J. Biochem. 122, 696-702 (1997)

Structure and Function in *Escherichia coli* of Plasmids Containing Pyrimidine/Purine-Biased Stretch Originated from the 5′-Flanking Region of the Basidiomycete *ras* Gene[1]

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Received for publication, March 27, 1997

The Basidiomycete *ras* gene possesses a pyrimidine-rich stretch (CT-motif) with a short (7 bases) mirror repeat in which its major transcription start point is contained. To analyze the tertiary structure induced by the CT/AG-biased sequence and its effect on gene expression in supercoiled plasmids in *Escherichia coli*, the DNA fragment containing the *ras* CT/AG sequence was inserted into the *Eco*RI site on pBR322 in both orientations and the resulting pBR322 derivatives, named pBR-CT[ras] and pBR-invCT[ras] were introduced into *E. coli* strains DM800 (*ΔtopA gyrB225*) and JM109 (*topA*$^+$ *gyrA96*). In pBR-CT [ras] the pyrimidine-rich sequence is on the pBR322 tetracycline-resistance gene (*tet*)-coding strand and in pBR-invCT[ras] the complementary purine-rich sequence is on this strand. DNAs of pBR-CT[ras] and pBR-invCT[ras] isolated from DM800 were frequently cleaved with single-strand-specific S1 nuclease within the CT/AG sequence, showing the formation of extended open structure. Compared with those carrying pBR322, DM800 and JM109 carrying pBR-CT[ras] showed much higher levels of tetracycline resistance (Tc^r), while both strains carrying pBR-invCT[ras] showed clearly lower levels of Tc^r. pBR-CT [ras] and pBR-invCT[ras], however, conferred reduced activity of β-lactamase on DM800 and JM109. pBR-CT[ras] derivatives lacking the counterpart of the mirror repeat did not form the S1-cleavable open structure within the CT/AG sequence and conferred pBR322-like Tc^r and β-lactamase activity. The tertiary structure formed in the CT/AG sequence *via* the mirror repeat was suggested to affect the expressions of pBR322-*tet* and -*bla* genes.

Key words: CT-motif, pBR322, regulation of gene expression, S1-sensitive sites, superhelical density.

Higher eukaryotic genomes often contain polypyrimidine/polypurine sequences upstream as well as in coding regions of genes (*1*). Some polypyrimidine/polypurine sequences have the capability of adopting non-B conformations, and in particular, the CT/AG-biased sequence having a mirror repeat can form an intramolecular triplex (also called H-form) (*1-3*). These sequences have been postulated to play a role in regulation of replication (*4, 5*) and gene expression (*6*), and in recombination (*7*). We have recently found that the basidiomycetous mushroom *Lentinus edodes ras* gene (*8, 9*) possesses a CT-motif which contains the transcription start point (tsp) and a short (7 bases) mirror repeat (see Fig. 4). The *ras* CT-motif extended over 62 nucleotides (55 are pyrimidines). Similar CT-rich sequences have also been found in the 5′ promoter regions of other filamentous fungal genes (*10*). However, structure-biological function relationships have not been well studied for these sequences. Here we investigated whether the *ras* CT/AG-biased sequence forms an unusual structure in pBR322 isolated from *Escherichia coli* strains DM800 (*ΔtopA gyrB225*) (*11, 12*) and JM109 (*topA*$^+$ *gyrA96*) (*13*) using S1 nuclease (*14*). It is known that pBR322 DNA isolated from DM800 carrying a deletion of the topoisomerase I gene is extremely heterogeneous in linking number and highly negatively supercoiled when compared with that from JM109. Formation of non-B DNA structures in naturally occurring sequences usually depends on negative supercoiling (*1*). The experimental results indicated that stable S1-sensitive nonbase-paired sites are formed within the CT/AG-biased sequence under high levels of negative supercoiling. Deletion analysis showed that the mirror repeat is involved in the formation of S1-cleavage sites. To assess the effect of the CT/AG sequence on the expressions

[1] This work was partly supported by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan, and research grants from Mishima Kaiun Memorial Foundation, Asahi Breweries Foundation, Agricultural Chemical Foundation of Japan, and the Kihara Memorial Foundation for the Advancement of Life Sciences.
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Abbreviations: *bla*, β-lactamase gene; CT-motif, pyrimidine-rich stretch; CT/AG, pyrimidine/purine; PADAC, pyridinium-2-azo-*p*-dimethylaniline chromophore; S1, single-strand-specific nuclease; Tc^r, tetracycline resistance; *tet*, tetracycline-resistance gene; tsp, transcription start point(s).

of pBR322-*tet* and -*bla* genes in *E. coli*, the CT/AG-biased sequence was inserted into the *Eco*RI site on pBR322 in both orientations, and it was found that the CT/AG sequence affects the expressions of both genes. The CT/AG sequence lacking the mirror repeat, however, had almost no effect. To understand these results, the topological structures of recombinant plasmid DNAs isolated from *E. coli* were analyzed.

MATERIALS AND METHODS

Construction of pBR322 Derivatives Carrying the CT/AG-Biased Sequence of ras Gene—The 129-bp *Eco*RI-*Rsa*I DNA fragment containing the *ras* CT/AG sequence (see Fig. 4) was inserted between the *Eco*RI and *Hinc*II sites on pUC19. The *Hin*dIII-linearized recombinant pUC19 was blunt-ended and ligated to the *Eco*RI adaptor (5′-GGAATTCC). The 155-bp *Eco*RI fragment with the *ras* CT/AG sequence was inserted into the *Eco*RI site of pBR322. Based on the orientation of insertion of the CT/AG sequence, two pBR322 derivatives with a single CT/AG-sequence were obtained and designated pBR322-CT[ras] and pBR-invCT[ras] (Fig. 1). In the pBR-CT[ras] the pyrimidine-rich sequence was on the pBR322 *tet*-coding strand and in the pBR-invCT[ras] the purine-rich sequence was on this strand. The plasmid DNAs carrying small deletions within the CT/AG sequence were constructed as follows. The pBR-CT[ras] DNA was partially digested with S1 to convert 50% of the DNA to a linear form. The S1-linearized DNA separated by agarose gel electrophoresis was blunt-ended by T4 DNA polymerase and recircularized by self-ligation. Two pBR-CT[ras] derivatives lacking the nucleotides from 33 to 45 and 33 to 51 were obtained and named pBR-CT[ras]⊿13 and pBR-CT[ras]-⊿19, respectively (see Fig. 4). The following pBR322 derivatives were also constructed as a control for the experiments. The *Bgl*I-fragment containing the *lacZ* sequence of pUC19, which contains no CT/AG-biased sequence, was blunt-ended and ligated to the *Eco*RI adaptor. This 154-bp *Eco*RI fragment was inserted into the *Eco*RI site of pBR322 and the resulting recombinant plasmids were named pBR-gal′ or pBR-invgal′. In the former, the *lacZ*-coding sequence was on the *tet*-coding strand. DNAs of the pBR322 derivatives were used to transform *E. coli* DM800 (*⊿topA gyrB225*) (*11, 12*) and JM109 (*topA*$^+$ *gyrA96*) (*13*). Restriction endonucleases, DNA modifying enzymes, and the *Eco*RI adaptor were purchased from Takara Shuzo and/or Nippon Gene and used according to the supplier's instructions.

Preparation of Plasmid DNAs—LB medium containing 50 μg of ampicillin per ml was used to grow all the *E. coli* transformants carrying the plasmid. Cells from an overnight (stationary) culture were diluted 100-fold into 300 ml of fresh growth medium, grown at 37°C with aeration to late exponential phase ($A_{600} \approx 0.7$), quickly chilled and centrifuged. Plasmid DNAs were extracted by an alkaline extraction procedure and purified by treatment with RNaseA and proteinase K as described previously (*15*).

S1 Nuclease Digestion—S1 nuclease provided by Sankyo Chem. was further purified as reported previously (*16*). Three micrograms aliquots of plasmid DNAs were incubated with 3 units of S1 at 37°C for 60 min in the reaction buffer (200 μl) containing 45 mM sodium acetate (pH 4.5),

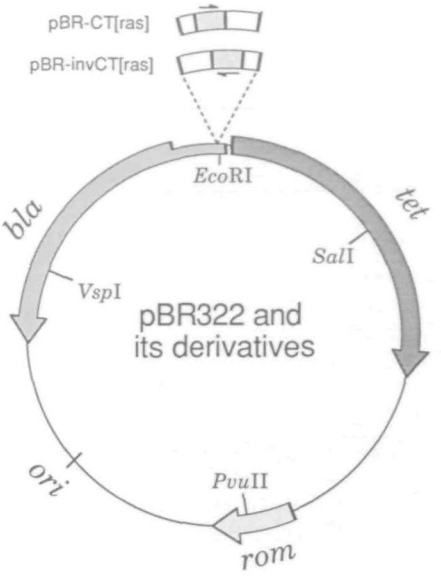

Fig. 1. **Structures of pBR322 and its derivatives carrying the CT/AG-biased sequence of the *ras* gene.** The *ras* CT/AG sequence is shown in Fig. 4. In pBR-CT[ras] the pyrimidine-rich sequence was on the pBR322 *tet*-coding strand and in pBR-invCT[ras] the purine-rich sequence was on this strand. Although not shown in the figure, the pBR322 derivatives carrying the *E. coli lacZ* sequence (154 bp) of pUC19 were also constructed as a control and named pBR-gal′ or pBR-invgal′. DNAs of the pBR322 derivatives were used to transform DM800 and JM109. Location and direction of transcription of the *tet* gene [initiated at nucleotide 45 (*27*)], the *bla* gene [initiated at nucleotides 36 and 4187 (*27*)] and the *rom* gene (*22*), location of the origin (*ori*) of replication, and restriction sites of *Eco*RI (4359), *Sal*I (651), *Pvu*II (2064), and *Vsp*I (3537) on the pBR322 genome (*22*) are indicated. The *ras* CT/AG-biased sequence (shaded)-containing fragments (open arc bars) inserted into the *Eco*RI site are shown, in which the direction of the arrow marks that of the CT strand. For more details, refer to "MATERIALS AND METHODS."

1.5 mM $ZnSO_4$, 1.5 mM $MnCl_2$, and 150 mM NaCl. The reactions were stopped by addition of Tris-HCl (pH 8.5) and EDTA to give final concentrations of 100 and 25 mM, respectively. One unit of S1 activity is defined as the amount of the enzyme that converts 50% of 3 μg of single-stranded pBR322 DNA to acid-soluble form under the above conditions.

Gel Electrophoresis—Agarose gel electrophoresis was carried out as described previously (*15*). Chloroquine-agarose gel electrophoresis was performed according to the method reported by Pruss (*17*).

Primer Extension Analysis—Restriction fragments (20 ng each) were incubated with 1 pmol of end-labeled synthetic primer at 95°C for 5 min and then at 65°C for 30 min in T7 DNA polymerase buffer [40 mM Tris-HCl buffer (pH 7.5), 20 mM $MgCl_2$, and 50 mM NaCl]. To the incubation mixture, T7 DNA polymerase (0.5 unit of Sequenase™, United States Biochemicals), dithiothreitol (final conc. 5 mM), and deoxynucleoside triphosphate (final conc. 1 mM each) were added and further incubated at 37°C for 10 min. After ethanol precipitation the reactions were electrophoresed on 8% polyacrylamide gels containing 7 M urea, followed by autoradiography. The sizes of the primer extension products were determined from the sequence ladders (ACGT) which were derived from the CT/AG-biased sequence of pBR-CT[ras] by the dideoxy chain-

termination method of Sanger *et al.* (*18*).

Tetracycline Resistance—Plasmid-harboring *E. coli* cells precultured in LB medium with 1 μg of tetracycline (Tc) per ml were transferred to fresh LB medium with 20 μg of Tc per ml and incubated at 37°C. At the indicated period, absorbance at 600 nm was measured. Late-exponential phase ($A_{600} \approx 0.7$) cultures were diluted 100-fold and spotted on LB agar plates containing increasing concentrations of Tc. The plates were observed for the presence or absence of growth.

Measurement of β-Lactamase Activity—Periplasmic β-lactamase fractions were prepared as follows. Late-exponential phase ($A_{600} \approx 0.7$) cultures of plasmid-harboring *E. coli* cells in 10 ml of LB medium with ampicillin (50 μg/ml) were harvested by centrifugation and washed with ice-cold 50 mM Na-phosphate buffer (pH 6.8), then resuspended in 2 ml of the Na-phosphate buffer containing 20% glycerol. The cells were disrupted by sonication and centrifuged at 16,000 rpm in a microcentrifuge at 4°C, and the supernatants were used as the periplasmic β-lactamase fractions. Protein concentration was determined by Coomassie dye-binding assay (Bradford assay) (*19*) with bovine serum albumin as a standard protein. The β-lactamase activity was assayed by measuring the degradation of pyridinium-2-azo-*p*-dimethylaniline chromophore (PADAC) (*20, 21*). Hydrolysis of PADAC (Calbiochem) was carried out in 1.4 ml of reaction mixture containing 50 mM Na-phosphate (pH 6.8) and 5 μM PADAC. The protein extract (2 μg) was added to the reaction mixture, and the decrease of absorbance at 570 nm was measured at 25°C. The amount of hydrolyzed PADAC was calculated using the molar absorption coefficient (45,000) of PADAC.

RESULTS AND DISCUSSION

Analysis of S1-Cleavability of pBR322 and Its Derivatives—To investigate whether the *ras* CT/AG-biased sequence forms S1-cleavable, nonbase-paired sites, DNAs of pBR322 and its derivatives isolated from *E. coli* topoisomerase I deletion mutant DM800 (*ΔtopA gyrB225*) (*11, 12*) were digested with S1 nuclease at pH 4.5. The full-length S1-linearized DNAs were further digested with restriction endonucleases which have a single unique cleavage site on the DNAs (see Fig. 1). Figure 2 shows the agarose gel electrophoretic patterns obtained for pBR322, pBR-CT[ras], and pBR-invCT[ras]. *Pvu*II-, *Sal*I-, and *Vsp*I-digests of the S1-linearized pBR322 (containing ~50% open circular DNA) yielded a number of discrete DNA bands (lanes 1, 4, and 7, respectively). These indicate that pBR322 possesses multiple S1-sites, although S1 cleaves the DNA once at each of these sites. There was diversity in the intensity of the DNA bands on the gels. The sizes corresponding to the two intense DNA bands observed in the digest by *Pvu*II (3.36 and 1.00 kb), *Sal*I (2.41 and 1.95 kb), or *Vsp*I (3.89 and 0.47 kb) summed up to the full length size (4,361 bp) of pBR322 (*22*). The results show that pBR322 possesses one site (nucleotide ~3,100) of highly preferential cleavage, supporting the previous observations by other investigators (*23, 24*). In all the cases of *Pvu*II-, *Sal*I-, and *Vsp*I-digests of the S1 linearized pBR-CT[ras] (lanes 2, 5, and 8) and pBR-invCT[ras] (lanes 3, 6, and 9), two additional intense DNA bands were observed at 2.35 and 2.16 kb, 3.76 and 0.75 kb, 3.64 and 0.87 kb, respectively, for the former and at 2.40 and 2.11 kb, 3.81 and 0.70 kb, 3.59 and 0.92 kb, respectively, for the latter. The sizes of the two fragments summed up to the nearly full-length size (4,512 bp) of the plasmid DNAs, showing the generation of another strong cleavage site (region) on both pBR-CT[ras] and pBR-invCT[ras]. This site (region) was found to fall into the CT/AG-biased sequence of both plasmid DNAs. DNAs of pBR-CT[ras] and pBR-invCT[ras] isolated from JM109 (*topA*$^+$ *gyrA96*) (*13*), on the other hand, were not preferentially cleaved by S1 within the CT/AG sequence, generating the corresponding two faint DNA bands in each of the cases of *Pvu*II-, *Sal*I-, and *Vsp*I-digestion (data not shown). pBR322 DNA isolated from DM800 has been reported to be extremely heterogeneous in linking number and highly negatively supercoiled when compared with that from JM109 (*17*). Therefore the CT/AG-biased sequence of the *ras* gene requires higher levels of negative supercoiling for formation of the preferentially S1-cleavable (stable), nonbase-paired structure. In the cases of DNAs of pBR-gal′ and pBR-invgal′ constructed by insertion of the *E. coli lacZ* sequence (containing no CT/AG-biased sequence) into the *Eco*RI site of pBR322 in both orientations (see "MATERIALS AND METHODS"), however, a preferentially S1-cleavable structure was not formed within/around the inserted sequence even if they were isolated from DM800 (data not shown).

Mapping of the S1-Cleavage Sites within the CT/AG-Biased Sequence of pBR322 Derivative pBR-CT[ras]—The S1-cleavage sites were analyzed by the primer extension method using the following two primers; primer R, 5′-GTATCACGAGGCCCTT identical with the sequence from 4333 to 4348 of pBR322 and primer H, 5′-GCAATTT-

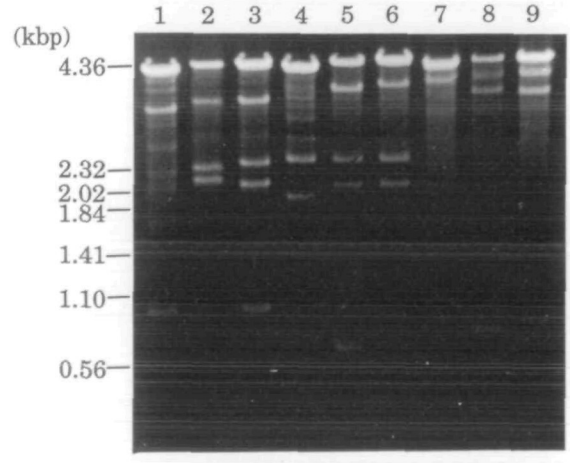

Fig. 2. **DNA fragments produced after digestion of the S1-generated unit-length linear pBR322 and its derivatives (containing ~50% open circular DNA) with restriction endonucleases.** Three micrograms aliquots of superhelical plasmid DNAs were digested with 3 units of S1 under the conditions described in "MATERIALS AND METHODS." The S1-digested DNAs were then digested with *Pvu*II (lanes 1-3), *Sal*I (lanes 4-6), and *Vsp*I (lanes 7-9). Electrophoresis was done in a 1% agarose slab gel under the conditions described previously (*15*). Lanes 1, 4, and 7, pBR322; lanes 2, 5, and 8, pBR-CT[ras]; lanes 3, 6, and 9, pBR-invCT[ras]. The *Hin*dIII-digest of phage λ DNA and *Hin*cII- and *Pvu*II-digests of pBR322 DNA were used as molecular size markers. Sizes (kb) are indicated to the left in the panel.

AACTGTGAT complementary to the sequence from 64 to 49 of pBR322 (see Fig. 3). In the *Pvu*II-digests of the S1-linearized pBR-CT[ras] (lane 2 of Fig. 2), DNA fragments at/around the two intense bands (2.35 and 2.16 kb) were subjected to analysis. In the extension of the CT-rich strand (the larger fragment and primer R), three intense and five faint bands were observed at nucleotides 45 (C), 46 (C), and 48 (T) and nucleotides 32 (T), 37 (C), 38 (C), 42 (T), and 55 (T), respectively, on the CT-rich strand (panel A of Fig. 3), indicating that the template (AG-rich) strand ended at the positions marked by arrows in Fig. 4, *i.e.*, S1 cleavages occurred at the arrow positions. In the case of the AG-rich strand (the smaller fragment and primer H), three intense bands were observed at nucleotides 60 (A), 57 (A), and 56 (A) (panel B of Fig. 3). The ends of the CT-rich template strand (the sites of S1 cleavage) are marked by arrows in Fig. 4. These results demonstrate that the *ras* CT/AG-biased sequence forms an extended open structure sensitive to S1 cleavage. The S1-cleavage sites determined by the extension of the CT-rich strand did not coincide with those of the AG-rich strand except for the cleavage between nucleotides 55 and 56. This suggests that S1 cleavages occurred simultaneously at site(s) on the CT-strand and site(s) on the AG-strand, and then second S1 cuts occurred on the opposite strand at or near the sites nicked first to yield linear DNA molecules slightly smaller than full length size.

A polypyrimidine/polypurine sequence with mirror repeat symmetry (*2*) has been reported to form an intramolecular triplex structure under conditions of negative supercoiling and either low pH (*25*) or neutral pH in the presence of divalent cations (*26*). So we constructed pBR-CT[ras]⊿13 and pBR-CT[ras]⊿19, which are the pBR-CT[ras] plasmids lacking the counterpart of the mirror repeat of the CT/AG sequence (see Fig. 4). The S1-digestion analysis of the two DNAs isolated from DM800 revealed that an S1-cleavable structure was not formed within the shortened CT/AG sequence (data not shown), showing that the mirror repeat is involved in the formation of S1-cleavage sites, and presumably in that of intramolecular triplex structure (H-y3 conformation) at pH 4.5.

Tc^r Levels of E. coli Carrying pBR322 and Its Derivatives—Although maximum Tc concentration permitting growth of DM800 (JM109) carrying pBR322 was 16 (125) μg per ml, DM800 (JM109) cells carrying pBR-CT[ras] could grow on agar plates at Tc concentrations up to 20 (175) μg per ml. DM800 (JM109) carrying pBR-CT[ras]-

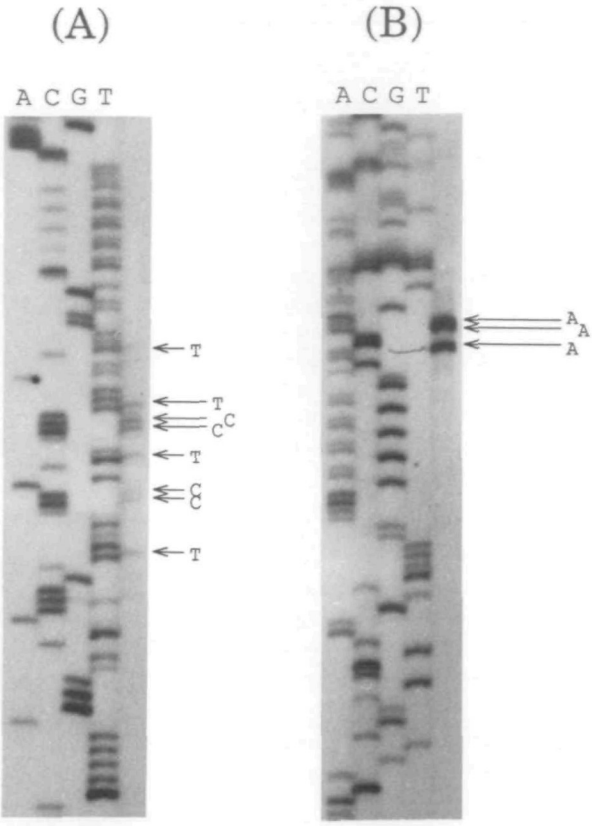

Fig. 3. **Determination of the S1-cleavage sites within the CT/AG-biased sequence of pBR322 derivative pBR-CT[ras] by the primer extension method.** The extensions of the CT-rich strand with primer R (panel A) and the AG-rich strand with primer H (panel B) were carried out using the larger or smaller fragment of the *Pvu*II-digests of pBR-CT[ras]. The nucleotides corresponding to the ends of extension products (marked by arrows) were determined from the sequence ladders (ACGT) which were derived from pBR-CT[ras] with primers R and H by the dideoxy chain-termination method of Sanger *et al.* (*18*).

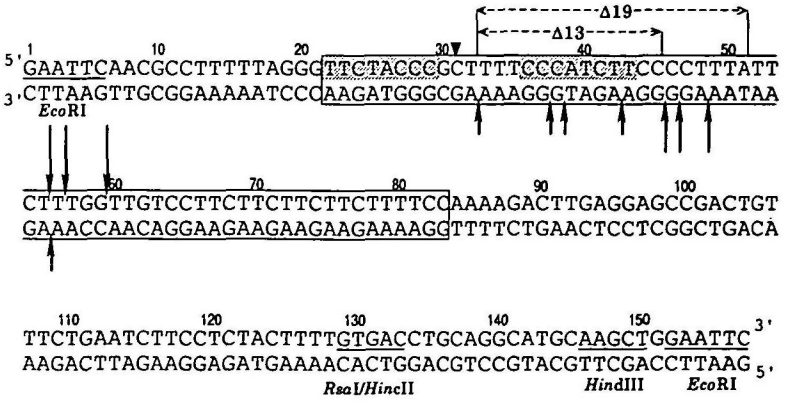

Fig. 4. **Summary of the S1-cleavage sites within the CT/AG-biased sequence of the *ras* gene.** The nucleotide sequence of the CT/AG-biased sequence (boxed)-containing region of the *ras* gene is shown. Mirror repeat sequences are shaded on the pyrimidine strand in the CT/AG box. Closed arrowheads are tsp. The S1-cleavage sites on the template strand in the extensions of the CT and AG strands are shown by vertical arrows. Their lengths correspond to the frequencies of S1 cleavages judged from the results of Fig. 3, with the most preferential sites marked by long arrows. Restriction sites used for the construction of recombinant plasmids are also shown. Two broken lines with arrowheads above the sequence indicate the regions of deletion in pBR-CT[ras]⊿13 and pBR-CT[ras]⊿19 (see "MATERIALS AND METHODS"). In pBR-CT[ras]⊿19 one end of the deletion does not match the position of the arrow of the S1-cleavage. This must be due to the occurrence of a second S1 cut on the opposite strand near to (not at) the site nicked first, yielding a 3'-protruding end. The sequence data will appear in the DDBJ/EMBL/GenBank nt sequence databases under accession No. D01208.

⊿13 and pBR-CT[ras]⊿19, however, could not grow at the same Tc concentration as pBR-CT[ras] though they were resistant to 18 (140) μg of Tc per ml. DM800 (JM109) carrying pBR-invCT[ras] could not grow in the presence of 13 (100) μg of Tc per ml, but it was resistant to 11 (75) μg of Tc per ml. DM800 (JM109) carrying the *E. coli lacZ* sequence (adopting usual B structure)-containing plasmids pBR-gal′ and pBR-invgal′ showed the same level of Tc^r as that carrying pBR322. The copy numbers of pBR322 and its six derivatives in DM800 (JM109) were almost the same, suggesting that the CT/AG sequence does not affect the regulation of replication of the plasmid DNAs. The results show that pBR-CT[ras] (pBR-invCT[ras]) confers higher (lower) levels of Tc^r on DM800 and JM109 than pBR322 does. pBR-CT[ras]⊿13 and pBR-CT[ras]⊿19 confer pBR322-like Tc^r on both strains. The plasmid-carrying DM800 cells, however, are highly sensitive to Tc when compared with the plasmid-carrying JM109 cells.

The growth of DM800 carrying pBR322 and its derivatives was examined in liquid medium. DM800 cells carrying pBR-CT[ras] showed almost no growth in the presence of 7 μg of Tc per ml and the difference in growth rate in the presence of 5 μg of Tc per ml between the DM800 cells carrying each of the plasmids was not clear. JM109 cells carrying each of the plasmids, on the other hand, grew well in liquid medium containing 20 μg of Tc per ml. So we examined the JM109 cells at this concentration of Tc. As shown in Fig. 5, the growth rate of JM109 carrying pBR-CT[ras] was clearly higher than that carrying pBR322 or either of the pBR-CT[ras] deletion derivatives. JM109 carrying pBR-invCT[ras] grew at the most reduced rate. Thus, the plasmids were ordered with respect to the expression of Tc^r in JM109 (DM800) as follows: pBR-CT[ras] > pBR-CT[ras]⊿13 ≥ pBR-CT[ras]⊿19 ≥ pBR322 > pBR-invCT[ras].

The CT/AG sequence of the *ras* gene is probably also widely single-stranded in supercoiled plasmids in *E. coli* cells; DNAs of pBR322, pBR-CT[ras] and pBR-invCT[ras] isolated from DM800 were found to be cleaved by single-strand-specific Bal31 nuclease (*14*) at neutral pH and under physiological conditions within regions similar to those cleaved by S1 (data not shown), although the restriction DNA bands were relatively diffused on gels because Bal31 possesses a higher quasi-processive exonuclease activity that simultaneously degraded both 3′- and 5′-termini of duplex DNA (*14*). So the upstream sequences of the pBR322-*tet* promoter region (nucleotides 10-45) (see Fig. 1) are expected to be easily unwound in the DNA of pBR-CT[ras], resulting in an efficient initiation of transcription of the *tet* gene. Even though in the DNA of pBR-invCT[ras] the unwound upstream region must be much closer to the pBR322-*tet* promoter, the plasmid confers lower levels of Tc^r on *E. coli* cells. Probably the CT/AG sequence does not adopt a canonical unwound structure in supercoiled plasmid in *E. coli*; it presumably forms an intramolecular triplex

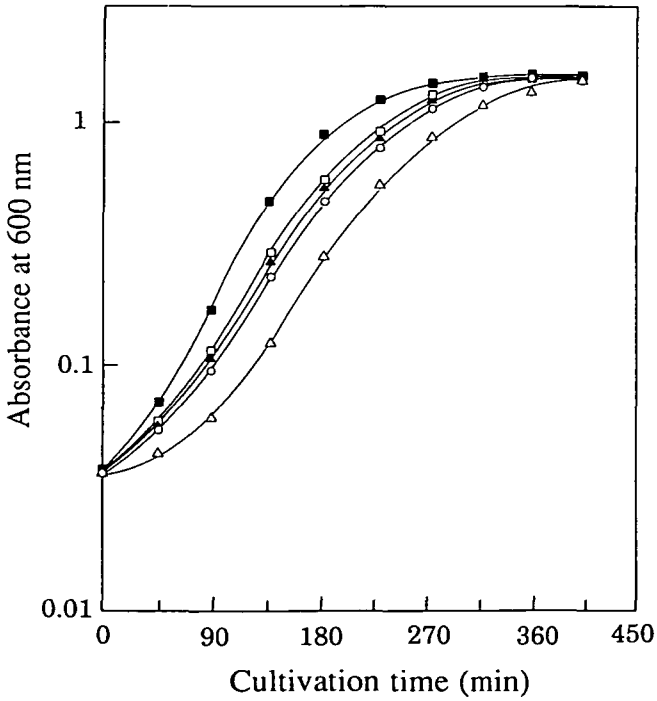

Fig. 5. **Growth curves of the JM109 cells carrying pBR322 and its derivatives.** Plasmid-harboring *E. coli* cells were cultured in LB medium containing 20 μg of Tc per ml at 37°C. At the indicated period, absorbance at 600 nm was measured. The plasmids carried were as follows: pBR322 (○), pBR-CT[ras] (■), pBR-invCT[ras] (△), pBR-CT[ras]⊿13 (□), pBR-CT[ras]⊿19 (▲). Absorbance values are the average of 3 independent experiments.

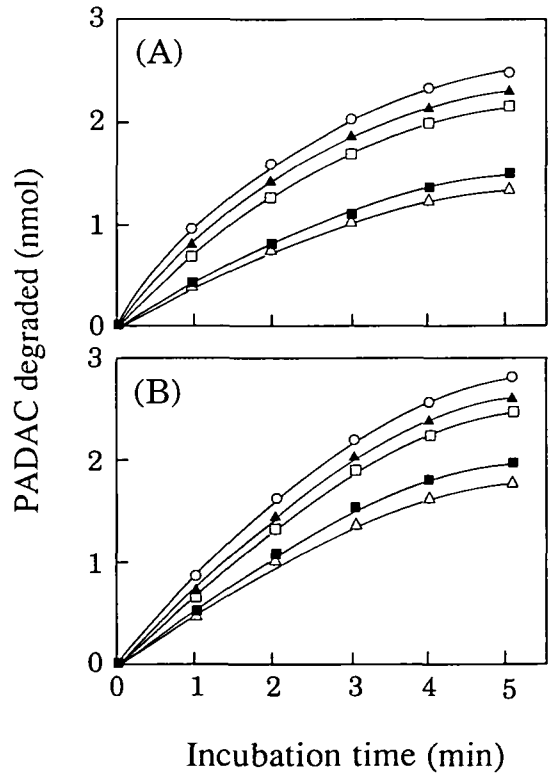

Fig. 6. **The periplasmic β-lactamase activities of DM800 (A) and JM109 (B) carrying pBR322 and its derivatives determined by measuring the degradation of PADAC.** The reaction was performed in 50 mM Na-phosphate buffer (pH 6.8) containing 5 μM PADAC at 25°C. The decrease of absorbance at 570 nm was measured at the indicated period and the amount of hydrolyzed PADAC was calculated. The plasmids carried were as follows: pBR322 (○), pBR-CT[ras] (■), pBR-invCT[ras] (△), pBR-CT[ras]⊿13 (□), pBR-CT[ras]⊿19 (▲). Absorbance values are the average of 3 independent experiments.

structure transiently *via* the mirror repeat. The sequence between the putative triplex portion and pBR322-*tet* promoter may be tightly double helical. This state is probably unfavorable for the initiation of transcription of the *tet* gene.

β-Lactamase Activity in E. coli Carrying pBR322 and Its Derivatives—The β-lactamase activity expressed in DM800 (JM109) carrying pBR-CT[ras] was similar to that carrying pBR-invCT[ras]. These activities were clearly lower than that carrying pBR322 (Fig. 6). DM800 transformants carrying pBR-CT[ras] or pBR-invCT[ras] showed lower levels of the activity than JM109 transformants carrying either of them. DM800 (JM109) carrying pBR-CT[ras]Δ13 or pBR-CT[ras]Δ19 showed clearly higher activities than that carrying pBR-CT[ras]. pBR-gal′ and pBR-invgal′ conferred very similar activities of β-lactamase to that of pBR-CT[ras]Δ19 on DM800 and JM109 (data not shown). Transcription of the *bla* gene is initiated at nucleotides 36 and 4187 and proceeds in the direction opposite to that of the *tet* gene (*27*) (see Fig. 1). The results, therefore, suggest that the ras CT/AG sequence-containing fragment inserted into the *Eco*RI site may inhibit the *bla* transcription started at nucleotide 36 in spite of the orientation of insertion of the fragment [RNA polymerase may stall at the unusual structure (intramolecular triplex?) formed in the CT/AG sequence], but it has almost no effect on the *bla* transcription initiated at nucleotide 4187, which is distant from the *Eco*RI site.

Negative Superhelicity of pBR322 and Its Derivatives Propagated in DM800—To understand the experimental results described above, the topological structures of the plasmid DNAs were analyzed. In the negative supercoiling distribution of pBR322 DNA isolated from DM800 (lanes 1 and 8 of Fig. 7A), the bottom band contains the most negatively supercoiled DNA, intermediate bands contain DNA with fewer supercoils (the topoisomers migrate more slowly with less supercoiling), and the top band contains nicked circular DNA (*17*). The two derivatives pBR-CT[ras] (lane 2) and pBR-invCT[ras] (lane 3) displayed supercoiling distributions wherein a smaller amount of highly negatively supercoiled DNA species remained, while the control four derivatives pBR-gal′ (lane 4), pBR-invgal′ (lane 5), pBR-CT[ras]Δ19 (lane 6), and pBR-CT[ras]Δ13 (lane 7) exhibited pBR322-like high levels of negative supercoiling. pBR-CT[ras] seems to contain a slightly more highly negatively supercoiled DNA species than pBR-invCT[ras]. To obtain more detailed information about the difference in negative superhelical density between pBR-CT[ras] and pBR-invCT[ras], two-dimensional chloroquine-agarose gel electrophoresis was done (panels B1, 2, and 3 of Fig. 7). Compared with pBR-CT[ras] (B2), pBR-invCT[ras] (B3) contained a larger amount of positively supercoiled DNA molecules (in the upper arc) which were derived from less negatively supercoiled DNA species propagated in DM800, confirming that the negatively superhelical density of pBR-invCT[ras] is lower than that of pBR-CT[ras]. Negative superhelical densities of DNAs of pBR322, pBR-CT[ras] and pBR-invCT[ras] isolated from JM109 were also analyzed. However, no clear difference in the densities could be detected between the three plasmid DNAs.

The high levels of negative supercoiling of pBR322 in DM800 are known to be attributed to the generation of twin

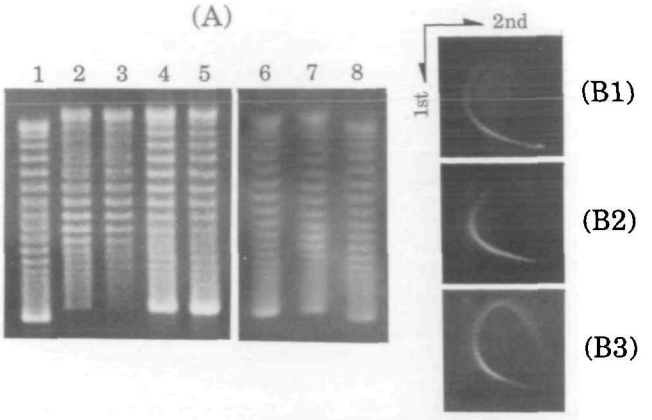

Fig. 7. **Chloroquine-agarose gel electrophoretic analysis of the degree of negative supercoiling of the plasmid DNAs isolated from DM800.** (A) One-dimensional electrophoretic patterns. Samples (2 μg each) of pBR322 (lanes 1 and 8), pBR-CT[ras] (lane 2), pBR-invCT[ras] (lane 3), pBR-gal′ (lane 4), pBR-invgal′ (lane 5), pBR-CT[ras]Δ19 (lane 6), and pBR-CT[ras]Δ13 (lane 7) were electrophoresed in a 1% agarose horizontal slab gel containing chloroquine (100 μg/ml) according to the method of Pruss (*17*). (B1-B3) Two-dimensional electrophoretic patterns. Samples (2 μg each) of pBR322 (B1), pBR-CT[ras] (B2), and pBR-invCT[ras] (B3) were loaded on gel. Chloroquine was present at 48 μg/ml in the first (vertical) dimension and at 196 μg/ml in the second (horizontal) dimension. During electrophoresis in the first dimension, topoisomers in the lower arcs of the three distributions were negatively supercoiled, while those in the upper arcs were positively supercoiled, causing the two arcs of each distribution to be superimposed. Electrophoresis in the second dimension resolved the superimposed topoisomers.

supercoiled domains during transcription of *tet*: in the absence of the topoisomerase I, the positively supercoiled domain is effectively relaxed by gyrase, resulting in a net accumulation of negative supercoils (*28, 29*). The presence of the *bla* gene is considered not to be required for high levels of negative plasmid supercoiling; the pBR322 deletion plasmid lacking the *bla* gene showed a pBR322-like high level of negative supercoiling (*30, 31*). As transcription proceeds, DNA in front of the transcription complex becomes positively supercoiled, and DNA behind the complex becomes negatively supercoiled (*29*). The inserted CT/AG-biased sequence-containing region present in the negative domain (*Eco*RI site) of the two pBR322 derivatives may form an altered structure, presumably such that introduction of negative turns into the derivatives is insufficient when compared with pBR322 DNA and/or some gyrase molecules do remove negative turns in the derivatives. As described earlier, the level of Tc^r of *E. coli* carrying pBR-CT[ras] is clearly higher than that carrying pBR-invCT[ras], showing more efficient initiation of the transcription of the *tet* gene in pBR-CT[ras]. This causes more frequent formation of twin supercoiled domains in pBR-CT[ras] than in pBR-invCT[ras], resulting in the increase of negative superhelical density of the plasmid.

We have shown above that the CT/AG-biased sequence with a short (7 bases) mirror repeat from the basidiomycete *L. edodes ras* gene forms an extended S1-cleavable open structure in pBR322 DNA and affects the expressions of the plasmid-coding *tet* and *bla* genes in *E. coli*. The open structure has been implied to be formed by the intra-

molecular triplex formation *via* the mirror repeat. Thus, we performed two-dimensional chloroquine-agarose gel electrophoretic analysis of the pBR322 derivatives containing the CT/AG-biased sequence under the following conditions. The DNAs were electrophoresed in low pH ($\leq$6.0) buffer containing no chloroquine in the first dimension and in neutral pH buffer containing chloroquine in the second dimension. If the CT/AG-biased sequence forms the stable intramolecular triplex structure, one would expect the detection of a break or irregular pulse in the topoisomer arc under these conditions (*3*). Unfortunately, a positive indication of the presence of intramolecular triplex structure was not obtained in the experiments (data not shown). Probably, the CT/AG-biased sequence does not form the intramolecular triplex structure under the conditions of the electrophoresis. It may form the triplex structure under more restricted conditions both *in vivo* and *in vitro*. In the basidiomycetous fungal cells, the potential exists to generate negative superhelical turns by the local removal of histone octamers from the chromosome. So the CT/AG-biased sequence is expected to form an unusual structure and to function also in the fungal cells.

We are indebted to Ms. M. Hayashi for typing the manuscript.

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