure 1 shows the pattern of cell envelope proteins of this set of strains after electrophoretic separation on an SDS-polyacrylamide gel (12% polyacrylamide). The DeoR protein exerts only a weak negative effect on Tsx synthesis, whereas CytR represses tsx expression more strongly. The amount of Tsx was greatest in the deoR-cytR double mutant. Our results thus support an earlier report by Krieger-Brauer and Braun (19), who concluded that synthesis of the Tsx protein increased in the following sequence: wild-type strain < deoR < cytR < deoR cytR mutants.

FIG. 1. Synthesis of Tsx protein in response to deoR and cytR mutations. Cell envelope proteins of LB medium overnight cultures of strains BRE2054 (deoR cytR tsx::Tn10) (lane 1), BRE2050 (deoR cytR) (lane 2), BRE2048 (cytR) (lane 3), BRE2049 (deoR) (lane 4), and BRE2047 (deoR cytR) (lane 5) were separated by SDS-PAGE (12% acrylamide). The positions of the outer membrane proteins OmpC, OmpF, OmpA, and Tsx are indicated by arrows. Note that the Tsx protein migrates above the heavy band that represents several unresolved polypeptides (see Fig. 2). The gel system used does not resolve the OmpC and OmpF proteins; the gel was stained with Coomassie blue.

FIG. 2. Effect of the Δ(crp)96 mutation on Tsx synthesis. Cell envelope proteins of LB medium overnight cultures of strains BRE2047 (deoR+ cytR+ crp+) (lane 1), BRE2055 [deoR+ cytR+ Δ(crp)96] (lane 2), BRE2050 (deoR cytR crp+) (lane 3), and BRE2058 [deoR cytR Δ(crp)96] (lane 4) were separated by SDS-PAGE (12% acrylamide), and the gel was stained with Coomassie blue.

It is well established that tsx expression is subject to catabolite repression (1, 20, 24), but it is unknown how this positive regulatory mechanism interacts with DeoR- and CytR-mediated regulation. To investigate such interaction, we introduced with phage P1 the Δ(crp)96 mutation into the four strains described above and analyzed the profile of cell envelope proteins by SDS-PAGE. No Tsx protein could be clearly detected in wild-type or deoR-cytR double-mutant strains carrying the Δ(crp)96 mutation (Fig. 2, lanes 2 and 4); the same result was obtained when this mutation was present in either the deoR or cytR single mutant (data not shown). Thus, the positive regulation of tsx expression mediated by the cAMP-CAP complex has a stronger effect than the negative regulation by the DeoR and CytR proteins. Although the Tsx protein was not detected in the above experiment, tsx expression was not completely abolished in the Δ(crp)96 mutants, since these strains were still sensitive to the Tsx-specific bacteriophage T6. From these data we conclude that Tsx synthesis is subject to double negative control by the DeoR and CytR repressors, but is most strongly affected by positive regulation through the cAMP-CAP complex.

Isolation of tsx-lacZ protein and operon fusions. In a wild-type strain, the Tsx protein is a relatively minor component of the outer membrane proteins. Additionally, on SDS-polyacrylamide gels it migrates to an area densely populated with other proteins (Fig. 1 and 2), making it difficult to quantitate changes in Tsx synthesis. To obtain a more sensitive tool to monitor tsx expression, we isolated tsx-lacZ protein and operon fusions. Such fusions were selected from pools of kanamycin-resistant insertions of λ placMu9 (lacZ protein fusion) and λ placMu55 (lacZ operon fusion) on the basis of their resistance to phage T6. One tsx-lacZ protein fusion strain and one tsx-lacZ operon fusion strain were subjected to the following tests to confirm the fusion of the tsx and lacZ genes. (i) The λ placMu insertions were transduced with phage P1 into strain BRE2047 by selecting for kanamycin-resistant colonies in the presence of X-Gal. LacZ′ Kan′ transductants were then tested by cross-
streaking against phage T6 and λ vir. All transductants tested were resistant to phage T6 but sensitive to λ vir. (ii) A tsx::Tn10 insertion was transduced with phage P1 into both fusion strains by selecting for tetracycline resistance in the presence of X-Gal. All transductants examined were Lac- KanR.

(iii) Cell envelope proteins from both fusion strains were analyzed by SDS-PAGE, and no Tsx protein was detected. This is shown in Fig. 3A (lane 2) for the Φ(tsx-lacZ)(Hyb) fusion strain GP4 (deor cytR) in comparison with its parent strain BRE2050 and strain BRE2054, a tsx::Tn10 control. Taken together, these tests provide proof that tsx-lacZ protein and operon fusion strains had been isolated.

The Φ(tsx-lacZ)(Hyb) protein fusion present in strain GP4 should encode a hybrid protein whose size depends on the insertion point of the λ placMu9 phage into tsx. This hybrid protein was detected in total cell extracts electrophoretically separated on an SDS-polyacrylamide gel (7% polyacrylamide) (data not shown) and reacted with an antibody directed against β-galactosidase (Fig. 3B, lane 1). It is slightly larger than β-galactosidase (Fig. 3B, lane 3), and we estimate its size to be approximately 120,000 daltons. Since the portion of such hybrid proteins encoded by the incomplete lacZ gene and the 117 base pairs from the S end of Mu is constant (approximately 117,000 daltons) (8), the fusion joint probably lies early in the tsx gene. It should be noted, however, that for large proteins, molecular size estimates based on SDS-PAGE are not precise.

**Regulation of tsx-lacZ expression by DeoR, CytR, and the cAMP-CAP complex.** To quantitate the influence of the various regulatory proteins on tsx expression, we transduced with phage P1 the Φ(tsx-lacZ)(Hyb) protein fusion and Φ(tsx-lacZ)2 operon fusion into an isogenic set of strains, carrying, in various combinations, mutations in the deoR, cytR, and crp genes (Table 2). When these strains were grown in minimal medium with glycerol as the carbon source, both the tsx-lacZ protein fusion and operon fusion showed the same regulatory pattern, as determined by β-galactosidase activity. Expression of the fused genes increased in the following order: wild-type < deoR < cytR < deoR cytR double mutant, where the absence of the DeoR repressor increased tsx-lacZ expression about 1.5- to 2-fold and the absence of CytR resulted in 6-fold-enhanced β-galactosidase activity. The level of β-galactosidase activity found in the deoR cytR strains GP4 and BRE2094 is approximately the sum of that observed in the single deoR (GP3 and BRE2093) and cytR (GP2 and BRE2092) mutants. Thus, the extent of the DeoR- and CytR-mediated repression of tsx expression appears to be additive.

To analyze the influence of the cAMP-CAP complex on tsx expression, we grew the various fusion strains in MMA containing glucose or glycerol and compared the β-galactosidase activities (Table 2). The deoR and cytR strains differed in their response to glucose in the growth medium. A strong reduction of tsx-lacZ expression in glucose-grown cultures could be measured only in strains carrying a cytR mutation, whereas the wild-type and deoR mutant strains showed only slightly reduced β-galactosidase activity. But, the high levels of β-galactosidase activity found in glycerol-grown cytR strains must be due to the activation of tsx transcription by the cAMP-CAP complex, which is prevented when the CytR repressor is present. In contrast, the DeoR-mediated regulation of tsx appears to be independent of the cAMP-CAP activator complex, since in deoR

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**TABLE 2. Effects of mutations in deoR, cytR, and crp on tsx-lacZ expression**

| Strain | Relevant genotype | β-Galactosidase sp act (μmol/min per mg of protein) |
|--------|------------------|-----------------------------------------------|
|        |                   | MMA with glucose | MMA with glycerol |
| GP1    | +                | 0.8                       | 0.8 |
| GP2    | +                | 5.4                       | 1.1 |
| GP3    | +                | 1.3                       | 1.1 |
| GP4    | +                | 6.6                       | 1.6 |
| GP5    | +                | 1.3                       | 0.5 |
| GP6    | +                | 0.9                       | 0.2 |
| GP8    | +                | 0.6                       | 0.4 |
| BRE2091| +               | 0.9                       | 0.7 |
| BRE2092| +               | 6.1                       | 1.7 |
| BRE2093| -               | 2.0                       | 1.6 |
| BRE2094| -               | 10.5                      | 3.3 |
| BRE2095| +               | 0.5                       | 0.2 |
| BRE2096| +               | 0.2                       | 0.9 |
| BRE2097| -               | 0.6                       | 0.5 |
| BRE2098| -               | 0.4                       | 0.5 |

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a Strains GP1 through GP8 carry the Φ(tsx-lacZ)(Hyb) protein fusion as a single copy in the chromosome. Strains BRE2091 through BRE2098 carry the Φ(tsx-lacZ)-2 operon fusion as a single copy in the chromosome. The symbol Φ indicates the presence of a lacZ fusion, and the abbreviation Hyb indicates that the fusion encodes a hybrid protein. The symbols lacZ+ and lacZ- denote lacZ genes with or without translational initiation signals, respectively.

b Symbols: +, intact chromosomally encoded deoR, cytR, and crp genes; -, mutated chromosomally encoded deoR, cytR, and crp genes.

c The strains were grown overnight in MMA with 0.4% glycerol or 0.4% glucose as the carbon source. Samples were withdrawn, and the specific β-galactosidase activity was determined (25). A parent strain (BRE2047) was always included as a control. The data shown are the mean values from two to three independent experiments.

d –, Strains with a mutation in crp do not grow on glycerol as the sole carbon source.
strains, tss-lacZ expression is increased to about the same extent regardless of the growth medium (Table 2). In agreement with the strongly reduced amount of Tss present in strains with the Δ(crp)96 mutation (Fig. 2), we found a further reduction of tss-lacZ expression in glucose-grown cells when the Δ(crp)96 mutation was present (Table 2). From these data, we conclude that Tss synthesis is controlled from two differently regulated promoters. The weaker promoter (P1) is repressible by DeoR, while the main promoter (P2) is subject to negative and positive control by CytR and the cAMP-CAP complex, respectively.

A CytR operator mutant in the hybrid tss-lacZ gene. Plasmid pCB008 carrying the cytR+ gene (3) strongly repressed the expression of the Φ(tss-lacZ)(Hyb) protein fusion regardless of the presence of the chromosomally encoded DeoR and CytR repressors (Table 3). Consequently, strain GP4 (deoR cytR9) harboring pCB008 formed only small colonies on lactose minimal plates. We took advantage of this weak tss-lacZ expression to obtain mutants with an altered response to CytR. Strain GP4(pCB008) (Table 3) was streaked on lactose minimal plates with ampicillin, and fast-growing colonies were isolated. Increased tss-lacZ expression in these strains could result from mutations in the cloned cytR+ gene that reduced or abolished CytR synthesis. Alternatively, this strong Lac+ phenotype could be due to a mutation in the tss regulatory region that prevents repression by CytR. To distinguish between these possibilities, we streaked the isolated mutant strains on minimal plates containing uridine as the sole carbon source. Efficient growth on uridine requires the expression of the CytR-controlled udp gene and the deo operon (14). Consequently, the isolated Lac+ mutants will form normal-size colonies on uridine minimal plates only if they carry mutations altering the expression of the cloned cytR+ gene or the function of the gene product. In contrast, strains with mutations in the tss regulatory region will show weak growth on these plates. Most (18 of 21) of the independently isolated Lac+ mutants grew well on uridine plates. The remaining strain (GP28) formed only very small colonies, indicating that it contained a mutation affecting the repression of the Φ(tss-lacZ)(Hyb) fusion by CytR.

The mutation in strain GP28 was shown by P1 transduction to be closely linked to the Φ(tss-lacZ)(Hyb) fusion. When kanamycin-resistant transductants in strain BRE2047 were (deoR cytR+) selected, all 10 transductants tested were still resistant to phage T6. They showed a much higher expression of the gene fusion on lactose MacConkey indicator plates than did the isogenic strain GP1, which carries the wild-type Φ(tss-lacZ)(Hyb) fusion. To analyze the regulation of the mutated fusion by the chromosomally encoded DeoR and CytR repressors, we transformed this fusion into deoR and cytR strains and subsequently measured β-galactosidase activity in glycerol- and glucose-grown cultures (Table 3). In glycerol minimal medium, the expression of the mutated Φ(tss-lacZ)(Hyb) fusion in GP25 (deoR+ cytR+) was about fivefold stronger than in the control strain, GP1 (Table 3). In a deoR strain, expression of the mutated and the wild-type fusion increased to the same extent: 1.2-fold and 1.3-fold, respectively. This indicated that the mutation did not affect DeoR-mediated repression of tss. However, the response to CytR was different in the mutant: a cytR mutation resulted only in a 1.3-fold increase in the expression of the fusion, whereas the corresponding value for the wild-type fusion was 6.6-fold (Table 3). This shows that in the isolated mutant the repression of tss-lacZ expression by CytR was strongly reduced but not completely abolished.

An interesting effect of the mutation is seen when the β-galactosidase values of glycerol- and glucose-grown cultures are compared (Table 3). In strains with the wild-type Φ(tss-lacZ)(Hyb) fusion, a significant glucose-mediated repression of gene expression was observed only when a cytR mutation is present. In contrast, in strains carrying the mutation, reduced expression of the fusion was also found in cytR+ strains and even in the presence of plasmid pCB008. This implies that the presence of the CytR repressor prevents activation of transcription initiation at P2 by the cAMP-CAP complex. Thus, the regulatory pattern of tss-lacZ expression in the mutant strain is exactly that predicted from the dual control of the P2 promoter by CytR and the cAMP-CAP complex (Table 2). Taken together, the properties of this mutant strain strongly suggest that it carries an alteration in the CytR operator region that reduces CytR binding to the tss P2 promoter. We have called this mutation O'-1.

Suppression of the operator mutation by a mutated cytR gene. Plasmid pCB008 was mutagenized in vivo by transformation into the mutD5 strain RM1036; transformants were grown overnight in LB medium (29). Isolated plasmid DNA was then retransformed into strain GP28 (deoR cytR) carrying the Φ(tss-lacZ)(Hyb) fusion with the O'-1 operator mutation. We looked for suppressors of this mutation by selecting the transformants on lactose plates containing ampicillin and screening the transformants for a Lac+ phenotype. Such Lac+ colonies can result from either a reversion of the O'-1 mutation or an alteration in the cytR+ gene that suppresses the O'-1 Lac+ phenotype. Several

**Table 3. Repression of tss expression by the cloned cytR+ gene and altered response of a tss-lacZ mutant (O'-1) to the CytR repressor**

| Strain | cytR+ plasmida | Chromosomal repressorb | β-Galactosidase sp act (µmol/min per mg of protein) after growth in c | MMA with glycerol | MMA with glucose |
|--------|----------------|------------------------|----------------------------------------------------------|------------------|------------------|
| GP1    | −              | +                      | 0.7                                                      | 0.9              |
| GP1    | +              | −                      | 0.1                                                      | 0.2              |
| GP2    | −              | +                      | 4.8                                                      | 2.2              |
| GP2    | +              | −                      | 0.3                                                      | 0.3              |
| GP3    | +              | −                      | 1.0                                                      | 0.9              |
| GP3    | −              | +                      | 0.9                                                      | 0.1              |
| GP4    | −              | +                      | 6.3                                                      | 1.7              |
| GP4    | +              | −                      | 0.4                                                      | 0.4              |
| GP25   | −              | −                      | 4.2                                                      | 1.4              |
| GP25   | +              | +                      | 2.3                                                      | 1.1              |
| GP26   | −              | −                      | 5.5                                                      | 1.1              |
| GP26   | +              | −                      | 2.1                                                      | 1.2              |
| GP27   | −              | −                      | 5.2                                                      | 1.0              |
| GP27   | +              | −                      | 4.0                                                      | 1.9              |
| GP28   | −              | +                      | 7.0                                                      | 2.6              |
| GP28   | +              | −                      | 4.7                                                      | 2.0              |

a Strains GP1 through GP4 carry the wild-type Φ(tss-lacZ)(Hyb) fusion as a single copy in the chromosome. Strains GP25 through GP28 carry the Φ(tss-lacZ)(Hyb) fusion with mutation O'-1 as a single copy in the chromosome.

b The cytR+ plasmid used was pCB008 (3); the other strains contained plasmid pBR322 (6) as a control. Symbols: +, presence of pCB008; −, absence of pCB008.

c Symbols: +, presence of intact chromosomally encoded deoR and cytR genes; −, presence of mutated chromosomally encoded deoR and cytR genes.

d The strains were grown overnight with 0.4% glycerol or 0.4% glucose as the carbon source. Samples were withdrawn, and the specific β-galactosidase activity was determined (25). The values shown are the mean values from two independent experiments.
Lac^- colonies were found in this experiment, and one of these carried a mutated pCB008 plasmid. This was ascertained by retransforming this plasmid (pGP1) into strain GP28. This plasmid most probably encodes an altered cytR gene (cytR*), since the expression of pGP1 strongly reduced the expression of the fusion in strain GP28 (Table 4). The cytR* mutation is not allele specific, since the expression of the wild-type tsx-lacZ fusion in strain GP4 is also repressible by pGP1 (Table 4). Both pCB008 and pGP1 showed the same restriction pattern after digestion with Clal, AvaI, and PvuII, indicating that no gross rearrangement had occurred in pGP1. We did not notice an increase in the amount of plasmid DNA isolated from strain GP28(pGP1) in comparison with strain GP28(pCB008), suggesting that the suppressor phenotype was not due to a mutation drastically increasing the copy number of pGP1 and thereby the amount of the CytR protein.

Direction of tsx transcription in the E. coli chromosome.

The tightly linked phoB, phoR, and tsx genes are located at 9 min on the E. coli linkage map (2), with about 15 kilobases separating tsx from the phoB-phoR operon (31). The tsx gene is distal to the phoB-phoR operon, and phoB is known to be transcribed in a clockwise direction (2). These data enabled us to use the Φ(tsx-lacZ)(Hyb) protein fusion to determine the direction of tsx transcription in the E. coli chromosome. The λ placMu9 prophage inserted into the tsx gene is genetically marked at both ends: at one end there is the Φ(tsx-lacZ)(Hyb) protein fusion, which confers a Lac^- phenotype, and the other end carries the Mu clts62 gene, whose gene product confers a temperature-sensitive immunity to superinfecting Mu phages (7). Depending on the direction of tsx transcription, the λ placMu9 prophage is inserted in one of two possible orientations relative to phoR (Fig. 4a and c). Upon UV irradiation, the λ placMu prophage can excise from the chromosome with variable endpoints, giving rise to specialized lambda transducing phages that can carry bacterial genes located near the original λ placMu insertion (8). We reasoned that it should be possible to isolate a specialized lambda transducing phage that carries the phoR+ gene and only one of the genetic markers flanking the original λ placMu9 insertion in tsx. The phenotypes of a lysogen harboring such a phoR+ transducing phage would then unambiguously indicate the direction of tsx transcription. If transcription is clockwise (Fig. 4a), the lysogen will be Lac^- and immune to phage Mu (Fig. 4b); if transcription is counterclockwise (Fig. 4c), the lysogen will be Lac^- and immune to phage Mu (Fig. 4d).

We isolated a collection of lambda specialized transducing phages by UV irradiation of strain GP1 [phoR+ Φ(tsx-lacZ)(Hyb)] and used this lysate to transduce strain

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**TABLE 4. Suppression of the operator mutation O^-1 by a mutated cytR gene**

| Strain* | Plasmid present | β-Galactosidase sp act (μmol/min per mg of protein) after growth in** |
|---------|-----------------|-------------------------------------------------------------|
|         |                  | MMA with glycerol | MMA with glucose |
| GP28    | pBR322           | 7.1               | 3.1               |
| GP28    | pCB008 (cytR*)  | 4.7               | 2.0               |
| GP28    | pGP1 (cytR*)    | 0.5               | 0.5               |
| GP4     | pBR322           | 6.1               | 1.2               |
| GP4     | pCB008 (cytR*)  | 0.2               | 0.1               |
| GP4     | pGP1 (cytR*)    | 0.1               | 0.07              |

* The chromosomal background of the strains was deoR cytR. Strain GP28 carries the wild-type (tsx-lacZ)(Hyb) fusion with the mutation O^-1 as a single copy in the chromosome; strain GP4 carries the Φ(tsx-lacZ)(Hyb) fusion without the mutation.

** The strains were grown overnight with 0.4% glycerol or 0.4% glucose as the carbon source in the presence of ampicillin (50 μg/ml). Samples were withdrawn, and the specific β-galactosidase activity was determined (25). The data shown are the mean values from two independent experiments.

* Plasmid pGP1 was isolated after in vivo mutagenesis in the mutD5 mutator strain RM1036.

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**FIG. 4. Direction of the tsx transcription in the E. coli chromosome.** The physical structure of the λ placMu9 insertion in the tsx gene is shown in the two possible orientations (clockwise [line a] and counterclockwise [line c]) relative to the phoB phoR operon. Lines b and d depict two possible excision events of the lambda prophage after UV induction. Line e indicates the determined orientation of tsx transcription. Segments from phage Mu are represented by the black boxes and flank the λ prophage positioned next to the Φ(tsx-lacZ)(Hyb) protein fusion. The right end Mu attachment site is designated S. The Mu cl gene of λ placMu9 carries the clts62 allele, which encodes a temperature-sensitive Mu repressor protein. The orientation of the λ prophage is shown by the position of the genes A, R, J, and the imm region. The direction of transcription of a particular gene is shown by an arrow, and its promoter region is designated (P). A prime indicates that a particular gene is not completely present or is interrupted by other DNA sequences. The figure is not drawn to scale.
BRE2101 (phoR::Tn10Δlac) to kanamycin resistance in the presence of XP. Strains carrying mutations in phoR produce alkaline phosphatase constitutively (32) and therefore form dark-blue colonies when plated with the indicator dye XP, whereas phoR+ strains form white colonies. We used this phenotype to identify lambda specialized transducing phages carrying the phoR+ gene. Of approximately 1,200 transductants inspected, 2 PhoR+ colonies were found. Both strains were lambda lysogens as judged by their immunity to phage λ, and they were Lac− when streaked on indicator plates containing X-Gal, and they were immune to superinfecting Mu phages at 32°C but not at 42°C. We therefore concluded that the direction of tsx transcription is counterclockwise (Fig. 4e). Since this conclusion is dependent on phoR+ being carried by the transducing phage, we ruled out the possibility that the PhoR+ phenotype in these lysogens resulted from fortuitous excision of the chromosomal phoR::Tn10 insertions. We isolated and tested several spontaneous PhoR− segregants and found that they were still resistant to tetra-cycline but were no longer lambda lysogens, were kanamycin sensitive, and were not immune to phage Mu at 32°C. This demonstrates that the PhoR+ phenotype of the above described lysogens was conferred by the lambda specialized transducing phage.

DISCUSSION

We have used tsx-lacZ protein and operon fusions to study the cis-acting transcriptional regulatory elements controlling synthesis of the E. coli outer membrane protein Tsx. Previous analyses have demonstrated that the cAMP-CAP complex positively affects tsx transcription (1, 20, 24), whereas the deoR- and cytR-encoded proteins act as repressors of tsx expression (19). These three regulatory proteins also control the expression of a number of genes involved in nucleoside uptake and metabolism (14, 26). This coregulation of tsx thus underlines the function of the Tsx protein in the permeation of nucleosides across the outer membrane (16, 19, 20, 27; Maier et al., in press).

The use of tsx-lacZ fusions has allowed us to study the interrelationship of the DeoR, CytR, and cAMP-CAP regulatory proteins and to quantify their contribution to the regulation of tsx. These experiments revealed interesting new features of tsx expression. The DeoR and CytR regulatory proteins repress tsx transcription to different extents. Synthesis of Tsx is strongly reduced by CytR, whereas DeoR influences tsx expression only weakly. In the cytR deoR double mutant, the amount of Tsx protein is greatest and the level of tsx expression is approximately the sum of those found in the single mutants. Thus, tsx expression increases in the following sequence: wild-type < deoR < cytR < deoR cytR. This regulatory pattern has been established by using several different approaches: the adsorption rate of phage T6, the rate of permeation of nucleosides across the outer membrane, the amount of Tsx protein (19), and, as shown here, the use of tsx-lacZ fusions. In both tsx-lacZ protein and operon fusion strains, the same regulatory pattern was found, indicating that tsx regulation occurs at the transcriptional level. It is of interest to compare the regulatory pattern of tsx expression with those of other DeoR- and CytR-controlled genes. The best-characterized system is the deoCABD operon, which encodes four nucleoside-catabolizing enzymes (14, 26). This operon is expressed from two differently controlled promoters, P1 and P2, which are separated by 599 base pairs; the former is regulated by the DeoR repressor, and the latter is regulated by both the CytR and DeoR repressors (10, 34). In contrast to tsx regulation, the DeoR repressor exerts the main effect on the synthesis of the deo-encoded enzymes, whereas the CytR repressor influences the expression of the deo operon only weakly. Furthermore, deo repression by CytR and DeoR is cooperative rather than additive (14). This is exemplified by the levels of the deoA-encoded thymidine phosphorylase: in a deoCytR double mutant, a 188-fold increase is observed in comparison with that in the wild type, whereas a single deoR or cytR mutation results in a 70- or 12-fold-higher enzyme level, respectively (34). Our data strongly indicate that such a cooperation between DeoR and CytR repressors does not play any role in tsx regulation. Therefore, the results presented in this study point to important differences in the regulation of tsx and the deo operon by CytR and DeoR.

Expression of tsx is most strongly affected by the cAMP-CAP complex. This positive regulation supercedes that of the two repressors: in a Δ(crp) deoR cytR mutant strain which lacks the CAP protein, Tsx protein was barely detectable. However, such strains are still sensitive to phage T6, indicating that some Tsx synthesis occurs independently of the activator complex. Indeed, a low β-galactosidase activity is found in tsx-lacZ fusion strains carrying the Δ(crp)96 mutation (Table 2). The amount of the cAMP-CAP complex present in the cell is strongly influenced by the available carbon source (11). Expression of tsx-lacZ fusions in deoR and cytR mutants is differently affected in glucose- and glycerol-grown cells. Derepression of tsx-lacZ fusions in deoR strains occurs to the same extent in glucose- and glycerol-grown cells, demonstrating that the DeoR-regulated tsx expression is independent of cAMP-CAP activation. This is in contrast to the strong reduction of tsx-lacZ transcription seen in glucose-grown cytR strains. Therefore, it is apparent that the CytR-controlled tsx transcription is also subject to activation by the cAMP-CAP complex. This regulatory pattern follows that for other CytR- and DeoR-controlled genes. All known promoters repressible by CytR are activated by the cAMP-CAP complex, while transcription occurring from DeoR-controlled promoters is independent of this activator complex (14, 26).

Our results suggest a simple model for the transcriptional control of Tsx synthesis. Expression of tsx is controlled at two separate and differently regulated promoters. The weaker of these, P1, is activated by the available carbon source, while the main promoter, P2, is activated by the cAMP-CAP complex and is negatively controlled by CytR. This model predicts that it should be possible to isolate tsx mutants that specifically alter the response to only one of the repressors. By taking advantage of the strong repression of tsx-lacZ expression in cells carrying the cloned cytR+ gene, we have been able to isolate a mutant that shows strongly decreased repression by CytR but no change in regulation by DeoR. This clearly demonstrates that the DeoR and CytR control of Tsx synthesis can be genetically separated. The regulatory pattern of this tsx mutant most probably results from an alteration in the CytR operator. The positive regulation of tsx expression by the cAMP-CAP complex still occurs in this operator mutant. In contrast to the wild-type Φ(tsx-lacZ) l(1Hyb) fusion strain, cytR+ strains are sensitive to glucose repression. This effect is most probably due to the strongly reduced CytR binding, emphasizing the dual control of the tsx P2 promoter by CytR and the cAMP-CAP complex. Our model suggests that Tsx synthesis should occur from two mRNAs that are different in length. This prediction can now
be experimentally tested, since the tsx gene has recently been cloned (13; E. Bremer, unpublished results).

Although the results presented here strongly suggest the existence of two tsx promoters, they do not provide any clues about the arrangement of these promoters at the 5' end of tsx. Recently, tsx regulation has also been studied by Valentine-Hansen and co-workers. The data obtained in this independent investigation are in agreement with our results and provide conclusive evidence that the DeoR-controlled P1 promoter precedes that of the CytR- and cAMP-CAP-regulated tsx P2 promoter (P. Valentine-Hansen, personal communication).

We are interested in studying the interaction of the CytR and DeoR repressors with their respective tsx operator sequences. As a first step, we were able to isolate a suppressor mutation on a plasmid carrying the cloned cytR gene that restored the negative control of the O'-1 operator mutant by CytR. This suppressor mutation is not allelic specific, because tsx-lacZ expression was reduced in both the wild type and the O'-1 operator mutant. Two mutational events could account for the suppressor phenotype. It could result from increased synthesis of the wild-type CytR protein, since in the operator mutant CytR binding is not completely abolished. Alternatively, the mutation could result from an altered repressor protein able to interact with both the wild-type and the mutated tsx P2 region. Clearly, it is necessary to isolate more operator mutants and correlate them with allele-specific suppressor mutations in cytR and deoR (35, 36) to identify the interactions of specific amino acid side chains of the repressor proteins with specific base pairs in the operator sequences (12). Such studies are currently in progress.

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