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Similarities between the regulatory sequences of the unrelated tetracycline genes of pBR322 and Tn10

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The regulatory regions of the tetracycline genes present in pBR322 (pSC101) and in the transposon Tn10 are compared. They show a low degree of nucleotide sequence similarity but a high level of structure similarity. Furthermore, analyses of RNAs transcribed in the opposite direction of the pBR322 *tet* gene show that there are two mRNA initiation sites separated by 29 nucleotides. This suggests the existence of two promoters for the *tet* repressor gene in Tn10. These features reveal a strong resemblance of the mode of regulation between the *tet* operons of Tn10 and pSC101.

Lactamase, β-; mRNA; Operon, *tet*; Plasmid pBR322; Transposon, Tn10

1. INTRODUCTION

Tetracycline resistance is encoded by a number of distinct genes that, while coding for proteins having the same function, do not have the same origin. They have been distributed in four genetic classes according to the degree of resistance to tetracyline or tetracycline analogues they confer to the bacteria and to sequence homologies as measured by DNA-DNA hybridization [1]. These tests put the tetracycline resistance (*tet*) gene of Tn10 in a different class (class B) from the *tet* gene of pSC101 (class C) which is the source of the *tet* gene present in pBR322. We report here observations which suggest that in spite of this different origin, the *tet* regulatory sequences present in pBR322 correspond to an operon which, as in Tn10, has two promoters directing the transcription of the repressor mRNA and that both participate in the synthesis of the β-lactamase (bla) mRNA.

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2. MATERIALS AND METHODS

2.1. *RNA isolation*

RNA was isolated from *E. coli* Hb101 harboring pBR322 as described [2].

2.2. *Determination of the mRNA 5'-ends*

10 μg RNA were hybridized to 0.01 pmol (8×10^4 cpm) of (5'-^{32}P)-end-labeled oligonucleotide A (see fig.1) in 20 μl hybridization buffer [80% formamide, 0.4 M NaCl, 0.04 M Pipes (pH 6.4), 1 mM EDTA] overnight at 47°C. Reverse transcription was carried out in 50 μl with 40 U enzyme (Anglian Biotechnology) in the presence of each deoxynucleoside triphosphate (400 μM) at 42°C for 60 min. The products were visualized by autoradiography after electrophoresis in a 6% polyacrylamide-7 M urea gel.

2.3. *Sequencing by primer extension*

The protocol of Zaug et al. [3] was followed. RNA (100 μg) was hybridized to 0.5 pmol (4×10^6 cpm) of the (5'-^{32}P)-end-labelled oligonucleotide C (see fig.1). The oligonucleotide was elongated as above in four separate reactions, each reaction containing one of the four chain terminator dideoxynucleotides (200 μM). A fifth reaction was run without chain terminator.

3. RESULTS AND DISCUSSION

In agreement with classification of the *Tet* genes of pBR322 and Tn10 into different genetic groups, comparison of the nucleotide sequences of the two gene promoter regions shows a low degree of

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similarity, mostly restricted to the hexanucleotide of the −35 box of the *Tet* gene and to a twice-repeated pentanucleotide TGATA (fig.1). However, the representation used in fig.1 shows that there is a high degree of overall structural similarity: both genes have two palindromes of similar sizes as well as similar relative positions and in all four the pentanucleotide is located at the same place.

The tetracyline gene that has been the most thoroughly studied is the *tet* gene operon of Tn10 [4]. It is composed of two genes, *Tet*A and *Tet*R, transcribed in opposite directions. The *Tet*A gene codes for resistance to the antibiotic, the *Tet*R gene coding for the *Tet*A repressor. The latter gene is controlled by two tandemly arranged promoters, P_{R1} and P_{R2}, separated by 20 nucleotides.

The tetracycline gene present in pBR322 originates from the insertion of a pSC101 *Eco*RI* fragment into the unique *Eco*RI site of pMB8, an ancestral pBR322 plasmid [5]. As a result of the strategy used to construct pBR322, the *Eco*RI site is the 5′-limit of the tetracycline gene domain. Analysis by electron microscopy of in vitro and in vivo transcription products has demonstrated the existence of at least three promoters, P1, P2 and P3, in the sequences flanking the *Eco*RI site [6]. Two of them, promoters P1 and P2, are close to one another; P2 directs the transcription of the tetracyclin gene and P1, oriented in the opposite direction, contributes to β-lactamase mRNA synthesis. The third, P3, is the β-lactamase gene promoter. By S_1 mapping analysis of pBR322 transcription products synthesized in vivo and in vitro, Brosius et al. [7] have determined the location of the three promoters. Fig.1 shows that while the location of the P1 promoter does not correspond to any of the Tn10 *tet* promoter's locations, P2 occupies the same position toward the large palindrome as the *Tet*A promoter in Tn10.

We have isolated and studied mutations of the pBR322 β-lactamase gene [2] and have used them to produce high levels of mouse dihydrofolate reductase [8]. In the course of these experiments, we have analysed the β-lactamase transcripts of the wild-type promoter. A synthetic oligonucleotide, oligonucleotide A (see fig.1C), complementary to the 5′-end of the β-lactamase coding sequence, was hybridized to RNA isolated from *E. coli* transformed by pBR322, extended using reverse

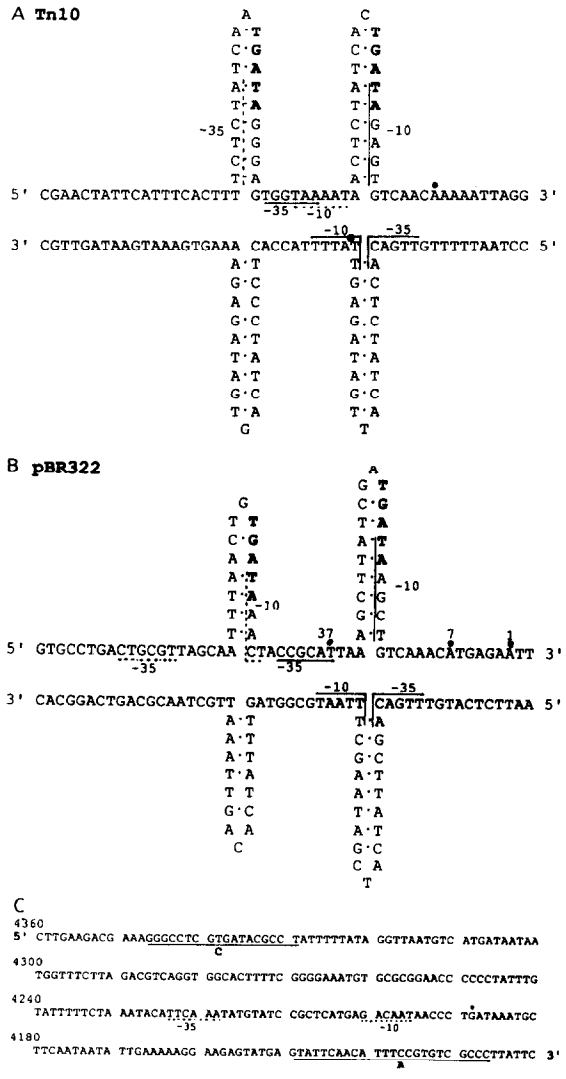

Fig.1. Comparison of the Tn10 and pBR322 *tet* gene regulatory regions. (A,B) The regions encompassing the *tet* regulatory regions of (A) Tn10 and (B) pBR322 genes, are displayed in such a way as to emphasize the structural analogy resulting from the presence of the palindromes, the basis of which is indicated by dots; it does not imply that the DNA assumes a stem-loop configuration. Nucleotides of the twice-repeated pentanucleotide are in bold type. (Upper strands) The two repressor gene promoters, *Tet* P_{R1} and P_{R2} of Tn10, are indicated by, respectively, dotted and continuous lines as are, respectively, P1 and the putative second repressor gene's promoter in pBR322. (Lower strands) The *tet*A gene promoters are indicated by a continuous line (C). Direct continuation of the pBR322 sequence toward the β-lactamase gene. The *bla* (P3) promoter is indicated by a dotted line. The length of the regions complementary to each of the two oligonucleotides, A and C, used to prime the reverse transcription reactions is underlined. Dots above nucleotides show transcription start sites. Numbering is that of Sutcliffe [11].

transcriptase and the products analysed by gel electrophoresis. Fig.2A shows that the polymerisation reaction gives rise to four cDNAs. The shortest is 63 nucleotides long, corresponding to an elongation of 39 bases. It places the 5′-end of the message at nucleotide 4189 (see fig.1C) which is the natural transcription initiation site of the *bla* gene [9]. The other three cDNAs that correspond to a large proportion of the β-lactamase mRNA give signals of equal intensity, two having a size of about 240 nucleotides and the third having a size of 290 nucleotides.

In order to determine precisely the 5′-end of these three additional messages, reverse transcription was repeated with an oligonucleotide (oligonucleotide C, fig.1) complementary to a region closer to the 5′-end of these mRNAs. The oligonucleotide was elongated and the sequence of the resulting cDNA determined (fig.2B). The results show the presence of cDNAs of 34, 40 and 70 nucleotides and set the position of the 5′-end of the mRNA at, respectively, nucleotides 1, 7 and 37 (see fig.1B). In addition to the S_1-protected fragment attributed to RNA transcribed from the promoter P1, Brosius et al. [7] have observed, but neglected, shorter protected fragments compatible with mRNAs initiating at or near, respectively, nucleotides 1 and 10.

Together, these and our results suggest the existence of two promoters. The mRNA initiating at or close to nucleotide 37 certainly corresponds to the promoter P1. The 5′-ends of the putative mRNAs initiating at nucleotides 7 and 1 are compatible with a second promoter with a -10 and a -35 region delimited by nucleotides 15–20 and 37–42, respectively. This assignment places this promoter in the same relative position as that occupied by PR2 in the *tet* gene of Tn10 (see fig.1). This strongly suggests that the *Tet* gene of pSC101 is an operon also regulated by a repressor gene controlled by two promoters in tandem.

The -35 and -10 sequences defined by these localizations present a very low degree of similarity to the consensus sequences [10], however, it has been precisely shown for the Tn10 *Tet* operon that a very poor similarity to the consensus which exists for the repressor gene promoters is associated with a transcription that is as efficient as that from the *Tet*A promoter which, in contrast, shows a very close similarity to the consensus [4].

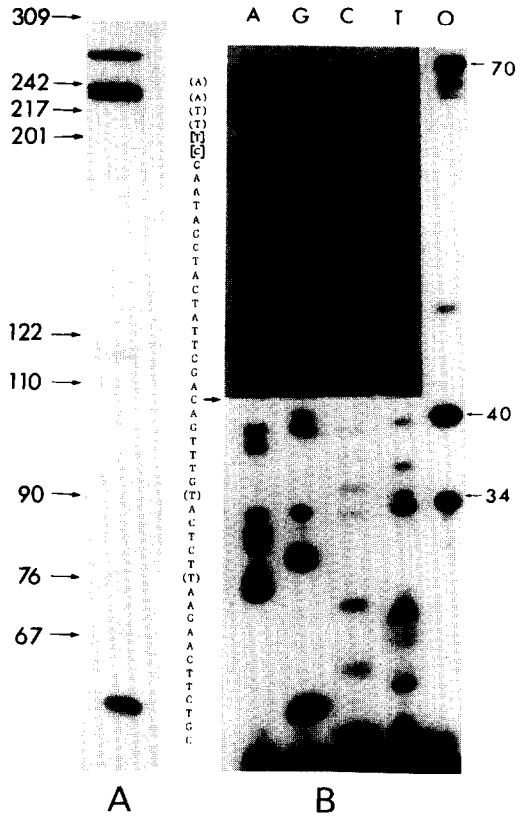

Fig.2. Determination of the 5′-ends of the *bla* mRNA initiating in the *tet* promoter region by primer extension. (A) Primer extension from oligonucleotide A. Numbers on the left indicate the size of *Hpa*II-cut pBR322. (B) Sequence determination by primer extension from oligonucleotide C. A, G, C, T indicate the sequence reactions run; O signifies no chain terminator added.

In conclusion, comparison between the *tet* regulatory sequences of Tn10 and pBR322 suggests that organisation of the regulation of these two different *Tet* genes is very similar. In addition, these observations show that interpreting the results from transcription analyses of pBR322 *bla* or *tet* gene promoters must take into account the complexity in regulation of this region.

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