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Transposon Tn*10* Contains Two Structural Genes with Opposite Polarity Between *tetA* and IS*10*$_R$

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The nucleotide sequence of the central part of Tn*10* has been determined from the rightmost *Hin*dIII site to IS*10*$_R$. This sequence contains two open reading frames with opposite polarity. The in vivo transcription start points in this sequence have been determined by S1 mapping. These results define one minor and two major promoters. The transcription starts of the two major promoters are only 18 base pairs apart, and the transcripts show different polarity and overlap by 18 base pairs. The nucleotide sequence reveals two regions with palindromic symmetry which may serve as operators. Their possible involvement in the regulation of transcription of both genes is discussed. Taken together these results allow for a maximal coding capacity of 138 amino acids directed toward IS*10*$_R$ and 197 amino acids directed toward *tetA*. The possible function of these gene products is discussed. The accompanying article (Braus et al., J. Bacteriol. 160:504–509, 1984) presents evidence that these genes are expressed.

Transposon Tn*10* mediates tetracycline resistance in enteric bacteria (6, 20). Two genes, namely, *tetA* and *tetR*, within the 6,700-base-pair (bp) long core region of Tn*10* are sufficient for the expression of tetracycline resistance (19, 29). *tetA* encodes a protein for active efflux of tetracycline from resistant cells (12, 19, 23, 24), whereas *tetR* encodes the TET repressor regulating the transcription of *tetA* and *tetR* (2, 10, 13, 29). The expression of *tetA* and *tetR* as well as a third gene leading to a 13- to 15-kilodalton protein is inducible by subinhibitory amounts of tetracycline (29, 30).

Aside from active efflux of tetracycline from resistant cells, two other mechanisms are thought to contribute to high-level tetracycline resistance encoded by Tn*10* (26). Preliminary evidence suggests that the gene loci encoding these mechanisms may be between *tetA* and IS*10*$_R$ on the Tn*10* sequence (5, 31). RNA polymerase binding sites have previously been mapped in this region (18).

To clarify the gene structure of Tn*10* between *tetA* and IS*10*$_R$ we determined the nucleotide sequence of approximately 960 bp spanning from the *Hin*dIII site to IS*10*$_R$, closing the gap between the previously published sequence data of Tn*10* (8, 12). Furthermore, we determined the transcriptional start points in vivo by S1 mapping. These data reveal the existence of two structural genes with opposite polarity in this part of Tn*10*.

MATERIALS AND METHODS

Materials. Restriction endonucleases, T4 DNA ligase, and S1 nuclease were purchased from Bethesda Research Laboratories, Bethesda, Md. Restriction digests were done as described previously. [^{32}P]phosphate was obtained from Amersham Corp. [^{32}P]ATP was synthesized from ADP and [^{32}P] as described previously (16). T4 polynucleotide kinase was a gift of V. Eckert and R. Heinzel.

Preparation of DNA. Tn*10* DNA was prepared from the plasmid pRT61. The 675-bp *Hpa*I fragment from pRT61 was subcloned into pUR222 (25), and the 420-bp *Hin*cII fragment from pRT61 was subcloned into pVH51 (9), yielding pWH201 to facilitate sequencing. The 1,050-bp *Hin*cII fragment subcloned into pFR51, now called pCB122, was a gift of C. Beck, Freiburg. Preparation of plasmids was done as described previously (11). DNA fragments were either eluted from polyacrylamide gels as described previously (21) or purified by RPC-5 chromatography (14).

Preparation of total RNA for S1 mapping. The preparation of total RNA was basically done as described previously (1). The respective cells were grown in 100 ml of L-broth containing 1% peptone, 0.5% yeast extract, and 0.17 M NaCl. At an optical density at 550 nm of 0.3 to 0.35 the cells were harvested and suspended in 3 ml of a solution of 0.02 M sodium acetate (pH 5.5), 0.5% sodium dodecyl sulfate, and 1 mM EDTA. After the addition of 3 ml of preheated redistilled phenol equilibrated with 0.02 M sodium acetate (pH 5.5), the mixture was gently shaken at 60°C for 5 min. After centrifugation the aqueous phase was extracted with preheated phenol. The RNA was precipitated by adding 3 volumes of ethanol and incubating for 30 min at −70°C. The RNA was collected by centrifugation, dissolved in 3 ml of the acetate-SDS buffer (see above), and precipitated two more times (as described above). The final precipitate was dissolved in 1 ml of distilled water. The RNA concentration was determined by measuring the optical density at 260 nm.

Determination of transcription start points by S1 mapping. Typically a DNA fragment with a 5′-^{32}P on the coding strand (ca. 40,000 cpm on 20 ng) was lyophilized together with 150 μg of RNA prepared from *Escherichia coli* R/R$_1$ transformed with pWH201 or *E. coli* CB471 containing pCB122 (C. Beck, personal communication). The dried nucleic acids were dissolved in 30 μl of hybridization buffer containing 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 6.4), 1 mM EDTA (pH 8.0), 0.4 M NaCl, and 80% formamide. The mixture was incubated at 75°C for 10 min and cooled gradually over 3 to 4 h to 37°C. Incubation was then continued for 2 h at 37°C. After the addition of 300 μl of S1 buffer containing 0.28 M NaCl, 0.05 M sodium acetate (pH 4.6), 4.5 mM $ZnSO_4$, and 20 μl of carrier single-stranded DNA per ml, the nucleic acids were treated with 50 to 250 U of S1 nuclease for 15 min. The reaction was terminated by two phenol extractions. After the phenol was removed twice

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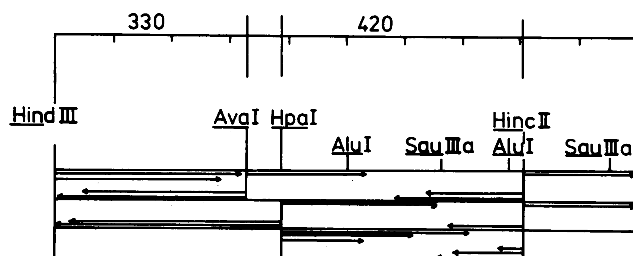

FIG. 1. Sequencing strategy. The arrows indicate the sequences determined in this study. Relevant restriction sites are indicated.

with ether, the reaction products were precipitated with ethanol and dried in vacuo. The probes were dissolved in 4 μl of the loading buffer containing 98% formamide, 0.05% bromphenol blue, and 0.05% Xylenxyanol and run on a 8% polyacrylamide (containing 1.3% bisacrylamide) gel with 8 M urea. The gel was exposed by autoradiography as described below.

Sequencing. The nucleotide sequence of DNA was determined as described previously (21). The reagents were obtained from NEN chemicals Dreieich. The reaction products were separated on 0.5-mm denaturing polyacrylamide gels, 80 cm in length with an acrylamide concentration of 12.5, 8, and 6%, respectively, and containing 8 M urea. The gels were exposed to Kodak X-Omat AR film. Generally about 300 nucleotides were readable. Restriction sites were found by using a computer program provided by L. Altschmied.

RESULTS

Nucleotide sequence of Tn*10* between the *Hin*dIII site and IS*10*$_R$. Figure 1 describes the sequencing strategy for approximately 900 nucleotides between the rightmost *Hin*dIII site on Tn*10* and IS*10*$_R$. These sequence data close the gap between two previous sequence determinations on Tn*10* (8, 12), such that the entire right part of Tn*10* starting with the *Xba*I site is known. Because the 675- and 420-bp *Hin*cII fragments (17) were cloned into suitable plasmids before sequencing (25), no experiments overlapping these restriction sites are needed.

The nucleotide sequence is displayed in Fig. 2. The numbering of the sequence is a continuation of the sequence given in reference 12. Analysis of the data in Fig. 2 reveals two open reading frames called ORFL and ORFR. ORFL starts at position 2047 and terminates at position 1379 and thus has a coding capacity of 222 amino acids. Its polarity is opposite to the one of *tetA* (12, 29), which is in agreement with references 5 and 31. ORFR starts at position 2060 and terminates at position 2476 and has a coding capacity of 138 amino acids. It has the same polarity as *tetA*, being opposite to ORFL. It is interesting to note that these reading frames with opposite polarity start only 12 nucleotides apart on the Tn*10* sequence. This finding would suggest that the mRNAs overlap and led us to define the transcriptional start points in both directions (see below).

Analysis of the codon usage in ORFL and ORFR reveals no striking differences from the codon usage of *tetA* (12) (analysis not shown). The only clear difference is the preferred use of the codon GCC for alanine, whereas in the *tetA* gene GCA was preferred. Aside from this minor difference there is no clear evidence from these data supporting the idea that the central DNA of Tn*10* may be assembled from different species.

It was suggested that tetracycline resistance encoded by Tn*10* involves a mechanism leading to reduced sensitivity of the ribosomes to the drug (26). Comparison of the primary amino acid sequence deduced from the nucleotide sequence of ORFL and ORFR with the known primary sequences of ribosomal proteins did not reveal homology to any of them. Hydrophobicity profiles of both possible polypeptides and the smaller part of ORFL, which is probably expressed (see below), show an even distribution of hydrophobic amino acids along the sequence. The general character of both polypeptides is rather hydrophilic as judged from this analysis.

Table 1 lists the restriction sites in the sequence determined here.

Determination of transcription start points. The transcriptional start points were determined by S1 mapping of the mRNA-DNA hybrids (1). Figure 3 displays the S1 maps for both DNA strands. We have determined the transcription starts between the *Hin*dIII site at position 1525 and the rightmost *Hin*cII site at position 2329. The three start points displayed in Fig. 3 are the only ones found on this DNA. As judged from the intensity of the hybrids two out of the three starts are the major ones. They are position 1985 from the rightward (toward IS*10*$_R$) mRNA and at position 2002 for the leftward mRNA. The minor transcript is directed to the right and starts at position 2021. Its significance is not clear due to

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A A G C T T T A A G T T G G T T C T C T T G G A T C A A T T T G C T G A C A A T G G C G T T T A C C T T A C C A   1580
          G T A A T G T A T T C A A G G C T A A T T T T T T C A A G T T C A T T C C A A C C A A T G A T A G G   1630
          C A T C A C T T C T T G G A T A G G G A T A A G G T T T T T A T T A T T A T C A A T A A T A T A A T   1680
          C A A G A T A A T G T T C A A A T A T A C T T T C T A A G G C A G A C C A A C C A T T T G T T A A A   1730
          T C A G T T T T T G T T G T G A T G T A G G C A T C A A T C A T A A T T A A T T G C T G C T T A T A   1780
          A C A G G C A C T G A G T A A T T G T T T T T T A T T T T T A A A G T G A T G A T A A A A G G C A C   1830
          C T T T G G T C A C C A A C G C T T T T C C C G A G A T C T C A T C T A T T G A A A C A G C T T G A   1880
          T A G C C T T T T T C A A C A A A C A A T A T T C G T G C T G A G T T A A C C A G T G A T T G A T A   1930
          G G T A C T C T T A A A A T T T T C T T G T T G A T G A T T T T T A T T T T C C A T G A T A G A T T   1980
          T A A A A T A A C A T A C C G T C A G T A T G T T T A T G G T A T C A T G A T G A T G T G G T C G T   2030
          G A C A A T C T T A A G A A C A T T T A G G T T A T T T T A T G T A T A T T G A A C A G C A T T C T   2080
          C G C T A T C A A A A T A A A G C T A A T A A C A T C C A A T T A G A A T A T G A T G A T A G A C A   2130
          G T T T C A T A C A A C G G T T A T C A A A G A T G T T C T A T T A T G G A T T G A A C A T A A T T   2180
          T A G A T C A G T C T T T A C T G C T T G A T G A T G T G G C G A A T A A A G C G G G T T A T A C C   2230
          A A G T G G T A T T T T C A G C G G C T G T T C A A A A A A G T A A C A G G G G T C A C A C T G G C   2280
          T A G C T A T A T T C G T G C T C G T C G T T T G A C G A A A G C G G C T G T T G A G T T G A G G T   2330
          T G A C G A A A A A A A C T A T C C T T G A G A T C G C A T T A A A A T A T C A A T T T G A T T C C   2380
          C A A C A A T C T T T T A C A C G T C G A T T T A A G T A C A T T T T T A A G G T T A C A C C A A G   2430
          T T A T T A T C G G C G T A A T A A A T T A T G G G A A T T G G A G G C A A T G C A C T G A G A G A T C   2482
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FIG. 2. Nucleotide sequence between the *Hin*dIII site and IS*10*$_R$. The nucleotide sequence starting from the *Hin*dIII site to IS*10*$_R$ is shown. The numbering of the nucleotides is continued from reference 12.

TABLE 1. Location of restriction sites

Restriction site	Position
*Alu*I	1874, 2095, 2282
*Sau*3	1547, 1856, 2183, 2353, 2479
*Rsa*I	1932, 2407
*Taq*I	2398
*Dde*I	1705, 1788, 1909, 2473
*Hin*fI	2375
*Fnu*4HI	1771, 2245, 2312
*Hin*dIII	1525
*Bgl*II	1855
*Ava*I	1851
*Afl*II	2037
*Hpa*I	1913
*Hin*cII	1913, 2329
*Aha*III	1808, 1979
*Afl*III	2393
*Ban*I	1826
*Hgi*AI	2292
*Nsp*BII	2243
*Xho*II	1855
*Bst*EII	1835
*Bbv*I	1771
*Fok*I	2104
*Hph*I	1837
*Mnl*I	2326, 2462
*Sfa*NI	1630, 1752
*Tth*111II	1894, 2196

the weak S1 mapping signal. However, the −10 and −35 promoter consensus sequences can be found in the sequence (22). In the following sections we will discuss only the two main start points.

Figure 4 shows the interpretation of the mRNA start points with regard to the nucleotide sequence. It may be concluded that all of the three start points determined in vivo by S1 mapping represent promoters as indicated by the consensus sequences shown in the figure (22). Several interesting aspects are clearly displayed in Fig. 4. The main two mRNAs start from a common, complex, and divergent promoter arrangement. This situation resembles the one of *tetR* and *tetA* on Tn*10* (2, 3, 12, 13, 29). The consensus sequences for the major promoters are separated by 26 bp, each located downstream from the other. The start points of

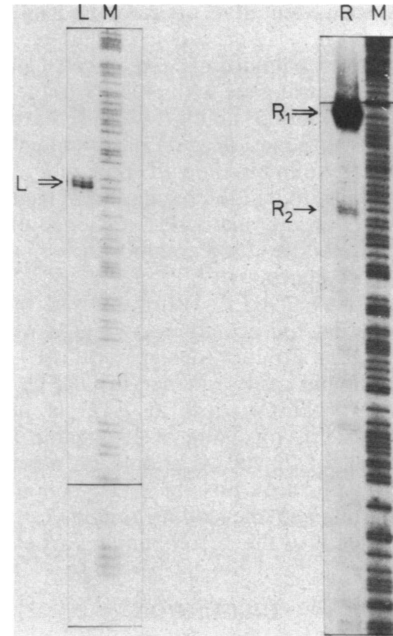

FIG. 3. S1 maps of the sequence between the *Hin*dIII site and IS*10*$_R$ on Tn*10*. The maps for both strands are shown. M denotes the marker lane originating from the purine reaction of the respective DNA fragments. R denotes the map of transcription toward IS*10*$_R$. R1 is the major start point, and R2 the minor start point. For this map the 420-bp *Hin*cII fragment (19) was labeled at the 5′ ends and then cut with *Rsa*I. L denotes the map of the mRNA directed toward *tetA*. To produce this map, pRT61 was linearized with *Ava*I (19), 5′ end labeled, and then cut with *Hgi*AI, and then the respective DNA was isolated.

the two mRNAs with opposite polarity are only 18 bp apart, and the resulting mRNAs overlap by this length (Fig. 4). Thus, the promoter arrangement is rather complex.

In addition, the nucleotide sequence implies interesting possibilities for the regulation of expression of these two genes. A 19-bp nearly palindromic sequence with a central base pair is located between the start points of the mRNAs with opposite polarity. Since this type of sequence arrangement often serves as a recognition site for regulatory proteins, it appears that the expression of both genes may be

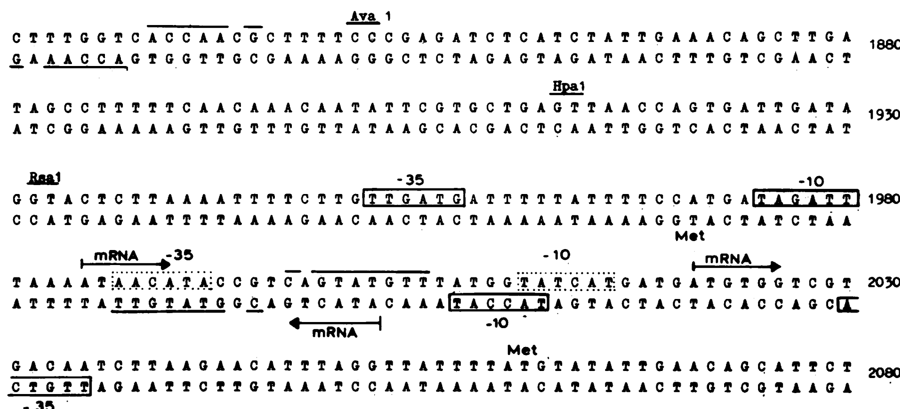

FIG. 4. Interpretation of the nucleotide sequence containing the gene starts. This figure displays the part of the DNA sequence where the transcription start points are located. Some restriction sites are indicated (19). The arrows denote the mRNAs as determined from the S1 maps. The boxes indicate the promoter consensus sequences (22). The dotted box denotes the consensus sequence for the minor mRNA start. The ATG codons serving as the potential translational start points are marked.

coregulated by this tentative operator and an unidentified regulator gene.

Another different palindromic sequence is located within the proposed structural gene with polarity to the left (Fig. 4), offering the additional possibility of a differential control of expression of both genes. Regulatory elements within the structural gene are found in the *galE* gene of *E. coli* and have been shown to be active in the regulation of transcription (7, 15). Taken together, the nucleotide sequence of the control region offers possibilities for a common as well as a different regulation of both genes.

The comparison of ORFL with the transcription start to the left reveals that the promoter is located within ORFL. Therefore, the gene product (designated OrfL) cannot contain the entire amino acid sequence of ORFL. Instead, the structural gene probably starts at ATG of position 1972 coding for a protein consisting of 197 amino acids with a molecular weight of 22,700. It should be noted, however, that this is only the first possible start codon; others are located downstream in the coding region, e.g., a GTG at position 1840 (see also the accompanying article [4]).

DISCUSSION

We have determined the nucleotide sequence between the end of the *tetA* gene and IS*10*$_R$ and mapped the transcription start points in this sequence. Although no function of the proposed gene products is known yet, it has been suggested that tetracycline-inducible proteins may be encoded in this region of Tn*10* (26, 31). The notion that these sequences of Tn*10* may be essential for the maintenance of the transposon is supported by the observation that no deletions occur even after many translocation events (C. Beck, personal communication) and that Tn*10*-like transposable elements isolated from different species are of roughly the same size (S. B. Levy, personal communication).

It is not surprising for these reasons that this part of Tn*10* contains, indeed, almost the maximal coding capacity. Of particular interest is the divergent polarity of the two genes and the complex arrangement of their regulatory sequence. Two promoters with opposite polarity are located next to each other such that the resulting two mRNAs overlap by 18 bp. This arrangement of promoters resembles the situation of the IS*10*$_R$ transposon promoter (27) where it leads to translational control of transposition (28).

A possible coregulation of both genes on the level of transcription is inferred by the palindromic sequence located between the two mRNA starts (Fig. 4). It is not clear, so far, where the regulatory gene may be located and what the mechanism of regulation may be. None of the plasmids used to produce the mRNAs in vivo for the S1 maps (pWH201 and pCB122, see above) contains the entire Tn*10*. Thus, a regulatory gene may be located on Tn*10* itself. It is rather unlikely to be *tetR* because the palindromic sequence between *orfL* and *orfR* shows no homology to the *tet* operators (3, 10, 13).

The S1 maps indicate that the entire coding capacity in the leftward open reading frame is not used. Instead, transcription starts within this open reading frame. Therefore, the second ATG within this open reading frame is the most likely translational start codon. It cannot be excluded that other GTG or ATG codons within this sequence are used. The molecular weight of the gene product is discussed in the accompanying article (4).

Figure 5 summarizes the genetic structure of the central part of Tn*10* known so far. The designation *orfL* and *orfR* for the two genes defined in this article is chosen, because the function of their gene products is still a subject of speculation. It has been assumed that *orfL* may encode an additional mechanism of tetracycline resistance (5, 26). On the other hand, *tetA* seems to be sufficient for high-level resistance to tetracycline (19). We compared the primary sequence of the *orfL* and *orfR* gene products to the amino acid sequences of the *E. coli* ribosomal proteins (courtesy of B. Wittmann-Liebold) and found no striking homology. It is thus unlikely that any of these genes codes for a protein replacing a ribosomal factor.

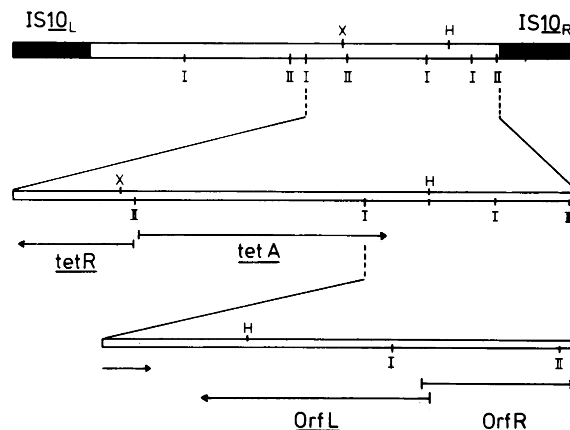

FIG. 5. Genetic structure of Tn*10*. This figure displays the genes known so far to be located in the core part of Tn*10*.

Codon usage analysis reveals no striking differences to the ones of *tetA* (12) and *tetR* (Postle and Bertrand, personal communication). Therefore, there is no reason to believe that *orfL* and *orfR* originate from a different source than *tetR* and *tetA*.

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