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Characterizing wild-type and mutant promoters of the tetracycline resistance gene in pBR313

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ABSTRACT

By employing a system of RNA polymerase binding and restriction endonuclease digestion, we demonstrate that the region in and around the Hind III site of pBR313 and pBR322 is the promoter for the tetracycline (Tc) resistance gene(s). Furthermore, it is shown that this region was transferred intact from pSC101 during the construction of the latter plasmids. The *in vitro* insertion of a few base pairs at the Hind III site produces a series of "down" promoter mutations in which the level of *in vivo* Tc resistance is reduced. Sequence analysis of the various promoter mutations revealed significant base pair rearrangements in the region between -40 and -12 of the promoter. While these base alterations do not appear to affect the firm binding of RNA polymerase, they do affect the ability of mutant promoters to initiate transcription. These observations suggest that the region from -40 to -12, previously designated as the "recognition region", is actually involved in the process of initiation of transcription.

INTRODUCTION

One of the important features distinguishing the plasmids pBR313 (1,2) and pBR322 (3) as cloning vectors is the tetracycline (Tc) resistance gene which permits the detection of cloned DNA fragments by insertional inactivation (4). This approach involves the insertion of a "foreign" DNA fragment into a restriction endonuclease cleavage site within the Tc resistance gene. The essential genes for this resistance mechanism have been transferred to the relaxed-replicating plasmids pBR313 and pBR322 from pSC101 (5). The proteins encoded by the genes have a molecular weight of 34,000 daltons and 18,000 daltons and occupy approximately 1 megadalton (md) of plasmid DNA (6,7). It has been shown that resistance to Tc can be abolished when DNA from *Drosophila melanogaster* (4) and *Neurospora crassa* (1) is cloned into the Hind III, Bam HI and Sal I restriction endonuclease cleavage sites of these plasmids. Presumably, this results from the creation of an elongated or truncated Tc resistance mRNA which can no longer function

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in the resistance mechanism effectively.

However, it is now known that some Hind III-generated recombinant plasmids retain some degree of Tc resistance (8,2,9). Preliminary experiments using the plasmid pBR313 indicate that the Hind III cleavage site is at the boundary of the Tc resistance gene and that it can be protected from cleavage by preincubation with E. coli RNA polymerase (10). This and other evidence (11,12) suggest that the Hind III cleavage site lies in or near the promoter for the Tc resistance genes and that insertional inactivation at this site is due to changes in the promoter sequence rather than to an alteration of the structural integrity of the Tc resistance genes.

In this paper we present a more detailed characterization of the Tc promoter as well as a description of a system for investigating the relationships between RNA polymerase and promoter recognition, binding and initiation sites. The experiments described here exploit a unique restriction site within the Tc promoter which can be protected by E. coli RNA polymerase from restriction endonuclease cleavage. This cleavage site can also be used to generate Tc promoter mutations by in vitro manipulations. These mutations involve the insertion or deletion of nucleotides which serve to uncouple the firm binding site from the recognition region (13,14). All mutations produced "down promoter" phenotypes with respect to the level of Tc resistance expressed by each plasmid. It will be shown that these mutations significantly affect the initiation of transcription rather than the ability of RNA polymerase to bind the mutant promoters. Furthermore, the strategic location of certain restriction enzyme cleavage sites within this promoter may provide a new method for investigating RNA polymerase-promoter interactions.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The bacterial strain used as a host for all plasmids examined in this paper was a derivative of E. coli K12, RR1 F^- pro leu thi lac Y str^r endo I^- r_K^- m_K^-. The ampicillin (Ap) and Tc resistant plasmids pBR313 and pBR316 have been described previously (1,2). The sigma-overproducing mutant of E. coli YN18 (a derivative of KY1125) was used as a source of DNA dependent-RNA polymerase (15). The plasmids Col E1 (16) and pML21 (17) were obtained from D. R. Helinski while

pSC101 was obtained from S. N. Cohen. Purified SV40 and pSC101 DNAs were kindly provided by H. M. Goodman and R. C. Tait respectively.

Media and Chemicals

Cultures of E. coli, RR1 were routinely grown in L Broth (1% Bacto Tryptone, 0.5% Bacto Yeast Extract, 1% NaCl, 0.1% glucose, pH 7.2) at 37°C. When cultures were grown for plasmid isolation, M-9 minimal medium (0.4% glucose, 0.4% casamino acids, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.7% Na_2PO_4, 0.3% KH_2PO_4, 0.5% NaCl, 0.1 NH_4Cl supplemented with 40 µg/ml thiamine hydrochloride) was used. Bacteriological supplies and chemicals were purchased from Difco Corp. and J. T. Baker Co., respectively.

Tetracycline resistance levels

The Tc resistance levels present in this paper were determined by the 50% efficiency of plating method (EOP) previously described by Tait et al., (6).

For Tc resistance levels less than 5 µg/ml Tc, cell growth in LB containing increasing amounts of tetracycline hydrochloride (Sigma) was monitored spectrophotometrically (A_{450}). Tc concentrations which inhibited the growth rate of exponentially growing cells by 50% were taken as the level of resistance. The plasmid free E. coli strain RR1 is sensitive to 1 µg/ml Tc during 3 hrs. of growth at 37°.

Preparation of plasmid DNA

Preparation of plasmid DNA by amplification with 180 µg/ml chloramphenicol (Sigma) was performed according to Clewell (18). The cleared lysate was extracted once with an equal volume of buffer saturated (50 mM Tris-HCl, 100 mM NaCl, pH 7.5) phenol, and again with the same volume of chloroform. The aqueous phase was ethanol precipitated and further purified according to the procedure described by Bolivar et al., (2).

Enzymology

Digestion of plasmid DNA with the restriction endonucleases Bam HI, Eco RI, Hae III, Hind III, Hpa I, Pst I, Sal I, and Sma I were all performed under conditions previously described (2). In this paper, the specific activities of the restriction endonucleases were adjusted by diluting with enzyme extraction buffer (20 mM KH_2PO_4 - K_2HPO_4, 1 mM EDTA, 1 mM NaN_3, 100 mM NaCl, 50% glycerol, pH 7.0) so that 1 µg of pBR313 DNA was digested with 1 µl of enzyme in 10 min. at 37°C. The reaction mixture was the same as that used to initiate transcription

in the presence of heparin (see next section). All restriction endonucleases were prepared according to the generalized procedure described by Greene et al., (19). The ligation of various restriction fragments was performed with T4 polynucleotide ligase purified to a concentration of 500 units/ml according to the procedure of Panet et al., (20). Reaction mixtures of 50 µl containing 66 mM Tris-HCl, pH 7.6, 6.6 mM $MgCl_2$, 10 mM dithiothreitol (Sigma) 0.6 mM ATP, 0.5 µg to 3 µg DNA, 1 µl T4 DNA ligase (0.5 units) were incubated at 12.5°C (2) for 12 hrs.

To remove the cohesive ends of Hind III digested plasmid DNAs a 50 µl reaction mixture containing 2-5 µg of DNA, 20 units of S1 nuclease (1200 units/ml, Miles) 0.3M NaCl, 4.5 mM $ZnCl_2$, 30 mM sodium acetate, pH 4.6 was incubated at 22°C for 30 min. The mixture was then phenol-chloroform extracted and dialyzed against 10 mM Tris-HCl, 1 mM EDTA pH 7.6 for 12 hr. (The Hind III ends of pBR313 were also made flush by polymerization with T4 DNA polymerase in a reaction mixture previously described by Goulian et al., (21). Two µl (1 unit) of T4 polymerase (also prepared according to Panet et al., 20) was used to polymerize 2 µg of DNA at 1° C for 2 hr.) Blunt-end ligation of S1 nuclease treated and polymerized DNA was carried out at 22°C in a 10 µl reaction volume containing the ligation buffer described above and 2 µl (1 unit) of T4 DNA ligase. Transformations of ligated DNAs were carried out with E. coli RR1 according to Bolivar et al., (3).

RNA Polymerase (RNAP) Protection

Protection experiments were carried out with sigma-saturated RNA polymerase (RNAP) prepared from E. coli YN18 according to the procedure of Burgess and Jendrisak, (22), and holoenzyme provided by M. J. Chamberlin. The enzyme was stored in a buffer containing 40 mM K_2HPO_4 - KH_2PO_4, pH 8.0, 10 mM $MgCl_2$, 0.1 mM EDTA, 1.0 mM dithiothreitol, 200 mM KCl, 50% glycerol. RNA polymerase (holoenzyme) from Bacillus subtilis was kindly provided by R. Doi.

RNA polymerase was routinely bound to plasmid DNA in an approximate molar ratio of 5:1 (RNAP/DNA). As described by Seeburg and Schaller (23) a reaction mixture of 15 µl contained 5% glycerol, 20 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 40 mM KCl and 0.2 µl of plasmid DNA. Upon the addition of 1 µl of RNA polymerase (1.2 mg/ml) the mixture was incubated at 37°C for 10 min. (Final KCl concentration was 66 mM.) One µl of restriction endonuclease was then

added to the reaction mixture and the incubation continued for an additional 10 min. To initiate RNA transcription of plasmid DNA, ribonucleoside triphosphates (Sigma) were added to the reaction mixture (final concentration of 100 μM ATP, GTP and 50 μM CTP, UTP) 1 min. after beginning the 37° incubation. In some instances, 1 μl of heparin (Sigma) was added to the reaction mixture (final concentration 10 μg/ml), 2 min. after the addition of RNA polymerase. All the reactions were stopped by the addition of 10 μl of 5% sodium dodecyl sulfate, 25% glycerol, 0.025% bromophenol blue (stop mixture) and then incubated at 65°C for 5 min.

Gel Electrophoresis

Reaction mixtures were subjected to electrophoresis in 1% agarose (Seakem) slab gels (15 cm x 12.5 cm x 0.6 cm) using the Tris-EDTA-borate buffer system (2). The gels were run at 130V for 90 min. and stained for 2-5 min. with a 4 μg/ml solution of ethidium bromide (Calbiochem). The DNA was visualized by photographing the gels with NP Type 55 Polaroid film, upon illumination with ultraviolet light. Using the same conditions for electrophoresis and staining some reaction mixtures were dialized against 10mM Tris-HCl, pH 7.5; 1mM EDTA and run on 4.5% polyacrylamide gels (Bio-Rad) (15 cm x 12.5 cm x 0.15 cm). Quantitative estimates of percentage cleaved versus uncleaved DNA were obtained by scanning the photographic negatives with the Quick Scan Jr. integrating densitometer (Helena Laboratories) using a 0.2 mm x 2 mm slit, without filter. Estimates of the percent protection by RNA polymerase are calculated as: 100 X circular DNA/(circular + linear DNA).

DNA Sequencing

Nucleotide sequence analysis of various DNA fragments was carried out according to the procedure described by Maxam and Gilbert (24). The 5' phosphates from approximately 50 μg of Eco RI digested DNA, were removed with 3 μl (0.7 units/μl) of bacterial alkaline phosphatase (Worthington) in a 200 μl reaction of 50 mM Tris pH 9.0, incubated at 65°C for 30 min. The reaction mixture was phenol-chloroform extracted twice and dialyzed against 10 mM Tris-HCl (pH 7.4). Before kinase treatment, the DNA fragments were then denatured by heating to 100° for 5 min. in the presence of 10 mM Tris-HCl, pH 9.5, 1 mM spermidine, 0.1 mM EDTA. The DNA was radioactively labeled in a 100 μl reaction mixture containing 50 mM Tris-HCl pH 9.5, 10 mM $MgCl_2$, 5 mM dithiothreitol,

1 mC [γ-^{32}P]ATP; 2500 C/mM (ICN), 50 μg DNA and 10 units T4 polynucleotide kinase. The mixture was incubated at 37°C for 30 min.

The reaction was terminated by a phenol-chloroform extraction and the excess [γ-^{32}P]ATP was removed by Sephadex G-50 column chromatography (1 x 13 cm) (50 mM Tris, 500 mM NaCl, 1 mM EDTA, 1 mM NaN_3 pH 8.0). At this point Eco RI cleaved pSC101 and pBR313-H DNA were digested with Bam HI while pBRH1 and pBRH3B were digested with Hae III. The double digested DNA fragments were extracted from preparative 7.5% polyacrylamide gels by elution in 3 ml of 0.5 M ammonium acetate, 10 mM magnesium acetate, 0.1 mM EDTA, 0.1% SDS for 10 hr. at 65°C. The eluant was precipitated with 2 volumes of cold 95% ethanol and after chemical modification and cleavage of the DNA fragments, the reaction mixtures were run on a 20% polyacrylamide gel. Gels were autoradiographed for 4 days with NS-2T (Kodak) X-ray film.

RESULTS

RNA Polymerase Protection

The use of DNA gel electrophoresis and restriction enzyme analysis have been shown to be very useful tools in the identification of promoters for E. coli RNA polymerase (25,26,10). Such an experimental approach is shown in Figure 1. As slot 4 clearly shows, when RNA polymerase was preincubated with pBR313 DNA in the presence of heparin, cleavage of the Hind III site by Hind III enzyme was prevented. Since heparin is known to attack nonspecific (closed) polymerase-DNA complexes, this observation suggests that protection was due to the formation of a heparin resistant (open) complex (27). Furthermore, when the RNA polymerase binding reaction was challenged with Hind III endonuclease after three hours of incubation, no decrease in protection was observed (data not shown). When RNA polymerase and the four ribonucleoside triphosphates were added to the reaction mixture before the Hind III treatment, protection by RNA polymerase was substantially reduced (Figure 1, slot 5). The release of protection in this instance indicates the movement of RNA polymerase away from the Tc promoter as a result of the initiation of RNA synthesis. No protection of the Hind III site was observed in either the absence of RNA polymerase (slot 6) or in the presence of B. subtilis RNA polymerase (Table 1, column G) which has been shown to selectively transcribe certain T7 promoters (28). With the exception of the Eco RI site which was partially protected

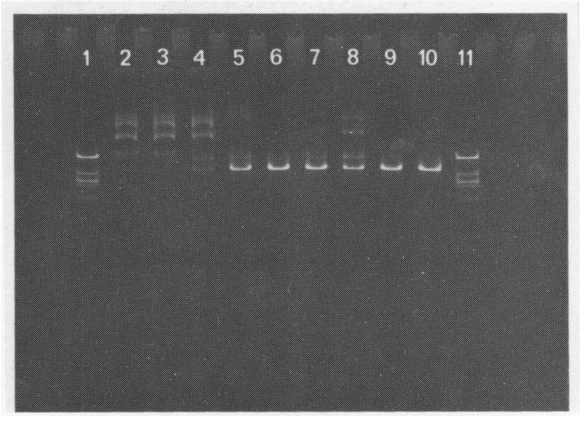

Figure 1. Agarose gel electrophoresis of RNA polymerase (RNAP protection experiments using pBR313 plasmid DNA. (slot 2 monomeric and dimeric forms of supercoiled and open circle pBR313 DNA, (slot 3) pBR313 incubated with RNAP, (slot 4) pBR313 digested with Hind III after preincubation with RNAP, (slot 5) pBR313 digested with Hind III after preincubation with RNAP and the 4 ribonucleoside triphosphates, (slot 6) pBR313 digested with Hind III in the absence of RNAP, (slot 7) pBR313 digested with Eco RI in the absence of RNAP, (slot 8) pBR313 digested with Eco RI after preincubation with RNAP, (slot 9) pBR313 digested with Bam HI, (slot 10) pBR313 digested with Sal I. Slots 1 and 11 contain Eco RI digested lambda DNA as molecular weight standards.

from digestion, neither the Bam HI (slot 9) nor the Sal I (slot 10) site was protected by RNA polymerase from endonuclease digestion. No protection by RNA polymerase was observed for the Hpa I and Sma I sites of pBR313 or the Pst I site of pBR322 (data not shown).

As shown in Figure 2 (A-F) the results obtained with pSC101 DNA were essentially the same as those for pBR313. Again, the Hind III site was protected by RNA polymerase (Fig. 2, B) and protection could be released by the addition of the four RNA precursors (Fig. 2, C). Quantitative estimates of the percent protection by RNA polymerase obtained from these and additional experiments are presented in Table 1.

Further investigations revealed that protection of the Hind III site is specific for pSC101 and its plasmid derivatives and not for the Hind III site present in bacterial plasmid pML21 (Table 1, column B). This observation is consistant with the notion that the Hind III site of pML21 is located within the structural region of the kanamycin

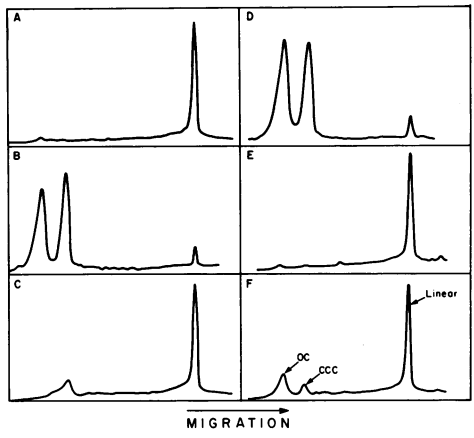

Figure 2. Densitometer tracings of the agarose-gel electrophoresis patterns of pSC101 DNA digested with; (A) Hind III in the absence of RNAP, (B) Hind III after preincubation with RNAP, (C) Hind III after preincubation with RNAP and the 4 ribonucleoside triphosphates, (D) Hind III after preincubation with RNAP and heparin, (E) Eco RI in the absence of RNAP, (F) Eco RI after preincubation with RNAP. The relative positions of linear, open circle (OC) and covalently closed circular (CCC) DNA with respect to the direction of migration are also shown in F.

resistance gene and not the promoter (11). As shown in Table 1, column F, protection of the Hind III site associated with the Tc promoter persists at a KCl concentration of 150 mM while cleavage of the Hind III site in pML21 is unaffected by either KCl (column E), RNA polymerase (column B) or both (column F). It should be noted that a KCl concentration of 200 mM or 40 μg/ml heparin and greater completely inhibited the Hind III and Eco RI cleavage reactions. As observed with pBR313, the Eco RI sites of pBR322 and pSC101 were partially protected from Eco RI digestion by RNA polymerase while the Eco RI sites of pMB9, Col E1, and SV40 show no protection (column I). Also, partial protection of the Eco RI sites of these plasmids was not significantly enhanced by the addition of a 20-fold excess of RNA polymerase to DNA (column L). This effect is probably due to the proximity of the Eco RI and Hind III sites (25 b.p.) since initiation of RNA synthesis releases partial and complete protection of both sites respectively (Table 1, columns K and D). The fact that the Hind III and Eco RI sites of pMB9 are separated by 340 b.p. (2) explains the lack of partial protection for the latter restriction site in this

Plasmid DNA	A: Hind III, NTPs, Heparin	B: RNAP, Hind III	C: RNAP, Heparin, Hind III	D: RNAP, NTPs, Hind III	E: 0.15M KCl, Hind III	F: 0.15M KCl, RNAP, Hind III	G: B. subtilis RNAP, Hind III	H: Eco RI, NTPs, Heparin	I: RNAP, Eco RI	J: RNAP, Heparin, Eco RI	K: RNAP, NTPs, Eco RI	L: 20:1 RNAP, Heparin, Eco RI	M: RNAP, Bam HI	N: RNAP, Sal I
pBR313	<1	95	90	3	<1	86	<1	<1	52	-	<1	55	<1	<1
pBR322	<1	96	95	1	<1	91	<1	<1	39	-	1	53	<1	<1
pSC101	<1	91	87	2	<1	90	<1	<1	48	-	-	-	<1	<1
pMB9	<1	93	90	4	-	88	-	<1	<1	-	-	-	-	-
pBR316	<1	91	89	1	-	89	-	-	-	-	-	-	<1	<1
pML21	<1	<1	-	-	<1	<1	-	-	-	-	-	-	-	-
pBR313-H	-	-	-	-	-	-	-	<1	45	-	-	-	-	-
pBRH3B	-	-	-	-	-	-	-	<1	42	35	15	91	<1	<1
pBRH1	-	-	-	-	-	-	-	<1	48	41	19	93	<1	<1
pBRH2	-	-	-	-	-	-	-	<1	40	35	16	91	-	-
pBRH4	-	-	-	-	-	-	-	<1	35	32	11	85	-	-
Col E1	-	-	-	-	-	-	-	<1	<1	-	-	-	-	-
SV40	-	-	-	-	-	-	-	<1	<1	-	-	-	-	-

Table 1. Percent protection as determined by densitometer tracings of agarose gels. The information displayed vertically above columns A-N represent the nature of each experiment and the order of addition of various reagents and enzymes. Each percentage is an average value obtained from experiments performed in triplicate. The experimental error associated with systems of analysis was ±5%. Dashes indicate that the experiment was not performed. RNAP = RNA polymerase; NTPs = ribonucleoside triphosphates.

plasmid (Table 1, column I).

Promoter Mutations and RNA Polymerase Protection

By exploiting the presence of a unique restriction site located in the Tc promoter, promoter-specific mutations were constructed *in vitro* which significantly affected the expression of Tc resistance. A preliminary

characterization of the plasmid pBR316 (an Ap-Tc resistance derivative of pBR312) has shown that it expresses the same level of Tc resistance as pBR313 despite the absence of the Eco RI site common to other pBR-plasmids (2). Because of this feature, pBR316 was used to construct promoter mutations involving the insertion of a small number of nucleotides into the Hind III site. This was accomplished by cleaving the Hind III site and removing the cohesive ends with the single-strand specific nuclease, S1. Chemically synthesized octanucleotides coding for the Eco RI cleavage substrate site (29) were inserted into the S1 treated Hind III site by blunt-end ligation (30). The products of this ligation were transformed into RR1 and the plasmid DNAs from several Ap resistant transformants were then examined for unique Eco RI cleavage sites by agarose gel electrophoresis. Two plasmids which had undergone the Hind III to Eco RI site conversion were designated pBRH1 and pBRH3B and are described in detail in another report (31). When the Tc resistance levels of these two plasmids were determined, it was found that pBRH1 and pBRH3B were resistant to approximately 2 μg/ml and 7 μg/ml Tc, respectively (Table 2). If the Eco RI-Bam HI restriction fragment of pBR322 is replaced by the corresponding fragment from pBRH1 and pBRH3B, two additional promoter mutations can be constructed (Fig. 3). Since the Bam HI sites in these three plasmids are identical with respect to their location in the Tc gene, the structural integrity of the

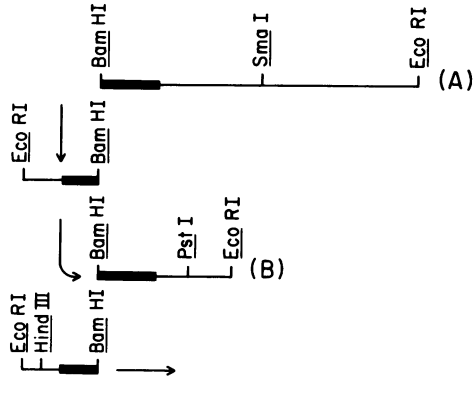

Figure 3. Diagrammatic representation of the construction of pBRH2 and pBRH4 promoter mutant plasmids (not drawn to scale). (A) Bam HI cleavage of either pBRH1 or pBRH3B. (B) Bam HI cleavage of pBR322. Arrows indicate the molecular cloning and replacement of the Eco RI-Bam HI fragments. Thick line represents the Tc resistance gene.

Tc resistance proteins will be retained. The resultant plasmids pBRH2 and pBRH4 (derived from pBRH1 and pBRH3B respectively) constructed in this manner were found to be resistant to no more than 2 μg/ml Tc in liquid culture. Another promoter mutation was constructed when the Hind III cohesive ends of pBR313 were made flush by polymerization with T4 DNA polymerase and joined by blunt-end ligation. The resulting plasmid (pBR313-H) was found to have no Hind III cleavage site and a reduced Tc resistance level (Table 2)(10).

The reduced levels of Tc resistance observed for each of the promoter-mutation plasmids may be explained by a reduction in efficiency of the Tc promoter. Whether this reduced efficiency is due to the lowered affinity of RNA polymerase for the promoter or to a decrease in the frequency of initiation can be tested by examining protection of the Eco RI site. The results of protection studies involving these plasmids are presented in Table 1. As indicated in columns I and J, heparin-resistant RNA polymerase protection of the Eco RI sites in pBRH3B, pBRH1, pBRH2 and pBRH4 is approximately equal but only about half that observed for the Hind III in pSC101 and its plasmid derivatives (columns B and C). The partial protection exhibited by the mutant plasmids could be observed within one minute after the addition of Eco RI enzyme and persisted for at least 3 hours (data not shown). Although initiation of transcription released protection of the wild-type promoter almost completely (column D), residual protection was observed for the promoter-mutation plasmids under similar conditions (column K). Furthermore, when the RNA polymerase to plasmid molecule ratio was increased to 20:1, protection was complete (column L) relative to the wild-type promoter. Complete protection of the Eco RI site could also be achieved by linearizing pBRH1 and pBRH3B with Sma I, and pBRH2 and pBRH4 with Pst I before the addition of RNA polymerase. These restriction sites were chosen because they are unique on these plasmids and the most distal to the Tc promoter (Fig. 3). Figure 4 shows that protection of the Eco RI sites on Pst I digested pBRH2 (slot 7) and pBRH4 (slot 11) is complete as compared to Hind III site protection on similarly digested pBR322. In these experiments, the lack of protection of the Hind III site (slot 2) or Eco RI sites (slots 6 and 10) results in the formation of two double digested fragments approximately 3600 and 750 b.p. in size (3,32). As with the circular forms of pBRH2 and pBRH4 DNA, residual protection was observed after the initiation of RNA synthesis (slots 8 and 12, respectively) while protection of the Hind III

Figure 4. RNA polymerase (RNAP) protection of Pst I digested pBR322, pBRH2 and pBRH4 as observed on a 4.5% polyacrylamide gel. (slot 1) 4300 b.p. linear pBR322 DNA plus RNAP; (slot 2) 3600 b.p. and 750 b.p. fragments of pBR322 generated after Hind III cleavage, minus RNAP; (slot 3) Hind III cleavage of pBR322, plus RNAP and heparin; (slot 4) Hind III cleavage of pBR322, plus RNAP and ribonucleoside triphosphates-NTPs; (slot 5) 4275 b.p. linear pBRH2 DNA, plus RNAP; (slot 6) 3575 b.p. and 750 b.p. fragments of pBRH2 generated by Eco RI cleavage, minus RNAP; (slot 7) Eco RI cleavage of pBRH2 plus RNAP and heparin; (slot 8) Eco RI cleavage of pBRH2 plus RNAP and NTPs; (slot 9) pBRH4 linear DNA plus RNAP; (slot 10) Eco RI cleaved pBRH4 minus RNAP; (slot 11) Eco RI cleavage of pBRH4 plus RNAP and heparin; (slot 12) Eco RI cleavage of pBRH4 plus RNAP and NTPs. The 4275 b.p. Pst I linear (A) and the 3575 b.p. (B) and 750 b.p. (C) Pst I-Eco RI fragments of pBRH4 are also indicated in slot 12.

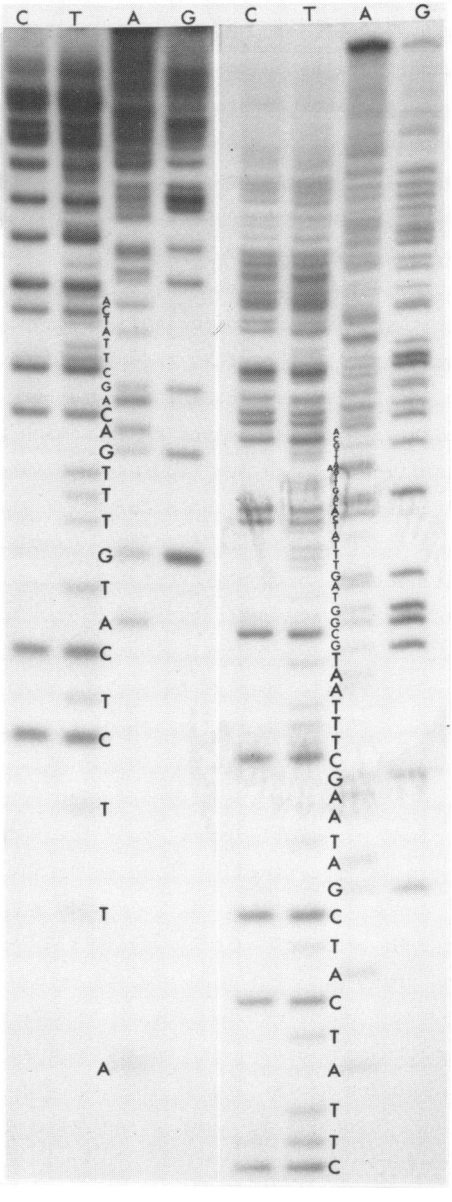

Figure 5. Autoradiograph showing the nucleotide sequence of the Eco RI-Bam HI fragment of pSC101. The terminal phosphate of the Eco RI end migrated off the gel in the reaction series on the left.

site of pBR322 was negligible (slot 4). Results obtained with Sma I digested pBRH1 and pBRH3B were identical to those observed for pBRH2 and pBRH4 (data not shown).

Nucleotide Sequence Analysis of the Tc Promoter and Promoter Mutations

Incorporating the essential elements of Tc resistance from pSC101 to pBR313 and pBR322 involved numerous in vitro manipulations. To insure that the nucleotide sequence of the Tc promoter in pBR313 (10) and pBR322 (32) is identical to that in pSC101, the corresponding region of the latter plasmid was also analyzed. To obtain sufficient quantities of pSC101 DNA for sequence analysis, a plasmid chimera was constructed in which a relaxed replicating plasmid was linked to pSC101 by the ligation of Eco RI cohesive ends (33). In this instance a 0.8 md plasmid, pBR345, which codes for the pMB1 origin of DNA replication was used (34). The resulting plasmid, pBR145, has a molecular weight of 6.6 md, and expresses a constitutive resistance to Tc of 75 μg/ml.

A previous restriction site analysis indicated that the Hind III sites of both pBR313 and pSC101 were located on a 375 b.p. Eco RI - Bam HI restriction fragment, just 25 b.p. from the Eco RI end (2). Therefore, pBR145 DNA was prepared by chloramphenicol amplification and used as a source of this restriction fragment. The nucleotide sequence starting at the Eco RI end of this fragment is shown in Figure 5. This sequence is identical to the corresponding regions in pBR313 and pBR322 and confirms pSC101 as the original source of the Tc resistance gene. The nucleotide sequence which best fits the requirements of a Pribnow box is shown in Figure 6b as a block of 7 nucleotides at positions -12 to -6. Our choice of these nucleotides was also influenced by in vitro transcription data (35) which indicated that the Tc resistance mRNA initiates from a 5'GTT corresponding to positions 1, 2, and 3 (Fig. 6b).

The nucleotide sequences of the promoter regions of pBRH1 and pBRH3B were determined by cleaving and radioactively labeling the 5' Eco RI ends of each plasmid. The labeled DNA was subsequently digested with Hae III endonuclease and the resulting fragments isolated. By sequencing both Eco RI - Hae III fragments, the arrangement of base pairs on both sides of the Eco RI site in pBRH1 and pBRH3B was determined. The DNA sequence of pBR313-H was determined unidirectionally from the Eco RI - Bam HI fragment as described above for pBR145. The DNA sequences of the promoter mutation plasmids (data not shown) are summarized in Figure 6c-e. It can be seen that the polymerization of the Hind III ends of pBR313 resulted in a 4

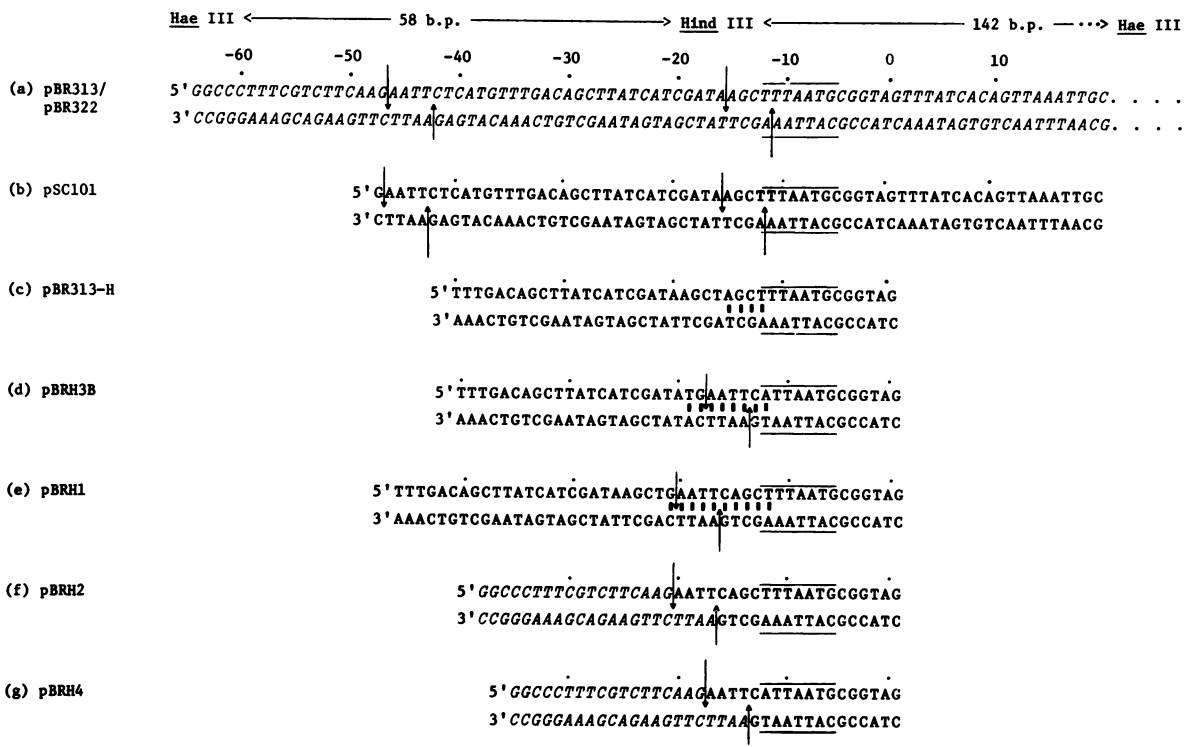

Figure 6. Nucleotide sequence in the region of the wild-type (a and b) and mutant (c-g) Tc promoters. Arrows in the sequences (d) through (g) represent the cleavage site for Eco RI while those between -47, -46 and -16, -15, of (a) and (b) represent Eco RI and Hind III sites respectively. Bars between the 5' and 3' strands (c, d and e) indicate nucleotide insertions. The Hae III sites depicted at the top of the figure were those used in the sequence analysis of pBRH1 and pBRH3B.

nucleotide insertion (Figure 6c) while the S1 treatment followed by blunt-end ligation of the Eco RI octanucleotide into pBRH3B, also produced a net gain of 4 nucleotides (Figure 6e). In the case of pBRH1 however, the S1 nuclease treatment appears to have removed only the external 5' deoxy-adenosine monophosphate from each of the Hind III ends. This resulted in a net gain of 10 nucleotides assuming that the ligation of the Eco RI octanucleotide to the cohesive ends was followed by in vivo repair of the single stranded gaps. The nucleotide sequences shown for pBRH2 and pBRH4 (Fig. 6f and g) were inferred from previous restriction site mapping and sequencing data.

Assuming that the point from which transcription initiates (position 1) remains the same in these mutant plasmids, it can be seen in Figure 6 that the insertions and deletions just described will affect the promoter sequence in the following ways: 1) The first nucleotide of the Pribnow sequence (position -12) is changed from a T to an A in pBRH3B (Fig. 6e). 2) The nucleotide sequence in the region between -13 and -40 of the wild-type promoter is displaced to the left by 4 base pairs in pBRH313-H and pBRH3B and 10 base pairs in pBRH1. This leftward displacement introduces different nucleotides at critical points in this region, such as position -35 (36,13,37). 3) In the plasmids pBRH2 and pBRH4, the region from -13 to -40 is substituted by the region between -64 and -47 in pBR322 (Fig. 6a).

The sequence of nucleotides to the right of position -12 is identical in all plasmids, therefore, a comparison between wild-type and mutant promoters sequences need only involve those base pairs remaining to the left of this position. Such a comparison (Table 2) indicates that all alterations of the Hind III site result in a reduction but not the elimination of Tc resistance. Upon examination of the base pair homologies between wild-type and mutant promoters, it can be seen that of all the positions associated with down mutational effects (Table 2, *), only the substitution at -35 is common to all promoter mutations. On the other hand, position -13, the site of a naturally occurring down promoter mutation in Shigella (38) is unchanged in all five promoter mutants. While pBR313-H and pBRH3B are quite similar with regard to their homology to pBR313, only homologies at positions -38, -33, and -21 are unique to these two plasmids. Of the three sites, only mutations at -33 are known to result in a down promoter phenotype (39). Positions -38 and -21 are points of little or no significance in the computer-generated promoter sequence of Scherer et al. (40). Furthermore, these two positions in the lac promoter

	-40 -35 -30 -25 -20 -15	Tc^r µg/ml	%AT	ΔH bonds	ΔG
	-gttGTTGAcATTTtt----ttggcGGTT a a a A + + * ***** + *				
pBR313	CATGTTTGACAGCTTATCATCGATAAGCT	75	62	-	-
pBR313-H	TTTGACAGCTTATCATCGATAAGCTAGCT	50	63	0	+4.7
pBRH3B	TTTGACAGCTTATCATCGATATGAATTCA	7	69	-2	-2.3
pBRH1	AGCTTATCATCGATAAGCTGAATTCAGCT	≤2	62	0	+4.7
pBRH2	GAGCCCTTTCGTCTTCAAGAATTCAGCT	≤2	52	+3	+8.4
pBRH4	CACGAGGCCCTTTCGTCTTCAAGAATTCA	≤2	52	+3	+6.6

Table 2. The important features of the wild-type and mutant Tc promoter plasmids are compared. The nucleotide sequence between -40 and -12 in pBR313 has a total of 69 hydrogen bonds and a free energy (G) value of -59.9 Kcals as determined by the system of Tinoco et al., (46). At the top of the table is a computer generated model promoter sequence where upper and lower case letters indicate the significance level for a particular nucleotide appearing at that position (40). Those positions where "down" promoter mutations (*) and protection from dimethylsulfate (+) (41) have been reported are also indicated. Changes in free energy and hydrogen bonds are indicated as ΔG and ΔH respectively. The base pairs from region -36 to -40 of pBRH2 and pBRH4 were obtained from the pBR322 sequence of Sutcliffe (32). The shaded nucleotides represent those not homologous to pBR313.

were not protected by RNA polymerase from dimethyl sulfate methylation (41).

DISCUSSION

Promoters consist of a particular arrangement of 40 to 50 base pairs of DNA which are recognized and bound by RNA polymerase. Within this sequence of DNA, three structural domains have been identified by genetic and biochemical studies: (1) position 1, the purine initiation nucleotide from which RNA synthesis begins; (2) position -6 to -12, the Pribnow box or firm binding site for RNA polymerase; (3) position -13 to -40, a region thought to be involved in the initial recognition of the promoter by RNA polymerase. Some models describing the transcriptional process envisage the formation of an "open" or "tight binding" complex at the Pribnow box after a "closed" complex involving the region between -13 and -40 is formed (42). However, there is no convincing evidence to date which demonstrates that the latter region is actually involved in recognition or that firm binding requires a site other than the Pribnow sequence. This paper presents

evidence which suggests that the sequence of nucleotides in the region between -13 and -40 is not essential for the firm binding of RNA polymerase but rather influences the process of initiation.

As mentioned earlier, the nucleotide sequence of pSC101 is identical to that of the corresponding region of pBR313 (Fig. 6) and furthermore, the region from -12 to -40 shares a 47% homology with the computer-generated promoter sequence of Scherer et al., (40) (Table 2). The longest stretch of homology involves the hexanucleotide (TTGACA) from -35 to -30. The importance of this region with regard to the process of transcription is underscored by the down promoter mutations which have been reported at each of the first five positions in this sequence (Table 2, *).

Further examination of the sequences represented in Table 2 reveal a possible correlation between the expression of Tc resistance and the placement of the hexanucleotide. For example, when sequence TTGACA is displaced to position -39 in pBR313-H and pBRH3B, a readily detectable level of Tc resistance can still be observed. However, the fact that this sequence is displaced beyond -40 in pBRH1 and completely missing in pBRH2 and pBRH4, may explain the near absence of Tc resistance in these plasmids. Alternatively, reduced levels of resistance may be the result of nucleotide substitutions at critical positions in the -13 to -40 region. Positions -38, -33 and -21, for instance, are the only positions in pBR313-H and pBRH3B homologous to the wild-type promoter but not conserved in the other promoter mutant plasmids. The construction of additional promoter mutations will be needed before one or both of these arguments can be substantiated. Nevertheless, it should be evident that a considerable portion of the -13 to -40 region can be altered (20% homology in pBRH3B) without abolishing Tc resistance completely. It is possible that normal transcription of the Tc gene depends on both the precise placement of a few nucleotides as well as a short sequence of bases, e.g. TTGACA, whose position may vary slightly. The latter variability may also reflect the flexible nature of that part of the polymerase molecule which interacts with this sequence.

With regard to thermodynamic considerations of helix stability, Table 2 shows that with the exceptions of pBRH2 and pBRH4, there appears to be no correlation between Tc resistance levels and changes in the calculated free energy of formation. Furthermore, since the substantial increase in free energy observed in pBRH2 and pBRH4 is also accompanied by considerable base pair rearrangement, it is difficult to evaluate the actual influence of

the former parameter on Tc resistance.

The fact that one can observe clear differences in the degree of protection between wild-type and mutant promoters (Table 1, columns B and I) suggests that the -13 to -40 region may be involved in promoter recognition and the firm binding of RNA polymerase. It is not unreasonable to assume that one or both of these possibilities could decrease the frequency of initiation and in turn reduce Tc resistance levels. However, as Figure 4 clearly shows, the respective cleavage sites of linear mutant and wild-type plasmid DNA are completely protected by RNA polymerase. What does distinguish mutant from wild-type promoter is the residual protection observed for the former after initiation of transcription (Table 1, column K, Fig. 4, slots 8 and 12). We conclude from these experiments that the base pair alterations in the -13 to -40 region have affected the ability of RNA polymerase to initiate transcription and not its ability to bind firmly to the mutant promoter. This conclusion is also consistent with other studies involving promoters with altered sequences or mutations in this region (43,44). Therefore, with respect to the formation of open promoter complexes, assigning the term "recognition" to this region could be misleading. How initiation, which occurs at position 1, can be affected by a DNA sequence on the other side of the firm binding site is not yet known. However, one could speculate that an altered sequence in the -13 to -40 region could work allosterically to decrease the rate of initiation by reducing RNA polymerase affinity for one or more of the ribonucleoside triphosphates.

The apparent discrepancy between the degree of protection observed for the linear versus circular forms of mutant plasmid DNA is not clear at this time. Superficially, it appears that there is a subpopulation of circular mutant plasmid molecules which are either not protected by RNA polymerase or more vulnerable to the action of Eco RI endonuclease. Whether or not this subpopulation represents the open circle or supercoil components present in all plasmid preparations, will be determined.

While the protection data presented here do not lend themselves to interpretations of association and dissociation kinetics, those experiments involving the release of protection may provide a means for determining the rate of initiation, i.e. the rate of transition from preinitiation to postinitiation complexes. This may be accomplished by determining the half-time for the rate at which protection of prebound plasmid DNA is released upon the addition of the four ribonucleoside triphosphates. In fact, the

incomplete release of protection observed with pBRH2 and pBRH4 (Fig. 4, slots 8 and 12) suggests a half-time for the rate of initiation on the order of minutes rather than milliseconds (45). Questions concerning rates of initiation and the nature of the mutant promoter transcripts will be investigated further.

Finally, we have ruled out the possibility that additional mutations in the mRNA leader sequence or structural portion of the gene are acting posttranscriptionally to reduce Tc resistance levels in these plasmids. It has been shown that when Eco RI DNA fragments, known to carry promoters, are cloned into the Eco RI sites of pBRH3B, pBRH1, pBRH2 and pBRH4, nearly wild-type levels of Tc resistance can be observed in some instances (31). These results confirm our belief that the mutational effects responsible for reduced Tc resistance are restricted to the promoter region. The ability of certain Eco RI fragments to complement the attenuated function of these mutant promoters has also provided a means for selectively cloning pieces of DNA that carry either; (1) intact promoters or; (2) nucleotide sequences needed to restore the original Tc promoter structure. Such fragments have already been cloned from a variety of prokaryotic and eukaryotic DNAs (12).

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