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Identification of Repressor Binding Sites Controlling Expression of Tetracycline Resistance Encoded by Tn*10*

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The regulatory region controlling the expression of tetracycline resistance and repressor genes contains two nearly identical regions of dyad symmetry. Deletions of this control region were isolated by digestion with S1 nuclease. The ability of these deletions to bind the *tet* repressor was determined by an in vivo repressor titration assay. The results indicate that repressor specifically binds both regions of dyad symmetry.

The tetracycline resistance determinant associated with the plasmid R100 is part of a transposable genetic element called transposon Tn*10* (16). Resistance does not result from the inactivation of the antibiotic (17). Instead, the resistance mechanism appears to involve the active efflux of tetracycline from the cell (1, 21). In addition, decreased ribosomal sensitivity to tetracycline may contribute to resistance (17, 28).

The expression of tetracycline resistance is regulated; preincubation with a subinhibitory concentration of tetracycline increases the level of resistance (13). Tn*10* directs the synthesis of two tetracycline-inducible proteins with apparent molecular weights of 36,000 and 25,000 (26–28). The largest is referred to as the TET protein and is known to be negatively regulated by a repressor which is inactive in the presence of tetracycline (27). The TET protein is membrane-associated and is essential for tetracycline resistance (15, 27). The 25-kilodalton protein is the repressor and has been shown to negatively regulate its own synthesis (2, 26). The location of the structural genes for both of these proteins has been determined (7, 15, 26) (Fig. 1).

These two genes are transcribed divergently from a common control region which has been shown to bind purified RNA polymerase (15) and *tet* repressor (9). The DNA sequence of this regulatory region has been determined and has revealed the presence of two nearly identical regions of dyad symmetry (4, 10). The experiments described in this report demonstrate that these symmetrical sequences are the repressor binding sites.

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MATERIALS AND METHODS

Materials. Growth media were prepared as described by Miller (24). Concentrations of antibiotics (Sigma Chemical Co.) used were 60 μg of kanamycin per ml and 100 μg of ampicillin per ml.

Restriction enzymes were purchased from Bethesda Research Laboratories or New England Biolabs and were used as described by the manufacturer. S1 nuclease and T4 polynucleotide kinase were obtained from P-L Biochemicals, Inc. T4 DNA ligase was a gift from R. Simoni.

Bacterial strains. All bacterial strains were derivatives of *Escherichia coli* K-12. Strain JD600 is *leu hsdM hsdR tonA supE lacY thi*. Strain MZ12 is *Δlac(ipozy)X74 trp thi*.

Plasmids and phage. Plasmids pBR322, pACYC177, and pRT86 have been described previously (5, 6, 26). Plasmid pRZ4045 contains a *lac* promoter fragment cloned between the *Eco*RI and *Sal*I sites of pBR322 (R. Johnson, M. Peterson, and W. Reznikoff, unpublished data). Plasmid DNA was isolated by the method of Humphreys et al. (12).

The bacteriophage λRS205 is a promoter cloning vector that contains a *lacZ* gene derived from a *trp-lac* fusion strain, W205, that deletes the *lacP0* region but leaves the *lacZ* gene intact. This phage contains a single *Eco*RI site and a single *Sal*I site located between the phage *att* site and the promoterless *lacZ* gene. A promoter-bearing DNA fragment inserted into these sites controls the expression of *lacZ*. The construction of this vector will be described elsewhere (K. P. Bertrand, K. Postle, L. V. Wray, Jr., and W. S. Reznikoff, manuscript in preparation).

Plasmid and deletion constructions. A plasmid clone of the repressor gene was constructed by digesting pRT86 and pACYC177 with *Hin*cII. After heat inactivation of the restriction enzyme, the DNA was treated with T4 DNA ligase and used to transform JD600 cells by the method of Mandel and Higa (18). The cells were inoculated into 4 ml of LB medium and incubated at 37°C with shaking for 90 min. Transformants were selected by plating on tryptone-yeast extract plates containing kanamycin. Since the *Hin*cII site of

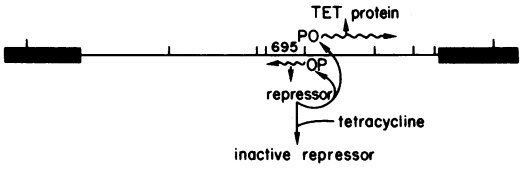

FIG. 1. Physical and genetic map of transposon Tn*10*. The thick lines represent the inverted repeat sequence of IS*10*, and unlabeled vertical lines are *Hin*cII cleavage sites. The structural gene for the repressor is located entirely within the 695-base-pair *Hin*cII fragment. P, Promoter; O, operator.

pACYC177 lies within the gene encoding β-lactamase, the transformants were screened for sensitivity to ampicillin. The presence of the *Hin*cII 695 fragment was confirmed by electrophoretic analysis of *Hin*cII digests of the resulting plasmids. The orientation of the insertions was determined by comparing the restriction patterns from a double digestion with *Bam*HI and *Xba*I. pRT241 contains the Tn*10* *Hin*cII 695 fragment inserted so that the *tet* repressor and β-lactamase genes are in opposite orientations.

Deletions of the *tet* regulatory region were constructed with pRT301 digested with either *Xba*I or *Sal*I. Linearized plasmid DNA (20 μg) was treated with 100 units of S1 nuclease at 28°C in a buffer consisting of 25 mM NaCl–3 mM $ZnSO_4$–25 mM sodium acetate (pH 5.0). Portions were removed after 5, 10, 15, 20, 25, and 30 min of incubation, and reactions were terminated by extraction with phenol. After extraction with ether, the samples were dialyzed against 10 mM Tris (pH 7.9) with Millipore VMWP filters as described by Marusyk and Sergeant (19). The DNA was self-ligated with T4 DNA ligase and used for transformation as described above. Transformants were selected by plating on tryptone-yeast extract plates containing ampicillin.

DNA sequencing. Deletions generated from the *Sal*I site were end labeled at the *Xba*I restriction site by treatment with [γ-^{32}P]ATP (Amersham Corp.) and polynucleotide kinase. After digestion with *Bgl*I, the labeled fragments were isolated and the sequence determined by the method of Maxam and Gilbert (20). The deletions generated from the *Xba*I restriction site were cloned as *Eco*RI-*Sal*I fragments into the single-stranded M13 cloning vector Mp8 (23) and were sequenced by the method of Sanger et al. (25).

β-Galactosidase assays. Cells were grown in M9 minimal medium supplemented with 0.2% glucose, 80 μg of tryptophan per ml, 4 μg of thiamine per ml, and antibiotics where appropriate. Cultures were assayed by the method of Miller (24).

RESULTS

Construction of recombinant plasmids and phage. In an earlier study (26), the *tet* repressor gene was mapped to the Tn*10* *Hin*cII 695 fragment. To construct a plasmid encoding the repressor, this fragment was inserted into the *Hin*cII site of pACYC177. The resulting plasmid, pRT241, is resistant to kanamycin and is compatible with ColE1 replicons such as pBR322. This plasmid does not contain the operator sites controlling the expression of the repressor, and thus, repressor synthesis is unregulated.

The plasmid pRT301 has the 160-base-pair

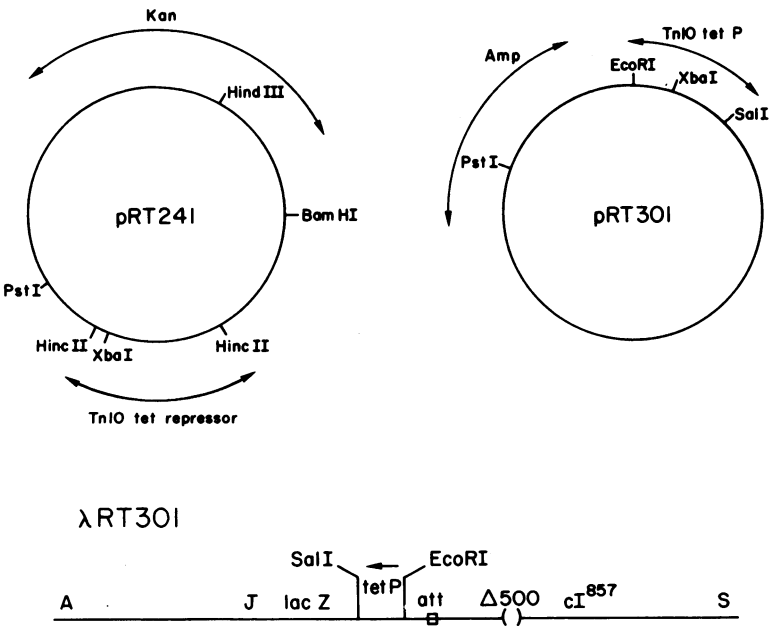

FIG. 2. Structure of plasmids and phage. The Tn*10* portions in the different plasmids are shown as well as the location of the kanamycin (Kan) and ampicillin (Amp) resistance genes.

TABLE 1. Repressor titration

Plasmids[a]	β-Galactosidase activity[b]
None	5,080
pACYC177	5,160
pRT241	206
pRT241 and pBR322	293
pRT241 and pRZ4045	209
pRT241 and pRT301	3,730
pRT241 and pRT315	3,870
pRT241 and pRT317	1,700
pRT241 and pRT320	202
pRT241 and pRT323	1,630
pRT241 and pRT331	3,590
pRT241 and pRT332	2,700
pRT241 and pRT333	1,210
pRT241 and pRT336	1,230
pRT241 and pRT339	208

[a] All assays were performed in strain MZ12 which is lysogenic for λRT301.

[b] β-Galactosidase activity is in units described by Miller (24). Values given are the average from three independent cultures. All assays were repeated at least twice.

*Taq*I fragment containing the *tet* control region cloned between the *Eco*RI and *Sal*I sites of pBR322 (Fig. 2). This *Eco*RI-*Sal*I fragment was cloned into the promoter cloning vector λRS205 to give λRT301. In this recombinant phage, the expression of β-galactosidase is controlled by the promoter for the *tet* resistance gene. The construction of this plasmid and phage will be described elsewhere (K. P. Bertrand, K. Postle, L. V. Wray, Jr., and W. S. Reznikoff, manuscript in preparation).

Assay for repressor binding. The binding of *tet* repressor was measured by repressor titration with a lysogen of the *tet* promoter-*lacZ* fusion phage λRT301. Repression of β-galactosidase was observed in the presence of a plasmid, such as pRT241, which encoded the *tet* repressor (Table 1). The introduction of a multicopy plasmid containing the *tet* control region into this strain resulted in a derepressed expression of *lacZ* due to the competition of the multiple operators for binding of the *tet* repressor. Thus, the plasmid pRT301 caused a large elevation in the level of β-galactosidase activity. It is interesting to note that plasmid pBR322 caused a slight increase in the level of β-galactosidase. When the pBR322 *tet* promoter region was removed, as in pRZ4045, this increase was not observed.

Isolation and sequence determination of deletions of the *tet* control region. To determine the DNA sequence required for repressor binding activity, a series of plasmids containing deletions of the *tet* control region in pRT301 were prepared. The endpoints of the various deletions are shown in Fig. 3.

The deletions 315, 317, 320, and 323 were generated by S1 nuclease digestion from the *Xba*I site, whereas deletions 331, 332, 333, 336, and 339 were generated from the *Sal*I site.

Effect of deletions on repressor binding. Plasmids containing the deletions of the *tet* control region were analyzed for their ability to bind repressor by the repressor titration assay (Table 1).

Plasmids pRT317 and pRT323 had an intermediate level of repressor titration. These plasmids contain deletions of all or part of O_1, a region of dyad symmetry, without extending into O_2, the second region of dyad symmetry. Plasmid pRT320, which contains a deletion extending into O_2, does not have the ability to bind repressor.

Plasmids pRT331 and pRT332 have deletion endpoints that differ by only two base pairs. The lower level of β-galactosidase observed for pRT332 suggests that these base pairs play a role in repressor binding. Plasmids pRT333 and pRT336 contain deletions of most of O_2 and showed an intermediate level of repressor titration. The deletion in pRT339 extends into O_1 and abolishes repressor binding activity.

DISCUSSION

A number of regulatory proteins have been shown to bind specifically to DNA sequences with dyad symmetries (3, 8, 14). In the present report, the *tet* repressor was shown to bind to the two regions of dyad symmetry that lie within the *tet* control region. The plasmids which contained deletions of O_1 had a higher level of derepression than did the plasmids which had deletions of O_2. This suggests that O_2 has a higher affinity for repressor.

FIG. 3. DNA sequence of the *tet* control region and deletions. The transcription start sites are shown by the arrows. The two regions of dyad symmetry, O_1 and O_2, are indicated.

It has been reported (9, 11) that the *tet* control region stoichiometrically binds four *tet* repressor molecules. Since repressor binds to two sites within this region, this suggests that each operator binds two repressor molecules, and thus, the functional form of the *tet* repressor is a dimer. The multiple operators at O_R and O_L in phage lambda are known to bind dimers of the *cI* and *cro* repressors (14).

The Tn*10* *tet* repressor has a weak affinity for the *tet* control region of pBR322. The tetracycline resistance determinants of Tn*10* and pBR322 have been placed in different classes based upon their different levels of resistance to tetracycline analogs and by DNA-DNA hybridization experiments (22). In spite of this, it has been noted that these two regulatory regions have significant sequence homology (4). The work presented here shows that there is some weak recognition between the regulatory elements of these two different resistance determinants.

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