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The Cyclic AMP (cAMP)-cAMP Receptor Protein Complex Functions Both as an Activator and as a Corepressor at the *tsx-p*$_2$ Promoter of *Escherichia coli* K-12

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The *tsx-p*$_2$ promoter is one of at least seven *Escherichia coli* promoters that are activated by the cyclic AMP (cAMP)-cAMP receptor protein (CRP) complex and negatively regulated by the CytR repressor. DNase I footprinting assays were used to study the interactions of these regulatory proteins with the *tsx-p*$_2$ promoter region and to characterize *tsx-p*$_2$ regulatory mutants exhibiting an altered response to CytR. We show that the cAMP-CRP activator complex recognizes two sites in *tsx-p*$_2$ that are separated by 33 bp: a high-affinity site (CRP-1) overlaps the −35 region, and a low-affinity site (CRP-2) is centered around position −74 bp. The CytR repressor protects a DNA segment that is located between the two CRP sites and partially overlaps the CRP-1 target. In combination, the cAMP-CRP and CytR proteins bind cooperatively to *tsx-p*$_2$, and the nucleoprotein complex formed covers a region of 78 bp extending from the CRP-2 site close to the −10 region. The inducer for the CytR repressor, cytidine, does not prevent in vitro DNA binding of CytR, but releases the repressor from the nucleoprotein complex and leaves the cAMP-CRP activator bound to its two DNA targets. Thus, cytidine interferes with the cooperative DNA binding of cAMP-CRP and CytR to *tsx-p*$_2$. We characterized four *tsx-p*$_2$ mutants exhibiting a reduced response to CytR; three carried mutations in the CRP-2 site, and one carried a mutation in the region between CRP-1 and the −10 sequence. Formation of the cAMP-CRP–CytR DNA nucleoprotein complex in vitro was perturbed in each mutant. These data indicate that the CytR repressor relies on the presence of the cAMP-CRP activator complex to regulate *tsx-p*$_2$ promoter activity and that the formation of an active repression complex requires the combined interactions of cAMP-CRP and CytR at *tsx-p*$_2$.

Selective repression of transcriptional initiation by DNA binding proteins is a widely used strategy for gene regulation in all cells (6). The most frequently observed repression mechanism in bacteria involves competition between a repressor protein and the RNA polymerase for a specific DNA sequence. This mechanism is based on the assumption that the bacterial repressors are by themselves fully capable of interacting with their operators. However, recent research has established that some eukaryotic repressors cannot independently regulate gene expression (35). These regulators rely on interactions with other DNA-binding proteins in order to locate and bind efficiently to their site of action. In *Escherichia coli* negative control of gene expression by the CytR repressor illustrates such a regulatory case (26, 30, 32, 33).

CytR regulates initiation of transcription from at least seven promoters that are engaged in expression of genes encoding proteins involved in transport and catabolism of nucleosides and deoxynucleosides (15, 24, 39). All CytR-regulated promoters studied so far are activated by the cyclic AMP (cAMP)-cAMP receptor protein (CRP) complex. The *deo-p*$_2$, *cdd*, and *cytR* promoter regions have been shown directly to contain two binding sites for cAMP-CRP (13, 36, 38), and sequence analyses suggest that also the *tsx-p*$_2$, *udp*, and *nupG* promoters contain tandem targets for the activator (4, 20, 23, 40). The present understanding of the mechanism responsible for CytR repression primarily originates from genetic and biochemical analyses of the *deo-p*$_2$ promoter. In vitro CytR interacts weakly with a sequence located between the two cAMP-CRP-binding sites separated by 53 bp (26). In the presence of cAMP-CRP, however, the affinity of CytR for this sequence is increased dramatically (approximately 1,000-fold), and CytR and cAMP-CRP bind cooperatively to *deo-p*$_2$, forming a nucleoprotein complex in which CytR is sandwiched between two DNA-bound cAMP-CRP complexes. Consistently, CytR represses transcription in vivo only in the presence of the cAMP-CRP complex (30, 32). Therefore, CytR relies on cAMP-CRP in order to interact strongly with the *deo-p*$_2$ promoter, and cAMP-CRP functions both as an activator and as a corepressor (32, 33).

To increase the level of understanding of the mechanism used by CytR to mediate repression of transcription, we have initiated an analysis of the protein-DNA interactions at the *tsx-p*$_2$ promoter. The *tsx* gene encodes a substrate-specific, channel-forming outer membrane protein, Tsx, which facilitates the permeation of nucleosides across this membrane (17, 19). The *tsx* gene is expressed from two differently regulated promoters, *tsx-p*$_1$ and *tsx-p*$_2$ (Fig. 1A). The weak *tsx-p*$_1$ promoter is negatively regulated by the DeoR repressor, whereas the main promoter, *tsx-p*$_2$, is subjected to both negative control by CytR and activation by the cAMP-CRP complex (3, 4, 17, 20). Here we demonstrate the presence of two binding sites for the cAMP-CRP complex, CRP-1 and CRP-2, and show that cAMP-CRP and CytR bind cooperatively to *tsx-p*$_2$. However, both the structure of the *tsx-p*$_2$ promoter and the formation of the cAMP-CRP–CytR nucleoprotein complex differ from that observed for the *deo-p*$_2$ promoter.

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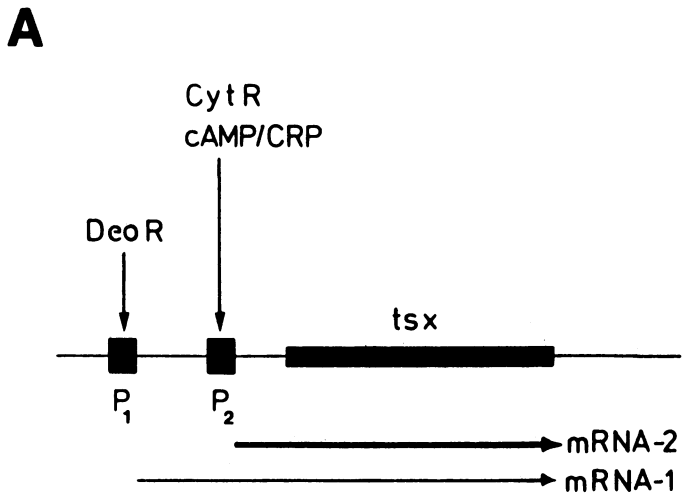
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cAMP/CRP
DeoR
tsx
P1
P2
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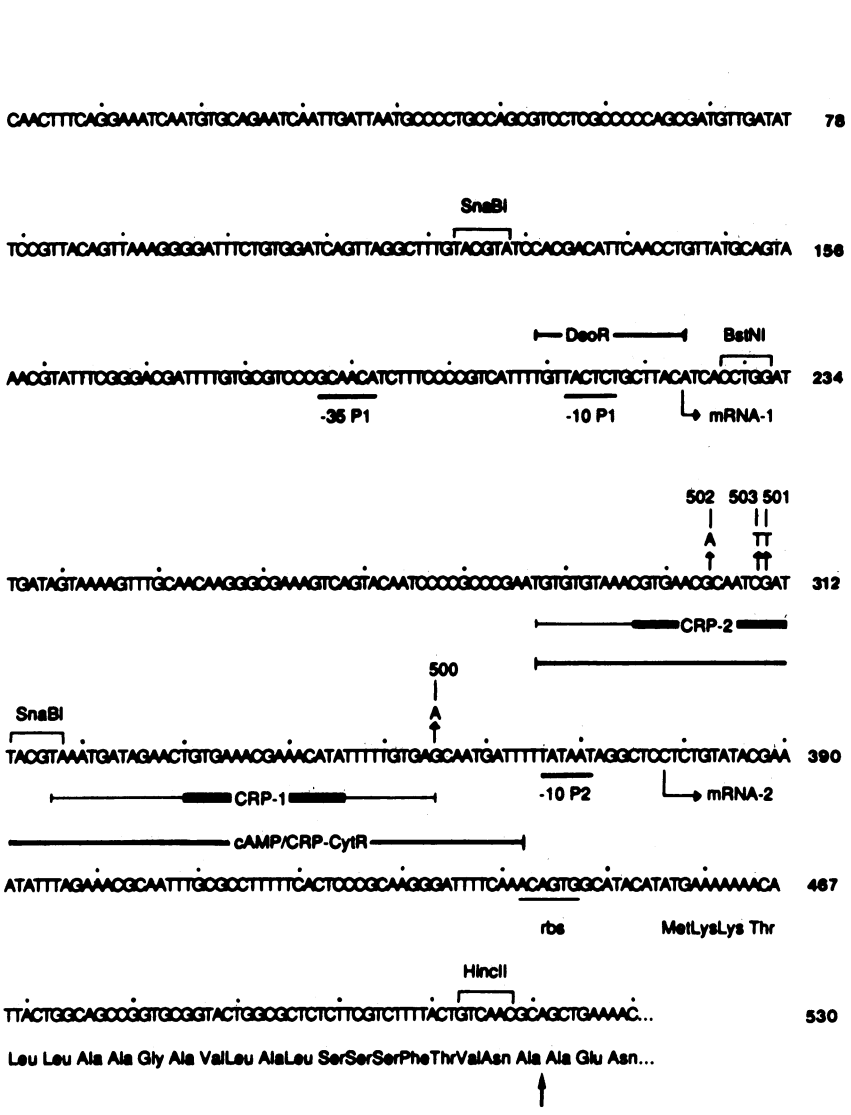
mRNA-1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DeoR
BstNI
AACGTATTTCGGGACGATTTTGTGCGTCCCGCAACATCTTTCCCCGTCATTTTGTTACTCTGCTTACATCACCTGGAT 234
-35 P1
-10 P1
mRNA-1
502 503 501
A TT TT
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CRP-2
500
A
SnaBI
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CRP-1
-10 P2
mRNA-2
cAMP/CRP-CytR
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rbs
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Leu Leu Ala Ala Gly Ala ValLeu AlaLeu SerSerSerPheThrValAsn Ala Ala Glu Asn...

MATERIALS AND METHODS

Materials. Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, S1 nuclease, DNase I, and calf intestinal alkaline phosphatase were obtained from Boehringer Mannheim, Bethesda Research Laboratories, or New England BioLabs and were used as recommended by the manufacturers. DNA sequencing was performed with the Sequenase 2.0 kit under the conditions recommended by the supplier (USB, Bad Homburg, Germany). The nonradioactive DNA labelling and detection kit used was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). γ-^{32}P-labelled nucleotides were from New England Nuclear Corp. (Boston, Mass.) and BND cellulose was from Serva (Heidelberg, Germany).

Growth conditions, bacterial strains, bacteriophages, and plasmids. Bacteria were grown aerobically at 37°C in liquid Luria-Bertani medium or minimal medium A with 0.4% glycerol or 0.4% glucose as the carbon source. When required, the minimal medium was supplemented with a solution of methionine, valine, leucine, and isoleucine to a final concentration of 0.02%. Minimal plates with lactose or uridine as the sole carbon source and Luria-Bertani plates were prepared as described previously (22, 29). The antibiotics tetracycline, kanamycin, and ampicillin were added to media at 5, 30, and 50 μg/ml, respectively. From a 10-mg/ml solution of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in dimethylformamide, 0.1 ml was added to agar plates or Luria-Bertani soft agar to distinguish between LacZ$^+$ and LacZ$^-$ phenotypes of bacterial strains and bacteriophages, respectively. λ Lac$^+$ specialized transducing phages carrying *tsx-lacZ* fusions and recombinant M13 phages were propagated as previously described (27, 29). Plasmid pCB008 (*cytR*$^+$) and the cloning vectors pBR322, pUC18, and pDK5 have all been described (1, 2, 16, 25). Plasmid pTZ18R was purchased from Pharmacia (Freiburg, Germany). Recombinant derivatives of phages M13mp18 and M13mp19 (25) were used for the preparation of single-stranded template DNA for nucleotide sequence analysis. All bacterial strains used are *E. coli* K-12 derivatives. Strain BRE2050 [F$^-$ *metB ilv rpsL* Δ(*argF-lac*)*U169 deoR8 cytR9*] and its derivative, strain GP4, carrying the chromosomal *tsx-lacZ* operon fusion positioned next to a λp*lac*Mu55 prophage (Kan^r), have been described (3). The *lac* fusion present in this strain has been described as a Φ(*tsx'-'lacZ*)*1*(Hyb) protein fusion (3). This is in error; analysis of the fusion junction has shown that this *tsx-lac* fusion is in fact an operon fusion (12). Strain TG1 [Δ(*lac-pro*) *supE thi hsd5*/F' *traD36 proA*$^+$ *B*$^+$ *lacI*^{q} *lacZ*ΔM15] (7) was used to propagate recombinant M13mp18 and M13mp19 phages. Strain GP78, a *tsx*::Tn*10* derivative of strain TG1, was constructed by P1*vir* transduction with a lysate prepared on the *tsx*::Tn*10* donor strain BRE2000.

Genetic procedures and isolation of CytR operator mutants. Standard techniques were used for generalized transduction with phage P1*vir* (22, 29). For the isolation of mutants showing reduced repression of transcription of a chromosomal *tsx-lacZ* operon fusion by CytR, the following selection procedure was used. Strain GP4 (*tsx-lacZ deoR8 cytR9*) was transformed with the high-copy-number *cytR*$^+$ plasmid pCB008 (1), rendering strain GP4 unable to grow on lactose-minimal plates (3). Lac$^+$ derivatives were selected on these plates after 2 days of incubation at 37°C. Mutants with alterations in the chromosomal *tsx-lacZ* fusion were identified by their inability to grow on uridine-minimal plates (15, 24). Linkage of the Lac$^+$ phenotype in the Lac$^+$ Uri$^-$ derivatives of strain GP4(pCB008) to the chromosomal *tsx-lac* operon fusion was verified after transducing the hybrid *tsx-lac* gene and the flanking λp*lac*Mu prophage with phage P1*vir* into strain BRE2050 (*deoR8 cytR9 tsx*$^+$) by selecting for kanamycin-resistant transductants. Representative transductants were then transformed with plasmid pCB008 (*cytR*$^+$), and their Lac and Uri phenotypes were retested. The λp*lac*Mu prophage positioned next to the *tsx-lacZ* fusion in strain GP4 and its mutant derivatives were induced with UV light (29), and the resulting lysates were plated onto lawns of strain BRE2050 in the presence of the β-galactosidase indicator dye X-Gal to detect LacZ$^+$ λ specialized transducing phages carrying the entire *tsx-lacZ* operon fusion (5).

Recombinant DNA techniques, DNA sequencing, and construction of plasmids. Routine manipulations of nucleic acids were all as previously described (27, 29). Radiolabelled restriction fragments were sequenced by the chemical modification procedure of Maxam and Gilbert (21). Sequencing of single-stranded DNA from recombinant M13mp18 and M13mp19 derivatives was performed by the method of Sanger et al. (28) with modified T7 DNA polymerase (Sequenase). DNA from the Lac$^+$ λ specialized transducing phage carrying the wild-type *tsx-lacZ* fusion was digested with the restriction enzyme *Sna*BI, the resulting DNA fragments were electrophoretically separated on a 1.4% agarose gel, and a 190-bp *Sna*BI fragment spanning the *tsx*-p_1 and part of the *tsx*-p_2 regulatory region (Fig. 1B) was isolated from the gel and cloned into the *Hin*cII site of the multiple cloning region of plasmid pTZ18R, resulting in plasmid pGP8. The cloned *Sna*BI fragment was excised from pGP8 by double digestion with *Eco*RI and *Pst*I and subsequently labelled with dioxygenin (Boehringer Mannheim GmbH); this restriction fragment was then used as a *tsx*-specific probe in DNA-DNA hybridization experiments. DNA from the Lac$^+$ λ specialized transducing phages de-

FIG. 1. Genetic organization of the *tsx* regulatory region and mutations altering CytR-mediated repression. (A) The *tsx* gene and its two promoters, *tsx*-p_1 and *tsx*-p_2, are schematically shown, and the two *tsx* mRNAs are indicated. The minor *tsx*-p_1 promoter is negatively controlled by the DeoR repressor, whereas the main promoter, *tsx*-p_2, is subjected to positive regulation by the cAMP-CRP activator complex and negative control by the CytR repressor. (B) Nucleotide sequence of the *tsx* regulatory region is given, and the −35 and −10 sequences of the *tsx*-p_1 promoter and the −10 region of the *tsx*-p_2 promoter are underlined. The start sites of both mRNA transcripts are indicated by arrows. The positions and nucleotide alterations in the CytR operator mutants are shown, and the numbers refer to the four *tsx* alleles characterized in this study; the *tsx-502* allele represents three different independent isolates. The 16-bp sequence in the *tsx*-p_1 promoter showing homology to known DeoR operator sites and two regions in the *tsx*-p_2 promoter with homology to cAMP-CRP DNA-binding sites are indicated; lines extending from the cAMP-CRP targets indicates those sequences which are protected by cAMP-CRP against DNase I digestion. The region protected by the cAMP-CRP–CytR repressor complex in DNase I footprinting experiments is indicated. The beginning of the *tsx* coding region is shown, and the presumed cleavage site for the Tsx precursor is indicated by a vertical arrow. The positions of restriction sites used for cloning and end labelling of the *tsx* promoter fragments are shown. Numbering of the nucleotide sequence follows that of Bremer et al. (4).

rived from the wild-type and six mutant *tsx-lacZ* fusions was digested with *Hin*cII. The DNA fragments were separated by agarose gel electrophoresis, transferred onto a nitrocellulose membrane (Schleicher & Schuell; pore size, 0.45 μm), and hybridized (34) to the *tsx*-specific dioxygenin-labelled probe isolated from plasmid pGP8. An approximately 950-bp *Hin*cII hybridizing fragment was detected in each case; one end of this fragment corresponds to a *Hin*cII site in the coding region from the Tsx signal sequence (Fig. 1B). These *Hin*cII fragments were cloned into M13mp18 and M13mp19 and used to sequence an approximately 360-bp DNA segment covering the *tsx*-p_1 and *tsx*-p_2 promoter regions. For each of the mutants, both DNA strands were sequenced to unambiguously identify the mutation. For further analysis, the chromosomal fragment was excised from the recombinant M13mp phages by double digestion with *Eco*RI and *Pst*I and cloned into plasmid pUC18. The cloned fragment from the wild-type was in the opposite orientation from those of the mutant *tsx* promoter regions. The wild-type and mutant plasmids were cut with *Eco*RI and *Hin*dIII, respectively, 5′ end labelled with ^{32}P, and digested with *Bst*NI (Fig. 1B). The radiolabelled *tsx*-p_2 fragments (304 bp for the wild-type and 323 bp for the mutant plasmids) were isolated from gel slices of a 7% polyacrylamide gel by electroelution. They were then further purified on a small (100 μl) BND cellulose column (equilibration buffer: 0.3 M NaCl, 10 mM Tris-HCl [pH 7.4], 1 mM EDTA; elution buffer: 1 M NaCl and 15% ethanol), precipitated with ethanol, dried, and resuspended in water. These uniquely end-labelled DNA fragments were then used for DNase I footprinting with CRP and CytR and for the S1 mapping of the *tsx*-p_2 transcriptional initiation site.

Isolation of RNA and S1 mapping. Strain GP78 (*tsx*::Tn*10*) was transformed with derivatives of plasmid pUC18 carrying the wild-type or mutant *tsx*-p_1 and *tsx*-p_2 promoter regions and was grown to late logarithmic phase in Luria-Bertani medium. Total RNA was purified from these cultures by extraction with hot phenol (60°C, pH 5). The RNA (120 μg) was hybridized to a 304-bp *Eco*RI-*Bst*NI fragment (uniquely 5′ end labelled with ^{32}P at the *Eco*RI site) that spans the *tsx*-p_2 promoter and part of the *tsx* coding region (Fig. 1B). Methods for hybridization and S1 mapping were as previously described (37).

Purification of the CytR and CRP proteins and DNase I protection. The CRP protein was purified by the method of Ghosaini et al. (14). Preparation of $CytR^+$ and $CytR^-$ protein extracts was carried out as described by Pedersen et al. (26). The $CytR^+$ extract was prepared from strain SO928 (Δ*deo* Δ*lac cytR*$^+$) carrying the CytR-overproducing plasmid p5-13. In this recombinant pDK5 (16) derivative, expression of the *cytR*$^+$ coding region is controlled by the transcriptional and translational elements of the *tac* promoter and the *lac* ribosome binding site, respectively. Expression of the *cytR*$^+$ gene was induced by adding 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were lysed in a French press, cellular debris was removed by centrifugation, and $(NH_4)_2SO_4$ was added to the crude extract to 25% saturation. Following centrifugation the $(NH_4)_2SO_4$ saturation of the supernatant was increased to 40%, the resulting pellet was dissolved in 1 ml of buffer A (60 mM potassium acetate, 10 mM Tris-acetate [pH 8.2], 5% polyethylene glycol, 1 mM dithiothreitol, and the extract was desalted on a Sephadex G-25 column equilibrated with buffer B (10 mM Tris-HCl [pH 7.8] 50 mM KCl, 1 mM EDTA, 50 μg of bovine serum albumin per ml, 1 mM dithiothreitol, and 0.05% Nonidet P-40). Approximately 30% of total protein in the extract is CytR (26). The same procedure was used to prepare a $CytR^-$ protein extract from strain SO929 (Δ*deo* Δ*lac cytR*) carrying the vector plasmid pDK5. DNase I footprinting was carried out as described by Galas and Schmitz (10). Various amounts of protein were mixed with radiolabelled wild-type or mutant *tsx*-p_2 DNA fragments and 15 μg of nonspecific competitor DNA per ml in reaction buffer (10 mM Tris-HCl [pH 7.8], 50 mM KCl, 1 mM EDTA, 50 μg of bovine serum albumin, 1 mM dithiothreitol, and 0.05% Nonidet P-40) in a total volume of 80 μl. A solution (20 μl) containing 1 ng of DNase I per μl was then added. The reaction mixture was incubated for 20 s at 37°C, and the reaction was stopped by adding 100 μl of a stop solution (20 mM EDTA, 0.3 μg of sonicated calf thymus carrier DNA per ml). The mixture was extracted twice with phenol; the DNA was precipitated with ethanol, dried, and resolved in loading buffer; and the DNase I reaction products were electrophoretically separated on a 5% polyacrylamide sequencing gel containing 8 M urea. The nuclease protection pattern was visualized by autoradiography.

β-Galactosidase activity measurements. β-Galactosidase activity in cultures grown overnight in minimal medium supplemented with either 0.4% glucose or 0.4% glycerol as the carbon source was assayed as described by Miller (22). Specific β-galactosidase activity is expressed as micromoles of substrate (ONPG; *o*-nitrophenyl-β-D-galactoside) cleaved per minute per milligram of protein.

Nucleotide sequence accession number. The nucleotide sequence of the *tsx* regulatory region has been assigned GenBank accession number M57685.

RESULTS

Binding of cAMP-CRP and CytR to the *tsx*-p_2 promoter region. In order to define the DNA-binding sites recognized by the cAMP-CRP complex, we performed DNase I protection experiments (10) using purified CRP protein and a 304-bp ^{32}P-end-labelled DNA fragment carrying the *tsx*-p_2 regulatory region (Fig. 2). Binding of the CRP protein is cAMP dependent and results in protection of two DNA regions that extend from position 287 to 312 bp (CRP-2) and from position 318 to 355 bp (CRP-1) (Fig. 1B). Protection of the CRP-2 region from DNase I digestion requires an approximately fivefold-higher concentration of cAMP-CRP than does protection of the CRP-1 site (Fig. 2, lanes 3 to 5), indicating that the CRP-1 target has a higher affinity for the activator complex.

In analogous experiments we investigated the binding of partially purified CytR protein to the *tsx*-p_2 regulatory region. The $CytR^+$ extract confers protection from DNase I digestion of the DNA from position 317 to 337 bp (Fig. 3A, lane 3). This region includes the sequence between the two CRP targets and overlaps the CRP-1 target. The protection observed is specific for the CytR repressor, as no protection is detected when a $CytR^-$ extract is used (see Fig. 5, lane 2). Surprisingly, the addition of cytidine or adenosine, the inducers for the *E. coli* CytR repressor (15, 24), did not prevent binding of CytR to the DNA (Fig. 3A, lanes 4 and 5).

Next, we performed footprinting experiments in the presence of both the CytR repressor and the cAMP-CRP activator complex. Compared with the footprints obtained when the regulatory proteins are present individually, a drastically changed protection pattern is observed (Fig. 3B). The protected region spans a continuous DNA segment of 78 bp that begins at position 287 bp and ends at position 364 bp, close to the −10 region of *tsx*-p_2 (Fig. 1B). The presence of both regulatory proteins results in the following alterations: (i) the

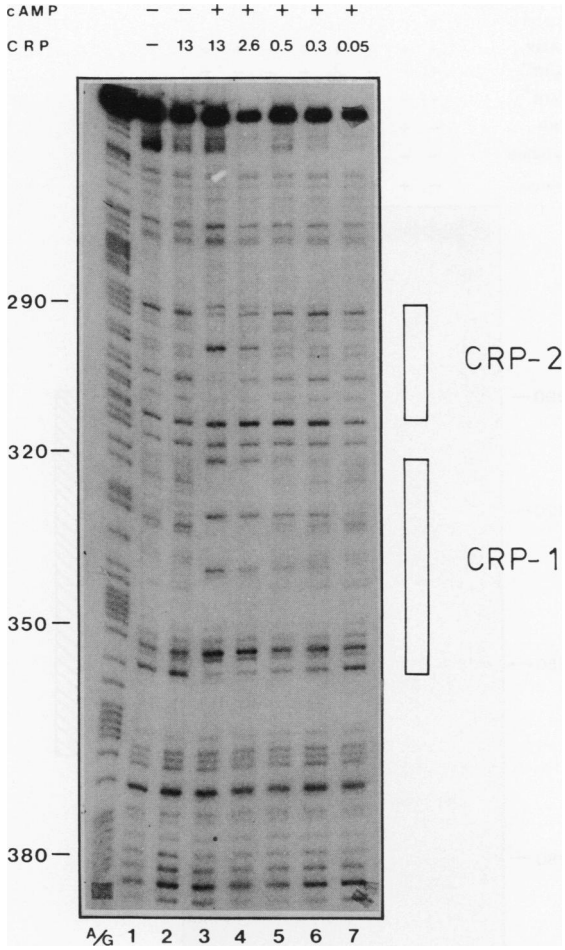

FIG. 2. DNase I footprinting analysis of cAMP-CRP protection of the wild-type tsx-p_2 promoter. A 304-bp *Eco*RI-*Bst*NI restriction fragment labelled with ^{32}P at its 5′ end and carrying the entire tsx-p_2 promoter was used as a template for DNase I footprinting at a concentration of 40 ng/ml. The reaction products were electrophoretically separated on an 8% sequencing gel, and the numbers on the left refer to the nucleotide positions given in Fig. 1B. − and +, absence or presence, respectively, of cAMP (50 μM) in the reaction mixture. The CRP concentration used is given in micromolar amounts. The two regions protected by the cAMP-CRP activator complex from DNase I digestion are indicated by the open boxes. An A + G sequencing reaction of the *Eco*RI-*Bst*NI fragment (leftmost lane) was used to calibrate the gel.

protection of CRP-2 is increased; (ii) the region between the two CRP targets is completely protected; and (iii) the protection pattern at CRP-1 is changed, and protection from DNase I digestion is extended close to the −10 region (Fig. 3B, compare lane 2 with lanes 5 and 7). Moreover, the two proteins cooperate in binding to tsx-p_2. A strong combined footprint can be detected at protein concentrations at which the individual components only occupy a minor fraction of their binding sites (Fig. 3A and B). Addition of cytidine to the reaction mixture results in a footprint which matches that of cAMP-CRP alone. This effect is specific for cytidine, since the addition of uridine, which functions as an inducer for CytR only in *Salmonella typhimurium* (15, 24), does not change the footprint (Fig. 3B, compare lanes 5 and 7 with lanes 6 and 8). Around position 270 bp, enhancements in DNase I digestion are observed (Fig. 3A and B). These changes in the digestion pattern are detected in nearly all gels (see Fig. 2, 3, 5, 6, and 7), irrespective of whether cAMP-CRP or CytR proteins were present in the assay. Hence, this is not a specific effect of these DNA-bound regulatory proteins but is likely due to variations in DNase I cleavage.

Characterization of tsx-p_2 mutants with a reduced response to CytR. To identify DNA sequences in tsx-p_2 that are important for CytR regulation, we isolated mutants of tsx-p_2 that show a reduced response to CytR. Strain GP4 (*deoR cytR*) carries a chromosomal fusion between the entire *tsx* regulatory region and the *lacZY* genes. Hence, in this construct, *lac* expression is directed both by the weak, DeoR-regulated tsx-p_1 promoter and by the main, cAMP-CRP-activated and CytR-regulated tsx-p_2 promoter (Fig. 1A). In the presence of the multicopy $cytR^+$ plasmid pCB008 (1), GP4 displays a Lac^- phenotype. We isolated 45 independent spontaneous Lac^+ derivatives of strain GP4 (pCB008) from minimal plates with lactose as the sole carbon source. Such colonies arise either by mutations in the plasmid-encoded $cytR^+$ gene or at the chromosomal *tsx-lacZ* operon fusion (3). From this collection of mutants, we identified five strains carrying mutations closely linked to the *tsx-lacZ* fusion which showed altered CytR-mediated regulation. In the presence of the CytR-overproducing plasmid pCB008, only a weak CytR-mediated repression of tsx-p_2 activity is observed (Table 1). Hence, CytR regulation of the mutant tsx-p_2 promoters is strongly reduced but not completely eliminated. However, CytR-mediated repression of tsx-p_2 is impaired to such an extent that the mutants no longer respond to the CytR repressor when expressed from the chromosomal $cytR^+$ gene (data not shown). Furthermore, the maximal level of *tsx-lacZ* expression in glycerol-grown cultures is reduced in all mutants isolated in this study (*tsx-501*, *tsx-502*, and *tsx-503*) (Table 1), whereas promoter activity of a previously isolated (3) CytR operator mutant (*tsx-500*) is not affected (Table 1). A comparison of the β-galactosidase activities in glycerol and glucose-grown cultures indicates that in all mutants transcription initiating from tsx-p_2 is still activated by the cAMP-CRP complex (Table 1).

Subsequent DNA sequence analysis of the tsx-p_1 and tsx-p_2 regulatory region showed that the *tsx-501*, *tsx-502*, and *tsx-503* alleles carried single-point mutations in the CRP-2 site of the tsx-p_2 promoter. The *tsx-500* allele carried a mutation downstream of CRP-1 (Fig. 1B). Taken together, these results demonstrate that the integrity of the CRP-2 target, which is only weakly recognized by the cAMP-CRP complex in vitro (Fig. 2), affects both the tsx-p_2 promoter activity as well as the CytR-mediated repression of transcription. In contrast, the *tsx-500* mutation, which is located outside of the two CRP targets, affects only CytR regulation of tsx-p_2 expression. To rule out the possibility that the lack of CytR regulation of tsx-p_2 was caused by the creation of a new promoter, high-resolution S1 mapping of the tsx-p_2 mRNA transcription initiation site (4) was carried out. All five mutants used the wild-type tsx-p_2 transcription initiation site (Fig. 4).

Independent binding of CytR and cAMP-CRP to the mutant tsx-p_2 promoters. Nuclease protection experiments were used to assess possible differences in the interactions of the two regulators with the wild-type and mutant promoters. In the presence of CytR alone, a specific DNase I footprint is detected in all four mutant promoters. The CytR protein appears to exhibit the same affinity for both the mutant and the wild-type promoters. Thus, at the conditions used here,

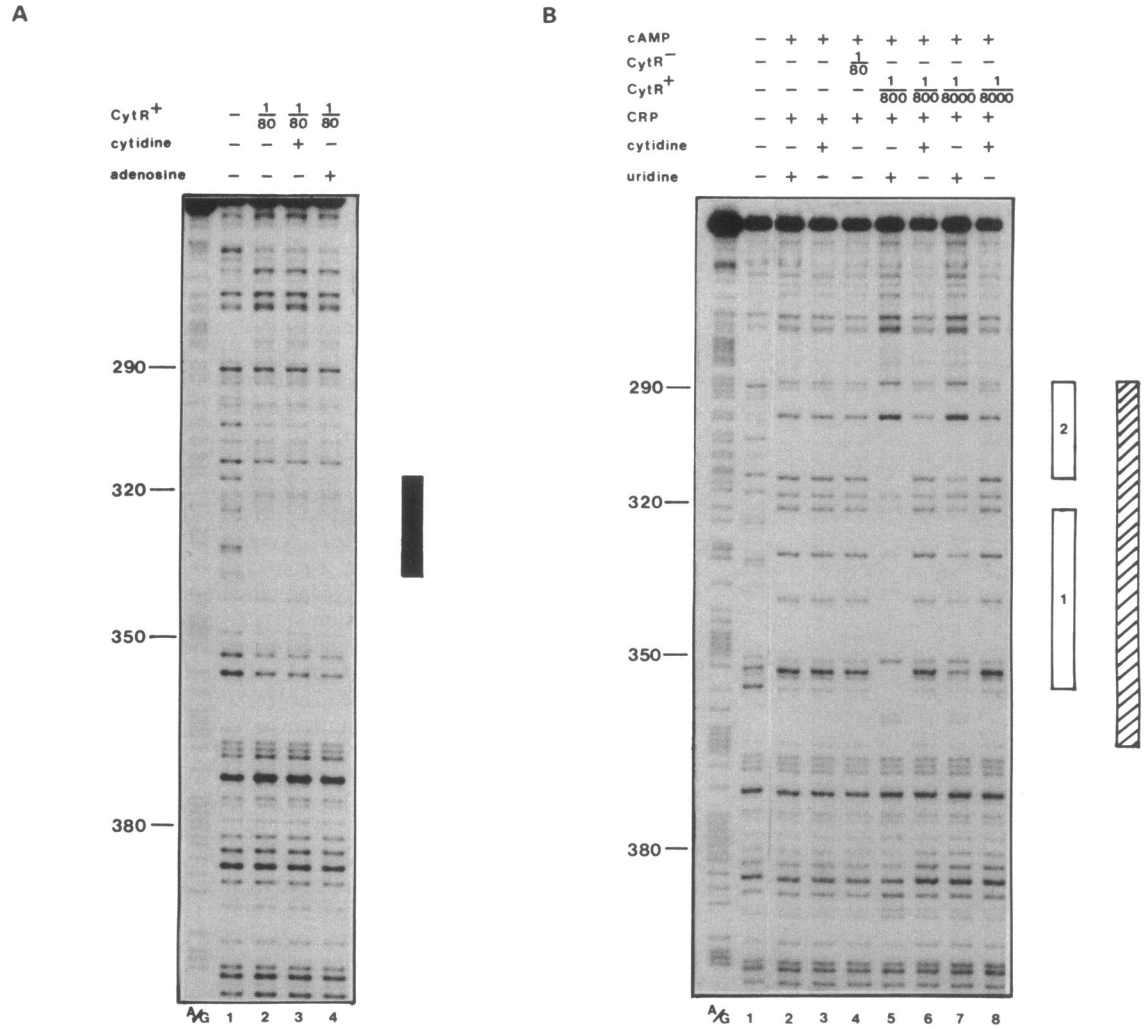

FIG. 3. Combined DNase I footprint of the wild-type *tsx-p*$_2$ promoter in the presence of both the CytR repressor and the cAMP-CRP activator complex. A 304-bp *Eco*RI-*Bst*NI restriction fragment carrying the *tsx-p*$_2$ promoter was labelled with ^{32}P at its 5′ end and was used as a template at a concentration of 40 ng/ml. The dilutions of the CytR$^+$ extract used are indicated. cAMP, purified CRP protein, and each of the nucleosides cytidine, adenosine, and uridine were added to the reaction mixture to final concentrations of 50 μM, 13 μM, and 10 mM, respectively. An A + G sequencing reaction of the template DNA was used to calibrate the gels, and the numbers on the left of each gel refer to the base pair positions shown in Fig. 1B. The open, hatched, and filled boxes indicate the DNA regions protected against DNase I digestion by CytR alone (A), in the presence of the cAMP-CRP complex alone (B), or by both regulatory proteins (B), respectively.

binding of CytR per se is not impaired by any of the point mutations (Fig. 5). For the *tsx-500*, *tsx-501*, and *tsx-503* alleles the DNase I protection pattern is very similar to that obtained with the wild-type promoter (Fig. 5). The CytR footprint of the *tsx-502* allele deviates slightly from that of the wild-type promoter around position 304 bp, however. An alteration in the DNase I digestion pattern was observed even when no protein was added to the reaction mixture (Fig. 5, compare lanes 1 and 3 with lanes 13 and 15).

The affinity of the cAMP-CRP complex for the two CRP targets of *tsx-500*, *tsx-501*, and *tsx-502* alleles seems to be very similar to that of the wild-type promoter, as determined from binding experiments in which the CRP concentration was varied from 50 nM to 13 μM (Fig. 6). In contrast, the *tsx-503* mutation at position 309 bp (Fig. 1B) has a severe effect on cAMP-CRP binding to the mutant CRP-2 site, whereas binding to the CRP-1 target remains unchanged (Fig. 6, compare lanes 3 and 4 with lanes 31 and 32). Hence, only one of the four mutants investigated in vitro manifests a clear change in independent binding of one of the two regulators.

Combined binding of cAMP-CRP and CytR to the mutant *tsx-p*$_2$ promoters. The effects of the four mutations on the formation of the cAMP-CRP–CytR–DNA complex were investigated by DNase I footprinting. To this end, we titrated in parallel the wild-type and mutant promoters with the cAMP-CRP complex and the CytR repressor in the presence of 50 μM cAMP. The *tsx-500* mutation at position 355 bp has a severe effect on the formation of the combined complex. Thus, protection of the DNA segment between the −10 sequence and the CRP-2 target, a characteristic feature of the nucleoprotein complex at the wild-type promoter, is essentially abolished by the *tsx-500* mutation (Fig. 7A, compare lanes 6 and 7 with lanes 13 and 14).

The G-to-T transversion at position 310 bp (*tsx-501*) significantly affects the formation of the nucleoprotein complex

TABLE 1. Expression of the wild-type and mutant *tsx-lacZ* operon fusions

tsx allele[a]	β-Galactosidase sp act[b] in the presence of:			Repression[c] (fold)
	pBR322		pCB008 (glycerol)	
	Glycerol	Glucose		
Wild type	5.6	1.0	0.2	28
tsx-500	6.0	1.0	2.8	2.1
tsx-501	1.8	0.8	1.1	1.6
tsx-502	1.7	0.9	1.2	1.4
tsx-503	1.9	0.6	1.5	1.3

[a] All strains carry the same chromosomal *tsx-lacZ* operon fusion and are isogenic (*deoR8 cytR9*); the *tsx* allele number refers to the mutation present in the *tsx-lacZ* hybrid gene. The *tsx-502* allele represents three different independent isolates carrying the same nucleotide exchange (Fig. 1B). The *tsx-500* allele was isolated previously and has been described as *tsx O*^{c}-1 (3).

[b] The cells were grown overnight in minimal medium A with 0.4% glycerol or 0.4% glucose as the carbon source. Specific β-galactosidase activity is expressed as micromoles of substrate (ONPG) cleaved per minute per milligram of protein; the data shown are the mean values of three independent experiments.

[c] Repression (fold) is calculated as specific activity of β-galactosidase in the presence of the *cytR*$^+$ plasmid pCB008 divided by the specific β-galactosidase activity in the presence of the vector pBR322.

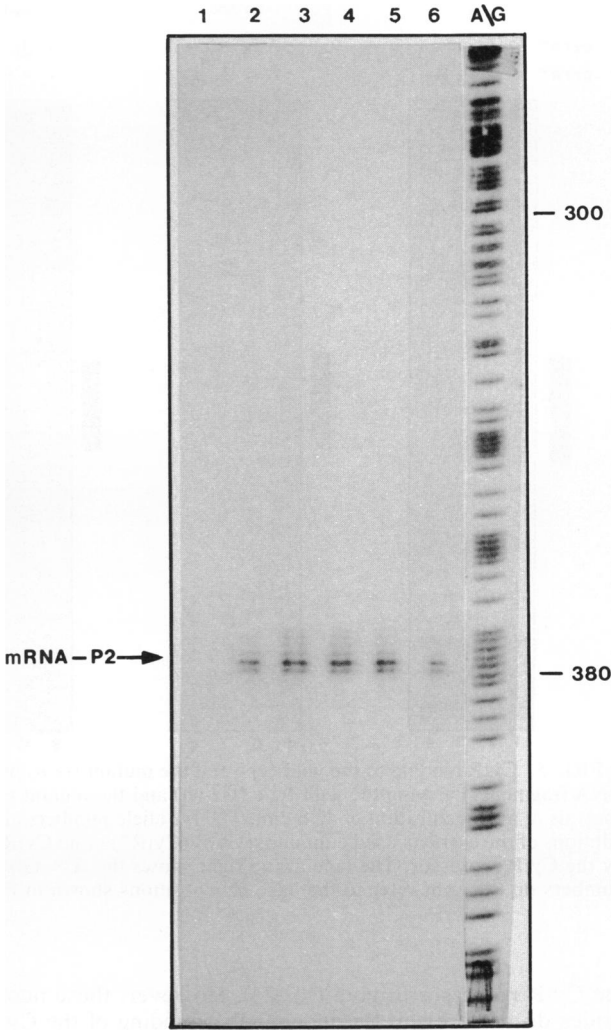

FIG. 4. S1 mapping of the transcription initiation site of the wild-type and mutant *tsx-p*$_2$ promoters. Total RNA was isolated from strain GP78 (*tsx*::Tn*10*) which has been transformed with plasmid pUC18 (lane 1) and its derivatives carrying the wild-type (lane 2) or mutant *tsx-500* (lane 3), *tsx-501* (lane 4), *tsx-502* (lane 5), and *tsx-503* (lane 6); hybridized with a radiolabelled *tsx*-specific probe; and digested with nuclease S1, and the reaction products were electrophoretically separated on a DNA sequencing gel. The lane at the right shows the A + G sequencing reaction of the DNA fragment from the wild-type promoter; the numbers on the right refer to the base pair positions shown in Fig. 1B.

at high cAMP-CRP concentrations (Fig. 7A, compare lanes 7 and 21). An even more pronounced effect of this mutation can be observed in experiments with a lower cAMP-CRP concentration (data not shown). Also, enhancement of DNase I digestion is specifically observed at positions 306 and 304 bp in the combined footprint (Fig. 7A, lanes 20 and 21). Therefore, it is conceivable that this mutation, which is located in one of the consensus CRP half-sites (8) (Fig. 1B), changes the structure of the nucleoprotein complex at CRP-2.

For the two other mutant promoters (*tsx-502* and *tsx-503*) that contain base changes in the CRP-2 target, the formation of the nucleoprotein complex is strongly reduced at low cAMP-CRP concentrations. However, at higher concentrations of cAMP-CRP, the complex can still be formed (Fig. 7B, lanes 3 and 4, 7 and 8, and 11 and 12; Fig. 7C, lanes 3 and 4, 7 and 8, and 11 and 12). Of particular interest in the combined footprint of the *tsx-503* allele is that the cAMP-CRP complex can interact strongly with the CRP-2 target in the presence of CytR (Fig. 7B, lane 11), although the affinity of cAMP-CRP for the mutated target is severely reduced (Fig. 6, lanes 31 to 33). This clearly shows that the two regulators cooperate in binding to the regulatory region of the *tsx-p*$_2$ promoter.

DISCUSSION

The mechanism used by the CytR protein to negatively control transcription deviates from that of other *E. coli* repressors. We have previously reported that the cAMP-CRP complex functions as an adaptor for the CytR repressor in the *deo* operon and that cAMP-CRP and CytR bind cooperatively to the *deo-p*$_2$ promoter (26, 30, 32, 33). In this report we used the *tsx-p*$_2$ promoter to investigate in detail the cAMP-CRP–CytR interplay. Like other CytR-regulated promoters in *E. coli* (13, 36, 38), the *tsx-p*$_2$ promoter contains two cAMP-CRP-binding sites which differ in their affinities for the activator complex. The high-affinity target, CRP-1, and the low-affinity target, CRP-2, are centered around positions −41 and −74 bp, respectively, in relation to the start site of transcription at the *tsx-p*$_2$ promoter (Fig. 1B). In the absence of cAMP-CRP, the CytR repressor interacts with a sequence extending from CRP-2 into CRP-1 (317 to 337 bp [Fig. 1B]). From the analysis of CytR interaction with the *deo-p*$_2$ promoter, we have recently proposed that the sequence motif 5′-tTTNCa-3′ (N = G, A, T, or C) might constitute an important part of the sequence recognized by CytR (26). Two copies of this motif are present in the *tsx-p*$_2$ sequence protected by CytR (315 to 320 bp and 332 to 337 bp [Fig. 1B]). However, CytR boxlike sequences are also present outside of the region protected by CytR, indicating that other sequence parameters (e.g., flanking sequences and relative orientation of the motifs) may be of importance for CytR binding. Cytidine and adenosine are the inducers for

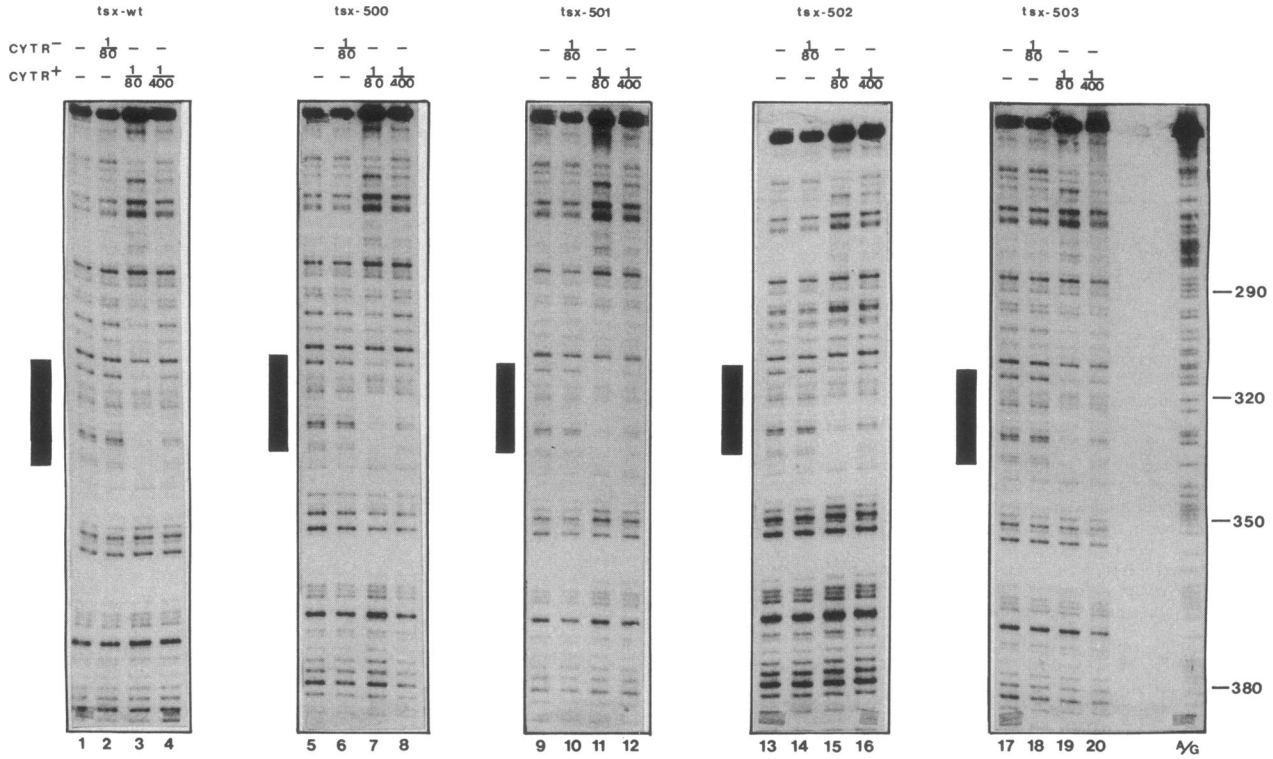

FIG. 5. CytR binding to the wild-type and the mutant *tsx-p*$_2$ promoter. ^{32}P-end-labelled *Eco*RI-*Bst*NI (304-bp) and *Hin*dIII-*Bst*NI (323-bp) DNA fragments carrying the wild-type (*tsx*-wt) and the mutant *tsx-p*$_2$ promoters, respectively, were used as templates in DNase I footprint analysis at a concentration of 40 ng/ml. The *tsx* allele numbers above the various panels represent the different CytR operator mutants. The dilutions of the extracts used containing CytR (CytR$^+$) or no CytR repressor (CytR$^-$) are indicated. The filled boxes mark the region protected by the CytR repressor. The lane at the right shows the A + G sequencing reaction of the DNA fragment from the wild-type promoter; the numbers on the right refer to the base pair positions shown in Fig. 1B.

the CytR repressor in vivo (15, 24). However, these nucleosides do not prevent the independent binding of the CytR protein to the *tsx-p*$_2$ promoter in vitro (Fig. 3A).

In the presence of both cAMP-CRP and CytR, a combined footprint is obtained. The footprint spans 78 bp and extends from CRP-2 close to the −10 sequence. Several important features should be noted. First, the protection pattern in CRP-2 corresponds to that obtained in the presence of cAMP-CRP alone. It thus seems reasonable to conclude that cAMP-CRP is bound to CRP-2 in the combined footprint. Second, the remaining parts of the footprint strongly deviate from those obtained in the presence of cAMP-CRP or CytR alone. Third, the protection extends close to the −10 sequence, i.e., beyond the protection obtained with cAMP-CRP or CytR individually. Finally, in the combined footprint, cAMP-CRP binding to CRP-2 is enhanced, and in the presence of cAMP-CRP, binding of CytR is enhanced (Fig. 3A and B). Hence, cAMP-CRP and CytR bind cooperatively to the *tsx-p*$_2$ promoter. This is also evident from the in vitro analysis of the *tsx-503* allele. Binding of cAMP-CRP to the mutated CRP-2 site is strongly reduced; however, in the presence of CytR, binding of cAMP-CRP is reestablished (Fig. 7B, lanes 10 and 11). The physiological importance of the cooperative DNA binding of cAMP-CRP and CytR to *tsx-p*$_2$ is emphasized by the observation that cytidine, the inducer of CytR, does not interfere with independent DNA binding of the repressor protein; rather, the cooperative binding of cAMP-CRP and CytR is interrupted. Release of CytR from the nucleoprotein complex in response to cytidine leaves the cAMP-CRP activator complex bound to *tsx-p*$_2$, which will allow immediate stimulation of transcription. Similar data have also been described for the interactions of the cAMP-CRP and CytR proteins with the *deo-p*$_2$ promoter (26).

We have characterized three mutations that reduce the CytR-mediated repression of transcription and have mapped them to the CRP-2 site of the *tsx-p*$_2$ promoter. The *tsx-503* mutation results in a strongly reduced cAMP-CRP binding to CRP-2. This mutation is located at a position that is highly conserved among cAMP-CRP-binding sites and is analogous to the *lacL29* and *deo-p*$_2$(89) alleles (9, 30). Therefore, the reduced repression by CytR observed for the *tsx-503* allele is likely to be a consequence of reduced binding of the cAMP-CRP complex to the CRP-2 site. In contrast, the affinity of the cAMP-CRP complex in vitro for the two mutant CRP-2 sites in *tsx-501* and *tsx-502* is very similar to that of the wild-type CRP-2 target (Fig. 6). These data indicate that changes at various positions in CRP-2 can affect the combined binding of the repressor and the activator to *tsx-p*$_2$ differently. The *tsx-502* mutation is located in the nonconserved center of the cAMP-CRP-binding site (8) and results in a changed DNase I digestion pattern (Fig. 5, lane 13). For the *gal-p*$_1$ promoter it has been suggested that mutations in the corresponding position in the cAMP-CRP-binding site reduce activation of transcription by reducing the bendability of this site (11). The *tsx-501* mutation is located in the right half of the cAMP-CRP-binding site at a position that has been proposed to be the bending center in the cAMP-

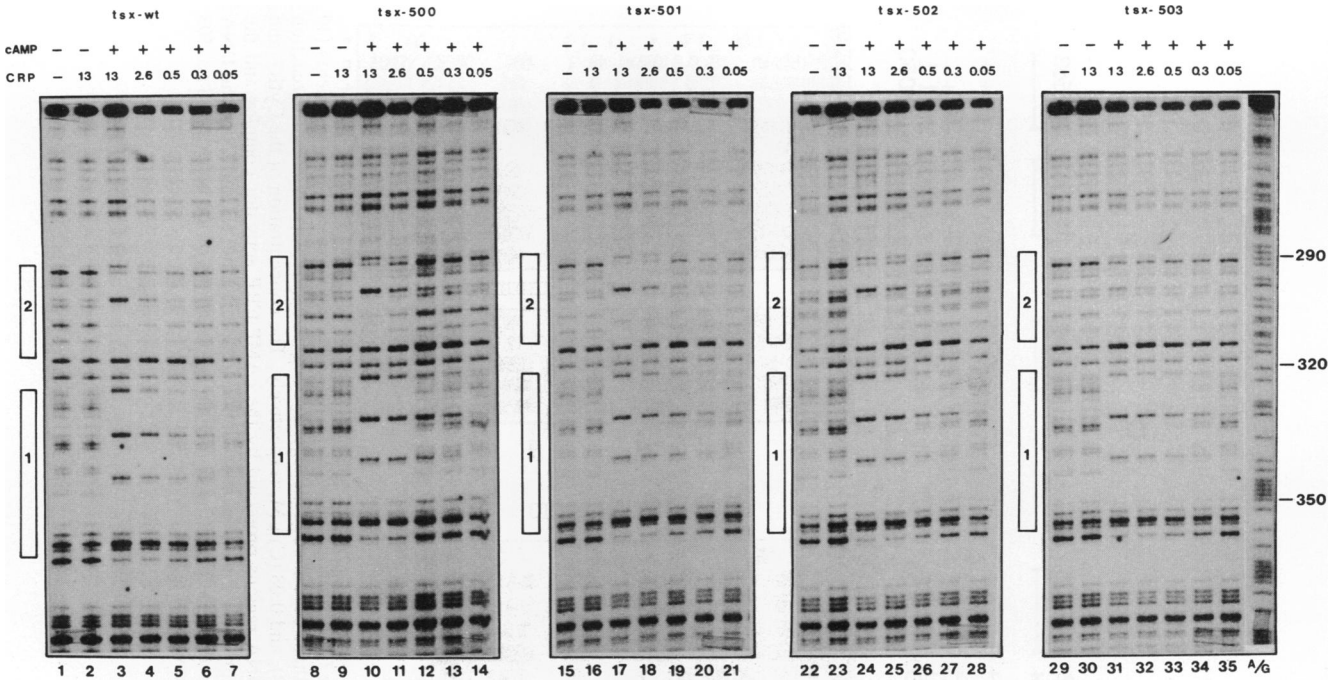

FIG. 6. DNase I footprinting analysis of the $tsx\text{-}p_2$ promoter and its mutant derivatives in the presence of the cAMP-CRP activator complex. The DNA templates (40 ng/ml) used are the same as described in the legend to Fig. 5. cAMP was added to the reaction mixture to a final concentration of 50 μM, and the CRP concentration present in the reaction is given in micromolar amounts. The open boxes indicate the positions of the DNA regions protected by the activator complex from DNase I digestion. The lane at the right shows the A + G sequencing reaction of the DNA fragment from the wild-type promoter; the numbers on the right refer to the base pair positions shown in Fig. 1B.

CRP–DNA complex in the *lac* promoter (18, 41). It is therefore possible that the *tsx-501* and *tsx-502* mutations result in a changed conformation of the cAMP-CRP–DNA complex and that this change is responsible for the reduced CytR-mediated regulation in vivo. Irrespective of which mechanism underlies the mutant phenotype, the mutations all point to the importance of a productive cAMP-CRP–DNA interaction at CRP-2 for CytR-mediated regulation. Finally, the recent isolation of CRP mutants that abolish CytR regulation at $tsx\text{-}p_2$ without affecting positive control (31) clearly shows that cAMP-CRP is a crucial component of the CytR repressor apparatus at this promoter.

Detailed investigation of a number of CytR-regulated promoters has shown that each promoter has individual features, and results obtained with one promoter may not be applicable to the others. In the $deo\text{-}p_2$ promoter, the combined cAMP-CRP–CytR footprint is the sum of the footprints obtained with the two regulatory proteins independently. At this promoter a heterologous nucleoprotein complex is formed in which CytR is sandwiched between tandem DNA-bound cAMP-CRP complexes (26, 33). For the CytR-mediated regulation of $deo\text{-}p_2$, there is a strict requirement of a 53-bp center-to-center distance between the two activator binding sites (30, 32). Our results with the $tsx\text{-}p_2$ promoter demonstrate that the tandem targets for the cAMP-CRP activator complex are only separated by 33 bp and that the CytR repressor binds independently to a DNA segment that includes the sequences between the CRP targets and overlaps with the CRP-1 site (Fig. 1B). The nucleoprotein complex formed at $tsx\text{-}p_2$ encompasses sequences downstream from CRP-1; however, the extent of the combined footprint of the activator and repressor equals that observed at $deo\text{-}p_2$ (33). At present, it is unclear whether the stoichiometry of the regulatory proteins in the nucleocomplex at $tsx\text{-}p_2$ corresponds to that at $deo\text{-}p_2$.

Several hypotheses can be envisioned to explain the combined footprint of the cAMP-CRP activator and the CytR repressor at $tsx\text{-}p_2$ (Fig. 8). In the first model, cAMP-CRP still binds to CRP-1 and CytR occupies the sequences on both sides of CRP-1. The second model hypothesizes a complex in which CytR alone occupies the region from CRP-2 close to the −10 sequence. In the third model, CytR interacts with the same DNA sequences in the combined footprint as in the independent footprint, and cAMP-CRP, rather than binding to CRP-1, binds to the region located immediately downstream of the CytR-binding sequence (Fig. 8). Inspection of the nucleotide sequence in this region of the $tsx\text{-}p_2$ promoter reveals the sequence 5′-TGTGAN$_6$AT TTT-3′ (Fig. 1B), the left part of which is the consensus sequence for a cAMP-CRP-binding site (8). An interesting feature concerning the last model is that the center-to-center distance between the CRP-2 target and this putative cAMP-CRP-binding site is 53 bp, which is exactly the distance found between the two cAMP-CRP-binding sites in $deo\text{-}p_2$. Furthermore, the *tsx-500* mutation, which only affects the formation of the combined cAMP-CRP–CytR nucleoprotein complex (Fig. 7A), is located in the N_6 sequence of the putative cAMP-CRP-binding site. Therefore, it could in principle decrease the binding of cAMP-CRP complex in a manner analogous to that of the *tsx-502* allele. However, the properties of the *tsx-500* mutation do not rule out the two other hypotheses, as this mutation may also be viewed as a mutation in a CytR box (5′-ATTGCT-3′ [the mutated nucleotide is underlined]). The determination of protein-DNA

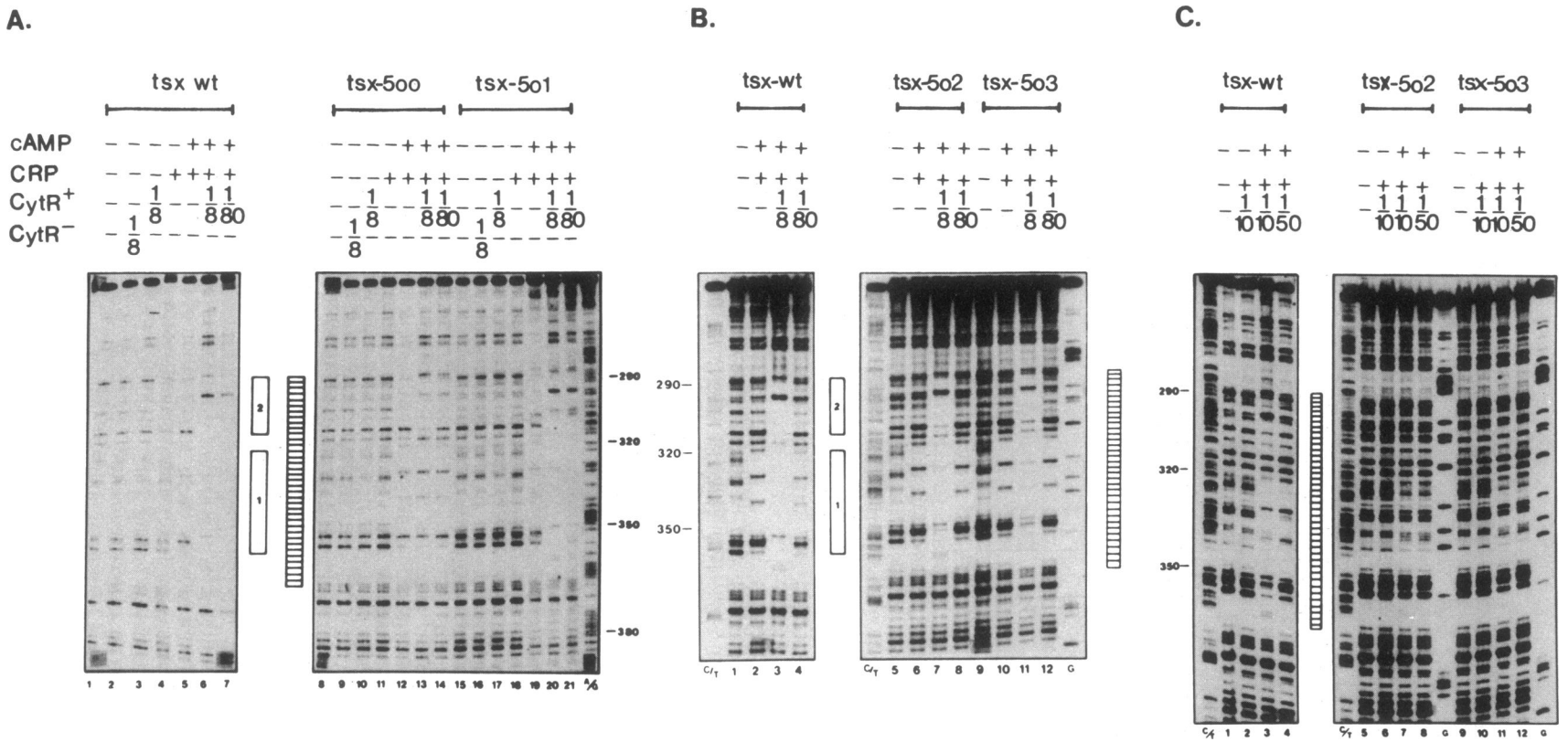

FIG. 7. DNase I footprint analysis of the wild-type and mutant *tsx-p*$_2$ promoters in the presence of both the CytR protein and cAMP-CRP. The radiolabelled DNA fragments (40 ng/ml) used as template DNA in DNase I footprints for the *tsx* wild-type and mutant promoters were identical to those described in the legend to Fig. 5. cAMP was added to the reaction mixture to a final concentration of 50 μM. Final CRP concentrations in the reaction mixtures were 2 (A), 1.7 (B), and 0.08 (C) μM, respectively. The concentration of the CytR$^+$ and CytR$^-$ extracts in the assay (indicated values, 10^{-2}) are shown. The open and hatched bars delineate the DNA regions protected by the cAMP-CRP activator complex and the cAMP-CRP–CytR repressor complex, respectively. The A + G sequencing reaction (rightmost lane) (A) and the C + T sequencing reaction (leftmost lane) (B and C) of the DNA fragment from the *tsx* wild-type promoter were used to calibrate the gels. (B and C) The rightmost panel shows the C + T and the G sequencing reactions of the *tsx-502* and *tsx-503* alleles, respectively. The numbers on the right (A) and on the left (B and C) refer to the base pair positions shown in Fig. 1B.

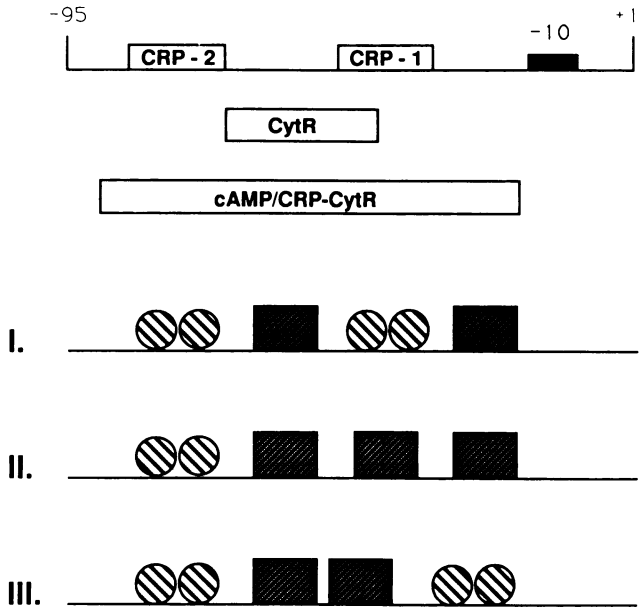

FIG. 8. Models for the cAMP-CRP–CytR DNA interactions at the tsx-p_2 promoter. The positions of the CRP-1 and CRP-2 targets upstream of the −10 region of the tsx-p_2 promoter are indicated. The regions protected from DNase I digestion by the CytR repressor alone and by the cAMP-CRP–CytR nucleoprotein complex are shown by the open bars. The CytR protein and the cAMP-CRP activator complex are represented by the hatched boxes and hatched circles, respectively. I, II, and III refer to the different models discussed in the text.

stoichiometries and detailed genetic analyses are required to distinguish between these models.

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