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Overlapping divergent promoters control expression of Tn*10* 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 Tn*10* tetracycline-resistance determinant in *Escherichia coli* K-12. The structural genes for *tetA*, the Tn*10* tetracycline-resistance function, and for *tetR*, the Tn*10* *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 Tn*10* *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 Tn*10* *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 Tn*10* *tet* regulatory region and the pBR322 *tet* regulatory region show significant DNA sequence homology (53%).

INTRODUCTION

The transposable element Tn*10* determines high-level Tc resistance in *E. coli* and other enteric bacteria (Foster et al., 1975; Kleckner et al., 1975). Expression of Tn*10* Tc resistance is regulated; exposure of resistant bacteria to subinhibitory levels of Tc induces maximal expression of resistance (Franklin, 1967; Robertson and Reeve, 1972). Although the mechanism of Tc resistance is not completely understood, recent studies indicate that active efflux of Tc is a major component of the resistance mechanism (McMurray et al., 1980; Ball et al., 1980). The Tn*10* Tc resistance determinant (*tet*) directs the synthesis of two proteins, a 36-kDal membrane protein that is essential for Tc resistance (Levy and McMurray, 1974; Levy et al., 1977, Jorgensen and Reznikoff, 1979) and a 25-kDa repressor protein that, in the absence of Tc, inhibits synthesis of the 36-kDa resistance protein (Yang et al., 1976; Wray et al., 1981). The repres-

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

sor protein also negatively regulates its own synthesis (Wray et al., 1981; Beck et al., 1982). The structural genes for the 36-kDal and 25-kDal proteins, designated *tetA* and *tetR*, respectively, have been localized within an approx. 2200-bp region of Tn*10* (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 sites(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 *Hin*cII restriction site at the junction of 695-bp and 1275-bp *Hin*cII restriction fragments (Fig. 1): (i) Deletions of the region to the right of the *Hin*cII 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 *Hin*cII 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 *Hin*cII site is partially protected from *Hin*cII 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 *Taq*I restriction fragment that spans the

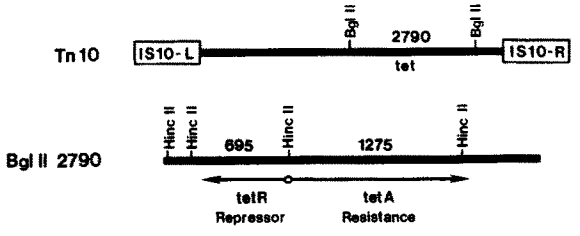

Fig. 1. Physical maps of the Tn*10* element. (Top) Tn*10* consists of two 1400-bp inverted repeat sequences (I*S10* left and I*S10* right) flanking a 6400-bp central region (Halling et al., 1982). (Bottom) The 2790-bp *Bgl*II fragment within the central region of Tn*10* spans the *tetR* repressor and *tetA* resistance genes; *tetR* is located within the 695-bp *Hin*cII fragment; *tetA* is located largely within the 1275-bp *Hin*cII fragment; *tetR* and *tetA* are transcribed from promoters located near the *Hin*cII site between the structural genes.

*Hin*cII 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 *Alu*I restriction fragment that spans the *Hin*cII 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 *Alu*I-*Hae*III restriction fragment that spans the *Hin*cII 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 Tn*10* *Hpa*I fragment that is comprised of 695-bp and 1275-bp *Hin*cII fragments (Fig. 1); the plasmid has a unique *Xba*I site within the *tet* regulatory region. Following treatment by bacterial alkaline phosphatase, the 5′ ends of *Xba*I-linearized plasmid DNA were labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The 3′ ends of *Xba*I-linearized plasmid DNA were labeled with the Klenow fragment of *E. coli* DNA polymerase I and [α-^{32}P]dATP in the presence of dCTP, dGTP, and dTTP. Following *Bsp*I digestion, the labeled fragments were isolated and subjected to sequence analysis. The sequence in the region of the *Xba*I site (bp 60–65, Fig. 2) was verified by analysis of the *Bsp*I-*Hin*cII fragment labeled at its *Hin*cII end (bp 87–92).

(b) S1 nuclease analysis of *tet* RNA

E. coli K-12 C600 harboring the Tn*10* *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 *Xba*I + *Nco*I and *Hin*fI, respectively. The 136-bp *Xba*I-*Nco*I fragment and the 1130-bp *Hin*fI fragment were eluted from 8% polyacrylamide gels, treated with bacterial alkaline phosphatase (Bethesda Research Laboratories) and 5′-end-labeled with [γ-^{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 *Hin*cII restriction site in the *tet* regulatory region, including the 158-bp *Taq*I restriction fragment that, on the basis of genetic evidence, contains the *tet*A 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-

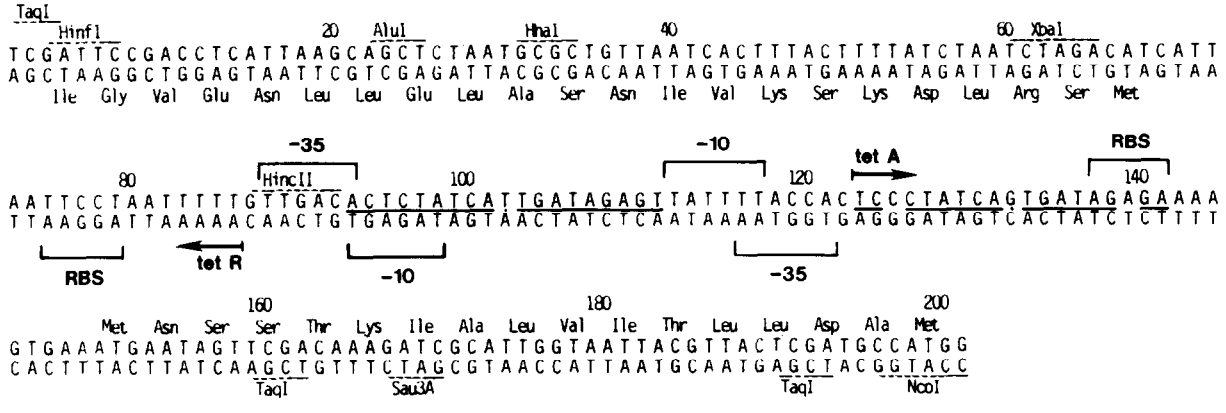

Fig. 2. DNA sequence of the Tn*10* *tet* regulatory region. Features of the *tetA* promoter, including the transcription start point (→), two regions of homology with other *E. coli* promoters (the −35 and −10 regions), the probable ribosome binding site (RBS), and the predicted amino-terminal amino acid sequence of the *tetA* gene product are indicated above the DNA sequence. The same features of the *tetR* promoter and structural gene are indicated below the DNA sequence. Two regions of dyad symmetry, the putative *tet* operator sites, are also indicated (lines between DNA strands at bp 93–111 and 123–141). The transcription start points were deduced by S1 nuclease mapping of *tet* RNA (Fig. 3); the assignments for probable translation initiation sites are discussed in the text. The DNA sequence is numbered from left to right starting at the *Taq*I site near the amino-terminus of *tetR*.

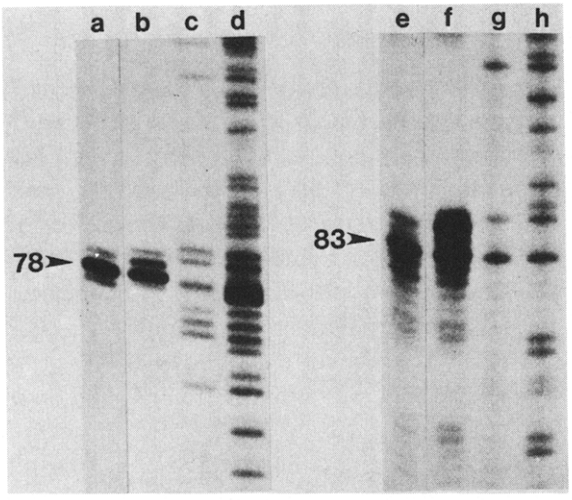

Fig. 3. S1 nuclease mapping of the 5′ ends of *tetA* and *tetR* RNA. Lanes a and b: Labeled S1 nuclease-resistant DNA protected by *tetA* RNA. The lower strand of the 136-bp *Xba*I-*Nco*I fragment (bp 65–200, Fig. 2) labeled at the 5′ *Nco*I end was annealed to unlabeled RNA in order to localize the 5′ ends of rightward (*tetA*) transcripts that cross the *Nco*I 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 *Hin*cII-*Nco*I fragment (bp 90–200, Fig. 2) labeled at the 5′ *Nco*I end. Lanes e and f: Labeled S1 nuclease-resistant DNA protected by *tetR* RNA. The upper strand of the 1130-bp *Hin*fI fragment (bp 4 to beyond 201, Fig. 2) labeled at the 5′ end in the *tetR* gene was annealed to unlabeled RNA in order to localize 5′ ends of leftward (*tetR*) transcripts that cross the *Hin*fI site in the *tetR* 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 *Hin*fI-*Nco*I fragment (bp 4–196, Fig. 2) labeled at the 5′ *Hin*fI end. Lengths of the most prominent S1 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 *Hin*cII restriction site (GTTGAC, bp 87–92), previously shown to be protected from *Hin*cII 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 *Hin*cII 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 λ (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 *Alu*I-*Hae*III 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 C-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 *c*I repressor and *cro* genes 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 *Xba*I site and the *Hin*cII site (bp 60–93, Fig. 2), and the *Hin*cII site and the *Taq*I 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 polypurine 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 Tn*10* 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 Tn*10* *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 Tn*10* *tetA* promoter region, including the conserved hexanucleotide TTGACA which is present in the −35 regions of both promoters. The pBR322 *tet* promoter, like the Tn*10* *tetA* promoter, is A + T rich (66% A + T for

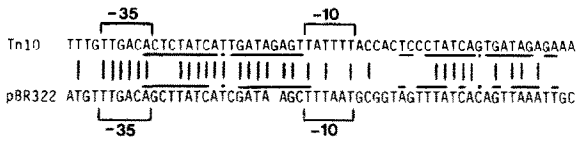

Fig. 4. Comparison of the Tn*10 tetA* promoter region and the pBR322 *tet* promoter region. The Tn*10* sequence corresponds to bp 84–143 in Fig. 2, and the pBR322 sequence corresponds to bp 6–64 of the published sequence (Sutcliffe, 1979). The −35 and −10 regions of the two promoters are indicated; regions 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 Tn*10*.

pBR322 vs. 67% A + T for Tn*10*). 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 Tn*10 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 Tn*10 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 Tn*10*, encodes inducible Tc resistance (Tait and Boyer, 1978). The similarities between the pBR322 *tet* and Tn*10 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 Tn*10 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 Tn*10 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 Tn*10 tetA* and *tetR* 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 Tn*10* TC resistance determinants may be quite similar.

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