Overlapping divergent promoters control expression of Tn10 tetracycline resistance

(DNA sequence; operator; repressor; regulatory region; dyad symmetry; plasmid pBR322; S1 nuclease mapping)

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SUMMARY

We have previously examined the genetic organization and regulation of the Tn10 tetracycline-resistance determinant in Escherichia coli K-12. The structural genes for tetA, the Tn10 tetracycline-resistance function, and for tetR, the Tn10 tet repressor, are transcribed in opposite directions from promoters in a regulatory region located between the two structural genes. Expression of both tetA and tetR is induced by tetracycline. Here we report the DNA sequence of the Tn10 tet regulatory region. The locations of the tetA and tetR promoters within this region were defined by S1 nuclease mapping of the 5' ends of in vivo tet RNA. The tetA and tetR promoters overlap; the transcription start points are separated by 36 bp. We propose that two similar regions of dyad symmetry within the Tn10 tet regulatory region are operator sites at which tet repressor binds to tet DNA, thereby inhibiting transcription initiation at the tetA and tetR promoters. The Tn10 tet regulatory region and the pBR322 tet regulatory region show significant DNA sequence homology (53%).

INTRODUCTION

The transposable element Tn10 determines high-level Tc resistance in E. coli and other enteric bacteria (Foster et al., 1975; Kleckner et al., 1975). Expression of Tn10 Tc resistance is regulated; exposure of resistant bacteria to subinhibitory levels of Tc induces maximal expression of resis-

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Abbreviations: bp, base pairs; kDa, kilodaltons; Tc, tetracycline.

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The structural genes for the 36-kDa and 25-kDa proteins, designated tetA and tetR, respectively, have been localized within an approx. 2200-bp region of Tn10 (Jorgensen and Reznikoff, 1979; Coleman and Foster, 1981); genetic analyses suggest that tetA and tetR are transcribed from divergent promoters located between the two genes (Wray et al., 1981). Here we report the DNA sequence of the region that contains the tetA and tetR promoters and the operator site(s) at which tetR repressor acts.

Several lines of evidence suggest that the tetA regulatory region and the tetR regulatory region are located in the immediate vicinity of a HincII restriction site at the junction of 695-bp and 1275-bp HincII restriction fragments (Fig. 1): (i) Deletions of the region to the right of the HincII site do not appear to alter the tetR structural gene, although these deletions influence the level of tetR expression (Wray et al., 1981). (ii) Deletions of the region to the left of the HincII site do not appear to alter the tetA structural gene, although these deletions, likewise, influence the level of tetA expression (Wray et al., 1981). (iii) The HincII site is partially protected from HincII cleavage by purified RNA polymerase (Jorgensen and Reznikoff, 1979) and purified tetR repressor protein (Hillen et al., 1982). (iv) Genetic fusions of the tetA regulatory region to the lacZ structural gene indicate that the tetA promoter and operator(s), are within a 158-bp TaqI restriction fragment that spans the HincII site (K.P. Bertrand, L.V. Wray Jr. and W.S. Reznikoff, in preparation); similar experiments indicate that the tetR promoter and operator(s) are within a 451-bp AluI restriction fragment that spans the HincII site (K.P. Postle, T. Nguyen and K.P. Bertrand, in preparation). (v) Finally, binding studies with purified tetR repressor indicate that the tet operator(s) are within a 287-bp AluI-HaeIII restriction fragment that spans the HincII site (Hillen et al., 1982).

**MATERIALS AND METHODS**

(a) DNA sequencing

The DNA sequence of the tet regulatory region of plasmid pRT29 (Jorgensen and Reznikoff, 1979) was determined by the procedures of Maxam and Gilbert (1980). pRT29 contains a single Tn10 HpaI fragment that is comprised of 695-bp and 1275-bp HincII fragments (Fig. 1); the plasmid has a unique XbaI site within the tet regulatory region. Following treatment by bacterial alkaline phosphatase, the 5' ends of XbaI-linearized plasmid DNA were labeled with T4 polynucleotide kinase and [γ-32P]ATP. The 3' ends of XbaI-linearized plasmid DNA were labeled with the Klenow fragment of E. coli DNA polymerase I and [α-32P]dATP in the presence of dCTP, dGTP, and dTTP. Following BspI digestion, the labeled fragments were isolated and subjected to sequence analysis. The sequence in the region of the XbaI site (bp 60–65, Fig. 2) was verified by analysis of the BspI-HincII fragment labeled at its HincII end (bp 87–92).

(b) S1 nuclease analysis of tet RNA

E. coli K-12 C600 harboring the Tn10 tet plasmid pRT29 was grown in LB broth containing 10 μg/ml tetracycline hydrochloride (Sigma). LB broth contained per liter: 10 g tryptone (Difco), 8 g NaCl and 5 g yeast extract (Difco). At an A_{550} of 0.35 the cells were rapidly chilled and, following addition of 20 μg/ml chloramphenicol (Sigma), harvested by centrifugation. The cells were lysed by addition of 300 μg/ml lysozyme (Sigma) and 1% sodium dodecyl sulfate, the lysate was ex-
tracted with phenol, and nucleic acids in the aqueous phase were precipitated with ethanol. The DNA probes for tetA and tetR RNA were prepared by digestion of pRT29 DNA with XbaI + NcoI and HinfI, respectively. The 136-bp XbaI-NcoI fragment and the 1130-bp HinfI fragment were eluted from 8% polyacrylamide gels, treated with bacterial alkaline phosphatase (Bethesda Research Laboratories) and 5'-end-labeled with \([\gamma-^{32}P]ATP\) (3000 Ci/mmol, Amersham). The DNA strands of the labeled fragments were separated in an 8% polyacrylamide gel as described by Maxam and Gilbert (1980), except that the sample buffer consisted of 10 mM HEPES, pH 8, 0.5% xylene cyanol, and 0.5% bromphenol blue in 98% deionized formamide. The separated strands were identified by DNA sequencing. Annealing of unlabeled RNA to 5'-end-labeled single-stranded DNA and S1 nuclease treatment were performed as described by Favaloro et al. (1980) and Treisman and Kamen (1981). In particular, the hybridizations were at 30°C for 16 h, and the hybrids were treated with 600 units/ml S1 nuclease (Sigma) at 15°C for 1 h. The S1 nuclease-resistant DNA and sequence reactions of appropriate 5'-end-labeled fragments were run in parallel on thin 8% polyacrylamide/urea gels (Maxam and Gilbert, 1980).

RESULTS

We determined the DNA sequence of a 201-bp region spanning the HincII restriction site in the tet regulatory region, including the 158-bp TaqI restriction fragment that, on the basis of genetic evidence, contains the tetA promoter and operator(s) (Fig. 2). The 5' ends of tetA and tetR RNA were localized within this region by S1 nuclease mapping (Fig. 3) (Berk and Sharp, 1977; Favaloro et al., 1980; Treisman and Kamen, 1981). Total unlabeled RNA was isolated from E. coli strain C600 containing plasmid pRT29, then annealed to single-stranded 5'-end-labeled DNA probes that span the tet regulatory region. The RNA-DNA hybrids were treated with S1 nuclease, and the DNA components of the S1-resistant hybrids were sized on polyacrylamide gels (Fig. 3). The slight heterogeneity (2-3 bases) in the lengths of the S1-resistant DNAs may reflect actual heterogeneity in the 5' ends of the tet RNAs. However, it seems more likely that this heterogeneity is primarily an artifact of the S1 nuclease procedure, since the relative amounts of the S1-resistant fragments vary with the concentration of S1 nuclease employed. Assuming that the 5' ends of the RNAs detected by the S1 nuclease procedure do define transcription initiation sites, the principal right-
**Fig. 3.** Sl nuclease mapping of the 5' ends of retA and retR RNA. Lanes a and b: Labeled Sl nuclease-resistant DNA protected by retA RNA. The lower strand of the 136-bp XbaI-NcoI fragment (bp 65-200, Fig. 2) labeled at the 5' NcoI end was annealed to unlabeled RNA in order to localize the 5' ends of rightward (retA) transcripts that cross the NcoI site. Lanes a and b show the results of independent experiments. Lanes c and d: G and G+A sequencing reactions for the double-stranded 107-bp HincII-NcoI fragment (bp 90-200, Fig. 2) labeled at the 5' NcoI end. Lanes e and f: Labeled Sl nuclease-resistant DNA protected by retR RNA. The upper strand of the 1130-bp HincI fragment (bp 4 to beyond 201, Fig. 2) labeled at the 5' end in the retR gene was annealed to unlabeled RNA in order to localize 5' ends of leftward (retR) transcripts that cross the HincI site in the retR gene. Lanes e and f show the results of independent experiments. Lanes g and h: G and G+A sequencing reactions for the double-stranded 193-bp HincII-NcoI fragment (bp 4-196, Fig. 2) labeled at the 5' HincII end. Lengths of the most prominent Sl nuclease-resistant DNA fragments are given in bp.

ward (tetA) transcript is initiated at bp 123 (Fig. 2) and the principal leftward (tetR) transcript is initiated at bp 86. Thus the tetA and tetR transcription initiation sites are separated by 36 bp.

Similar analyses of the 5' ends of tet RNA synthesized in vitro gave a more complex result. Rightward in vitro RNA starts were observed near nucleotides 123, 103, and 86; and leftward in vitro RNA starts were observed near nucleotides 86 and 106 (data not shown). The significance of the additional transcription initiation sites observed in vitro is unclear.

**DISCUSSION**

*E. coli* RNA polymerase can protect a region of 70–75 bp from DNase I digestion (Schmitz and Galas, 1979). Within this extended promoter region, sequences in the "−10 region" and "−35 region" appear to have particular functional significance, as indicated by the regions of greatest homology between different *E. coli* promoters, the locations of promoter mutations, and the locations of inferred contact sites between RNA polymerase and several well-characterized promoters (Reznikoff and Abelson, 1978; Scherer et al., 1978; Rosenberg and Court, 1979; Siebenlist et al., 1980). The most striking feature of the tetA and tetR promoters is the manner in which they overlap in the −10 and −35 regions (Fig. 2). Recent comparisons of *E. coli* promoters indicate that the −10 and −35 sequences are generally homologous to TATAAT and TTGACA, respectively (Rosenberg and Court, 1979; Siebenlist et al., 1980). Thus the probable −10 sequence of the tetA promoter is TATTTT (bp 112-117, Fig. 2) and for the tetR promoter it is TAGAGT (bp 93 98, Fig. 2). The −35 sequence of the tetA promoter is identical to the consensus sequence (TTGACA, bp 88-93, Fig. 2), whereas the probable −35 sequence of the tetR promoter is less homologous to the consensus sequence (TTGTAA, bp 116-121, Fig. 2). Like other *E. coli* promoters, the tetA and tetR promoters are comparatively A + T rich (tetA, bp 83-142, 67% A + T; tetR bp 67-126, 70% A + T). Moreover, the entire 201-bp region that spans the tet regulatory region is 65% A + T. The high A + T content of this region is consistent with its low thermal stability (Hillen and Unger, 1982). The HincII restriction site (GT-GAC, bp 87–92), previously shown to be protected from HincII cleavage by RNA polymerase (Jorgensen and Reznikoff, 1979), is located within the −35 region of the tetA promoter; however, it is likely that RNA polymerase poised to initiate transcription at either the tetA or tetR promoter would protect the HincII site from cleavage. It is also noteworthy that the tetA RNA transcript is initiated within a run of pyrimidine nucleotides (CTCCCT, bp 122–127), a feature that has been reported for relatively few promoters (Rosenberg and Court, 1979; Siebenlist et al., 1980).
The physical overlap between the tetA and tetR promoters suggests that RNA polymerase interaction with these promoters is mutually exclusive, and it raises the question of whether or not some mutations that reduce the efficiency of one promoter might thereby increase the efficiency of the other promoter by affecting the competition between the two promoters for RNA polymerase binding. The bioA and bioB promoters in E. coli (Guha et al., 1971; Otsuka and Abelson, 1978) and the $p_R$ and $p_{RM}$ promoters in phage $\lambda$ (Ptashne et al., 1980) are additional examples of divergent overlapping promoters. In neither of these cases is the overlap as extensive as it is for the tetA and tetR promoters. There does not appear to be strong competition between the bioA and bioB promoters; however, this may reflect the fact that the bio promoters are relatively weak (Barker et al., 1981).

Expression of both tetA and tetR is regulated by Tc and the tetR repressor (Levy and McMurray, 1974; Yang et al., 1976; Wray et al., 1981; Beck et al., 1982). Hillen et al. (1982) purified the tetR repressor, demonstrated that it binds to tet DNA in the absence of Tc but not in the presence of Tc, and localized repressor binding site(s) within the 187 bp Alul-HaeIII region (bp 24 to beyond 201 Fig. 2). Since regulatory proteins have, in general, been found to bind DNA sequences that exhibit varying degrees of dyad symmetry (Gilbert and Maxam, 1973; Bennett et al., 1976; Musso et al., 1977; Ptashne et al., 1980), it is highly probable that one or both of the regions of dyad symmetry within the tet promoter region (bp 93–111 and 123–141) are the operator site(s) to which tetR repressor binds. The two regions of dyad symmetry show extensive homology; the sequence 5'-CTATCA-TGATAG-G is present in both regions. The DNA sequence suggests a model in which tetR repressor binds to tandem operators in the tetA-tetR promoter region. Furthermore, the topology of the tet promoters and the putative operator sites suggests that repressor bound at either operator site would almost certainly inhibit tetA transcription. Repressor bound at the leftward operator site (bp 93–111) would, likewise, almost certainly inhibit tetR transcription; however, it is difficult to predict what the effect of repressor bound at the rightward operator site (bp 123–141) would be on tetR expression. Thus, it appears that transcription of the tetA and tetR structural genes may be regulated by repressor binding to shared operator sites. Multiple operators have previously been described for the cla repressor and shared sites of phage lambda (Ptashne et al., 1980) and the lexA protein of E. coli (Little et al., 1981).

Predicted amino-terminal amino acid sequences for the tetA and tetR gene products are shown in Fig. 2. Genetic analyses (K. Postle, T. Nguyen and K.P. Bertrand; K.P. Bertrand, L.V. Wray and W. Reznikoff, in preparation) suggest that the amino-terminal coding regions for the tetR and tetA structural genes are located between the XbaI site and the HincII site (bp 60–93, Fig. 2), and the HincII site and the TaqI site (bp 88–162), respectively. DNA sequence data (Hillen et al., 1983; K. Postle, T. Nguyen and K.P. Bertrand, in preparation) indicate that the designated translational reading frames are the only open reading frames that extend through the tetA and tetR structural genes. The predicted translation initiation codons are, in each case, preceded by polyuridine sequences that resemble the sequences of known ribosome-binding sites (Shine and Dalgarno, 1974; Atkins, 1979; Scherer et al., 1980; Gold et al., 1981). Assuming the predicted translation initiation sites are correct, the tetA and tetR structural genes are separated by a non-translated regulatory region of 81 bp.

Tc-resistance determinants have been classified by several criteria, including the level of Tc resistance conferred, the level of resistance conferred to Tc analogs, and DNA homology as judged by DNA-DNA hybridization experiments (Mendez et al., 1980). By these criteria the Tc-resistance determinants of Tn10 and the plasmid pBR322 represent two distinct classes of Tc-resistance determinant (Mendez et al., 1980). Therefore, it is of interest to compare the DNA sequences of the Tn10 tetA and pBR322 tet regulatory regions (Fig. 4). The pBR322 tet promoter region, defined by mutational studies (Rodriguez et al., 1979) as well as its sequence homology with other E. coli promoters (Sutcliffe, 1979), shows significant sequence homology (53%) with the Tn10 tetA promoter region, including the conserved hexanucleotide TTAGAC which is present in the -35 regions of both promoters. The pBR322 tet promoter, like the Tn10 tetA promoter, is A + T rich (66% A + T for
Fig. 4. Comparison of the Tn10 tetA promoter region and the pBR322 tet promoter region. The Tn10 sequence corresponds to bp 84-143 in Fig. 2, and the pBR322 sequence corresponds to bp 6-64 of the published sequence (Sutcliffe, 1979). The -35 and -10 regions of the two promoters are indicated; regions of dyad symmetry are indicated with horizontal lines, and homologies between the two sequences are indicated with vertical lines. A 1-bp gap was introduced into the pBR322 sequence to maximize the homology with Tn10.

pBR322 vs. 67% A + T for Tn10). However, the most striking similarity between the two tet promoters is in the regions of dyad symmetry that are situated in nearly identical locations between the -35 region and -10 region and distal to the transcription initiation sites (Fig. 4). As in the Tn10 tetA promoter region, the two dyad-symmetry elements in the pBR322 promoter region are similar to each other; the pentanucleotide TATCA is present in both symmetry elements in both promoter regions. We speculate that the symmetry elements in the pBR322 tet promoter region are, like their homologues in the Tn10 tetA promoter region, operator sites at which tet repressor binds.

The pBR322 tet region is derived from the plasmid pSC101 (Bolivar et al., 1977) which, like Tn10, encodes inducible Tc resistance (Tait and Boyer, 1978). The similarities between the pBR322 tet and Tn10 tetA promoter regions suggest that the two promoter regions, and presumably, the two Tc-resistance determinants, evolved from a common ancestral sequence. Although there has been significant divergence at the nucleotide level (47%), essential structural features of the promoters and putative operators have, apparently, been conserved. Indeed, the similarity between the pBR322 and Tn10 tet regulatory regions raises the question of whether or not there might be some recognition between the regulatory elements of the two systems.

By analogy with the genetic organization of Tn10 tet, the promoter for the putative pSC101 tet repressor gene could be present in pBR322. Stuber and Bujard (1981) detected divergent overlapping promoters in the tet promoter region of pBR322 by analyzing the lengths of short in vitro transcripts. Their data suggest, however, that the divergent promoters in the pBR322 tet region are arranged somewhat differently than the Tn10 tetA and terR promoters; the RNA transcripts produced from the pBR322 promoters appear to overlap by 10-40 nucleotides. Nevertheless, the overall genetic organization and regulation of the pSC101 and Tn10 Tc resistance determinants may be quite similar.

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