The presence of the region on pBR322 that encodes resistance to tetracycline is responsible for high levels of plasmid DNA knotting in *Escherichia coli* DNA topoisomerase I deletion mutant

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ABSTRACT

Plasmid pBR322 DNA isolated from *Escherichia coli* DNA topoisomerase I deletion mutant DM800 is estimated to contain about 10% of the knotted forms (Shishido et al., 1987). These knotted DNA species were shown to have the same primary structure as usual, unknotted pBR322 DNA. Analysis of the knotting level of deletion, insertion and sequence-rearranged derivatives of pBR322 in DM800 showed that the presence of the region on pBR322 encoding resistance to tetracycline (*tet*) is required for high levels of plasmid knotting. When the entire *tet* region is present in a native orientation, the level of knotting is highest. Inactivating the *tet* promoter is manifested by a middle level of knotting. For deletion derivatives lacking various portions of the *tet* region, the level of knotting ranges from lowest to high depending on the site and length of the *tet* gene remaining. Inverting the orientation of *tet* region on the pBR322 genome results in a middle level of knotting. Deleting the ampicillin-resistance (*bla*)gene outside of its second promoter does not affect the level of knotting, if the entire *tet* gene remains. A possible mechanism of regulation of plasmid knotting is discussed.

INTRODUCTION

Duplex DNA knots have been reported to be formed *in vitro* reaction systems of phage T4 DNA topoisomerase II (1), *Drosophila* DNA topoisomerase II (2), *Escherichia coli* DNA topoisomerase I (3), the resolvase protein encoded by transposon Tn*3* (4) and phage λ Int protein (5,6). *E. coli* DNA gyrase has been described to do occasionally knot DNA *in vitro* (7). Information on the *in vivo* DNA knotting is scant, although majority of the DNA prepared from tailless capsids of phage P2 has been reported to be knotted duplex rings (8).

We have recently found that plasmid pBR322 DNA propagated in *E. coli* strain DM800 carrying deletion of the DNA topoisomerase I gene (Δ*topA*) with a compensatory mutation of the DNA gyrase B gene (*gyrB225*) (9,10) contains about 10% of the knotted forms (11). Analysis of the knotted DNA molecules of pBR322 reveals that predominantly knots with an odd number of nodes are produced (see figure 1). Almost all the trefoils and 5-noded knots tested have a negative topological sign (11). The knotted molecules are considered to be formed by the DNA gyrase reaction. Some control, however, seems to be involved in the DNA knotting. The studies on the knots formed in nicked DNA rings by *E. coli* DNA topoisomerase I have shown that the enzyme produces every knot theoretically possible in terms of the number of nodes and topological sign (3). The 4-noded and 6-noded knots made in supercoiled DNA by Tn*3* resolvase during the *in vitro* site-specific recombination have been shown to possess two positive and two negative sign and four positive and two negative sign, respectively (4). Analysi1s of the knots produced in supercoiled DNA by

the *in vitro* phage λ integrative recombination have shown that only the knots with an odd number of nodes with positive sign are produced (5,6).

We have analyzed the primary structure of the knotted pBR322 DNA isolated from DM800. Using a recombinant DNA technique, it has been examined whether there are sequence-dependent differences in the level of knotting among the DNAs of pBR322 derivatives isolated from DM800. We describe in this paper that the knotted plasmid DNA species possess the same arrangement of base sequences as usual, unknotted pBR322 DNA. The presence of the tetracycline-resistance region (*tet*) is responsible for high levels of plasmid knotting. Based on the experimental results obtained, we discuss a possible mechanism of generation of the knotted pBR322 molecules in *E. coli* DM800.

MATERIALS AND METHODS

Construction of pBR322 derivatives. For construction of deletion and insertion derivatives, pBR322 was digested with one or two restriction endo-nucleases (figure 2), followed by digestion with *Bal*31 nuclease, if necessary, and single-stranded DNA ends were filled in using the Klenow fragment of *E. coli* DNA polymerase I. The DNA was recircularized with T4 DNA ligase and used to transform *E. coli* strain DM800. Construction of sequence-rearranged derivatives was as follows. The pBR322 *Eco*RI-*Pvu*II fragment, containing the *tet* transcription unit and the major part of the *rom* (RNA one inhibition modulator) transcription unit, was inverted after the conversion of the *Eco*RI sites to blunt ends by repair with the Klenow fragment (figure 2). Restriction fragment was isolated from the region encoding resistance to tetracycline of pBR322, treated with the Klenow fragment and then, inserted into the blunt-ended *Pvu*II site of pBR322 deletion plasmid which lacks the region from the *Eco*RV site to the *Ava*I site (figure 2). The resulting DNA yielded a transformation of DM800. Restriction endonucleases and DNA modifying enzymes all were purchased from Takara Shuzo Co. (Kyoto, Japan) and used according to the supplier's instructions. Published procedure (12) was used for transformation of *E. coli*. Plasmid DNA was prepared from the transformants showing the expected pattern of antibiotic resistance and subjected to restriction analysis. Transformants carrying plasmids with the expected structure were used for analysis of knotting. These plasmids are shown schematically in figure 2.

Other methods. E. coli W3110 derivative DM800 [Δ(*topA cysB*)*204 gyrB225 acrA13*] (9,10) was used. Plasmid DNA was isolated from cells grown at 37°C with aeration to late exponential phase ($A_{600} \approx 0.7$) in LB medium containing L-cystein (60 μg/ml) and ampicillin (50 μg/ml) or tetracycline (15 μg/ml) according to the published procedure (11). Agarose and polyacrylamide gel electrophoreses were performed according to the published procedures (11 and 12, respectively). Chloroquine-agarose gel electrophoresis was performed according to the method reported by Pruss (13). DNA was recovered from agarose gel with GENECLEAN KIT of Bio101 Inc. (La Jolla, CA) according to the maker's recommendation. Published procedure (11) was used for electron microscopic analysis of DNA molecules coated with RecA protein.

RESULTS

Primary structure of knotted pBR322 molecules produced in E. coli DNA topoisomerase I deletion mutant DM800

pBR322 isolated from *E. coli* DM800 was nicked by pancreatic DNase I and electrophoresed in an agarose gel, resulting in separation of the knotted nicked DNA species with various

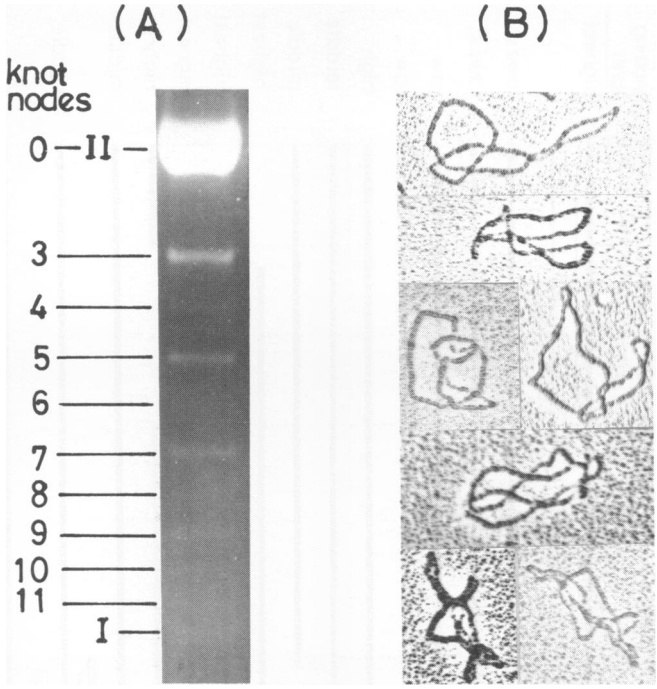
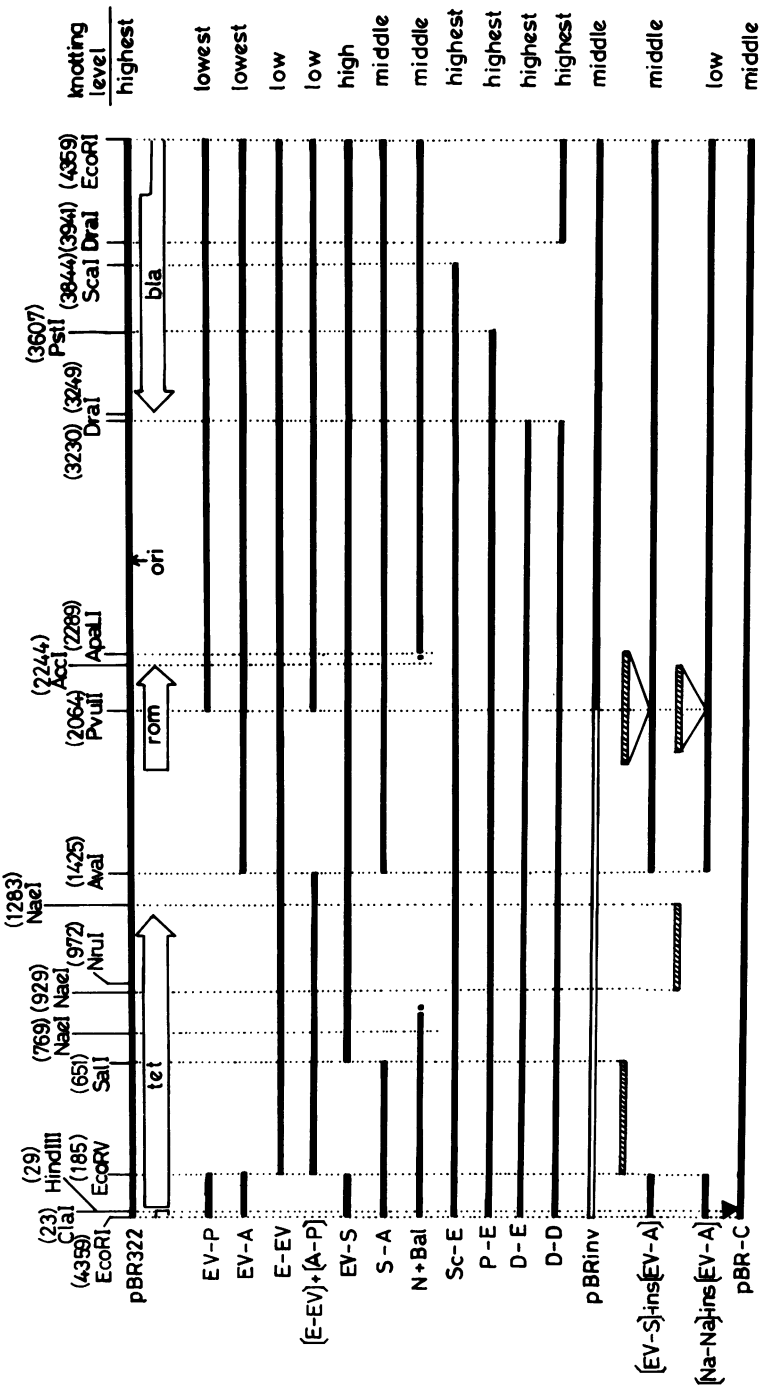


Figure 1. Isolation of the knotted DNA species from pBR322 preparation from *E. coli* strain DM800. (A) Separation of the knotted DNA species by agarose gel electrophoresis. pBR322 (800 μg) prepared from DM800 was nicked by pancreatic DNase I in the presence of ethidium bromide as described by Hsieh and Wang (23), extracted 5 times with phenol to remove ethidium bromide, precipitated with ethanol, dissolved in 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA, and dialyzed against the same buffer. The nicked pBR322 DNA was electrophoresed in a 1% agarose horizontal slab gel in Tris-acetate/EDTA buffer, pH 8.2 (40 mM Tris base, 20 mM sodium acetate, and 1 mM EDTA) at 2 V/cm for 16 h at room temperature, resulting in the formation of bands of knotted DNA species which were separated from usual, unknotted DNA. The number of knot nodes assigned in the published paper (11) are given to each the DNA bands. Unknotted nicked circular (II) form of pBR322 and a trace amount of unknotted supercoiled pBR322 DNA (I) that escaped the DNase I nicking treatment are indicated. (B) Electron micrographs of representative knotted DNA molecules. The DNA species in knot nodes 3−11 were recovered from gels, re-electrophoresed, and purified using a standard procedure. DNA was coated with RecA protein, mounted and photographed in the electron microscope according to the published procedure (11).

numbers of nodes from the unknotted nicked circular form (figure 1-A). As proved in previous paper (11), neighboring rungs of a knot ladder differ by one node and the knotted DNA with an odd number of nodes is a predominant DNA species. All the knotted DNA bands were isolated from the gel and confirmed to be knotted by electron microscopy after coating the DNA with RecA protein (figure 1-B). A mixture consisting of multiple knotted pBR322 molecules with three to eleven nodes (figure 1) was cleaved with one or two restriction endonucleases: *Nae*I, *Hin*fI or *Eco*RI + *Ava*I, *Dra*I + *Pvu*II, *Apa*LI + *Acc*I, *Bam*HI + *Pst*I. The unknotted open circular pBR322 DNA was isolated and digested in parallel. Agarose gel electrophoretic analysis showed that the restriction digests of the knotted pBR322 molecules are the same as those of the unknotted pBR322 control (data not shown).


knotting level
highest
lowest
lowest
low
low
high
middle
middle
highest
highest
highest
highest
middle
middle
low
middle
(4359) EcoRI
(3844)(3941) ScaI DraI
(3607) PstI
(3230) (3249) DraI
(2244) AccI
(2289) ApaLI
(2064) PvuII
(1425) AvaI
(1283) NaeI
(972) NruI
(929) NaeI
(769) NaeI
(651) SalI
(185) EcoRV
(29) HindIII
(23) ClaI
(4359) EcoRI
bla
ori
rom
tet
pBR322
EV-P
EV-A
E-EV
(E-EV)+(A-P)
EV-S
S-A
N+Bal
Sc-E
P-E
D-E
D-D
pBRinv
(EV-S)-ins(EV-A)
(Na-Na)-ins(EV-A)
pBR-C

The knotted nicked pBR322 molecules isolated were treated with T4 DNA ligase and the resulting DNA was used to transform *E. coli* DM800. Transformants showing resistance to ampicillin or tetracycline were picked up 100 each and their resistance to the other antibiotic was examined. All the transformants tested were found to have resistance to both of the antibiotics. There existed no difference in the efficiency of transformation between the knotted and unknotted circular DNAs ($3-4\times10^4$ transformants per microgram of DNA).

The results, taken together, showed that the knotted pBR322 molecules possess the same primary structure as usual, unknotted pBR322 DNA; they are unlikely to be the product of an intramolecular recombination of the DNA.

Sequences in pBR322 cause high levels of plasmid DNA knotting

It was examined whether particular sequences of the pBR322 genome are associated with high levels of plasmid DNA knotting in *E. coli* DM800. Various deletion derivatives of pBR322 (figure 2) were systematically constructed using unique restriction sites in order to localize the region required for DNA knotting. Plasmid DNAs propagated in DM800 were isolated according to the procedure reported previously (11), nicked by DNase I and electrophoresed in an agarose gel to analyze the level of plasmid knotting (figure 3). The negatives of the agarose gel electrophoretic patterns yielded a densitometric measurement of the fraction of knotted DNA species. The levels of knotting were classified into highest, high, middle, low, and lowest (see the right of figure 2). pBR322 DNA propagated in DM800 was electrophoresed in lane 1 (control for lanes 2−8), lane 9 (control for lanes 10−14) and lane 15 (control for lanes 16−18) of figure 3: DNA samples of lanes 1−8, lanes 9−14, and lanes 15−18 were separately electrophoresed, stained and photographed. Deletion plasmids which carry a deletion from the *Eco*RV site to the *Ava*I site (figure 3, lane 3) or that from the *Eco*RV to the *Pvu*II site (figure 3, lane 2) exhibited lowest level of knotting. Deleting the *Eco*RI-*Eco*RV fragment (containing the promoter and beginning of the coding region of the *tet* gene, and second promoter of the *bla* (resistance to ampicillin) gene) and deleting both the *Eco*RI-*Eco*RV fragment and the *Ava*I-*Pvu*II fragment (containing the promoter and major part of the coding region of the *rom* gene) were manifested by low levels of plasmid knotting (figures 3, lanes 4 and 5, respectively). The deletion plasmid lacking the *Eco*RV-*Sal*I fragment displayed high level of knotting (figure 3, lane 6). The deletion plasmid lacking the *Sal*I-*Ava*I fragment (figure 3, lane

Figure 2. Deletion, insertion, and sequence-rearranged derivatives of pBR322 constructed in this study. A restriction map of pBR322(24−26) linearized at the *Eco*RI site is presented at the top of the figure. Another *Nae*I site and *Acc*I site are located at nucleotide 401 and 651, respectively. Two other *Apa*LI sites are at nucleotide 2787 and nucleotide 4033. Transcription of the *tet* gene is initiated at nucleotide 45 (16) and transcription of the *bla* gene is initiated at both nucleotide 36 and nucleotide 4187 (16,26). Location and direction of the *rom* transcription unit (17) and location of the origin (*ori*) of replication are also shown. Plasmid designations are shown on the left and correspond basically to the first letters of the restriction endonucleases used in the construction. Deletions are presented with interruptions of closed bars. For construction of derivative N+Bal, the *Nru*I-linearized unit-length pBR322 DNA was shortened by ca.1.4 kilobase pairs by treatment with *Bal*31 nuclease. The putative ends of deletion of the plasmid are shown (*Bal*31 was shown to remove nucleotides from both 3′ and 5′ ends of the DNA at a considerably different rate). Derivative pBRinv shows the pBR322 of which *Eco*RI-*Pvu*II region (open bar) is inverted. Details for sequence-rearranged deletion derivatives [EV-S]-ins[EV-A] and [Na-Na]-ins[EV-A] are presented in the text. Derivative pBR-C is the pBR322 which possesses the insertion of two base pairs at the *Cla*I site (closed triangle); this insertion was created by filling in the *Cla*I-derived single-stranded ends, and religating. A summary of the results of analyzing the level of knotting found in various pBR322 derivatives is given on the right. Based on a densitometric tracing of the negatives of the agarose gel electrophoretic patterns pictured in figure 3, the level of knotting is classified arbitrarily with the wordextent.

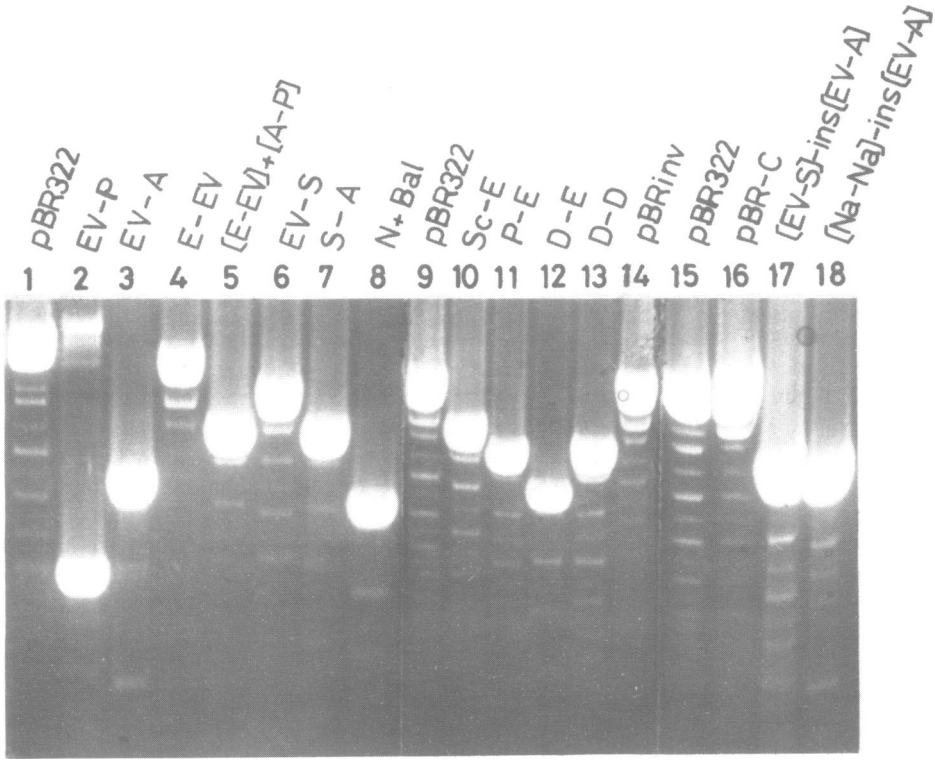


Figure 3. Agarose gel electrophoretic analysis of the level of plasmid DNA knotting found in various derivatives of pBR322. Samples (40 μg each) of pBR322 derivatives isolated from DM800 were subjected to agarose gel electrophoresis after DNase I nicking as in figure 1. Derivatives of pBR322 are designated as in figure 2. A DNA band just below that of unknotted open circular DNA in lanes 1, 4, 5, 6, 7, 9, 10, 11, 13, 14, 15, and 16 is full-length linear DNA generated by accumulation of random nicks. In lanes 3 and 18, a faint DNA band of unknotted supercoiled DNA that escaped the nicking treatment is visible at the bottom.

7) and that having deletion from the internal site of the *tet* gene to the end of the *rom* gene (figure 3, lane 8; figure 2, sample N+Bal) both displayed middle levels of knotting. Four *bla* deletion plasmids constructed by deleting the *Sca*I-*Eco*RI fragment (figure 3, lane 10), the *Pst*I-*Eco*RI fragment (figure 3, lane 11), the *Dra*I (nucleotide 3230)-*Eco*RI fragment (figure 3, lane 12), and the *Dra*I (nucleotide 3230)-*Dra*I (nucleotide 3941) fragment (figure 3, lane 13) from pBR322 all displayed pBR322-like knotting. The high levels of plasmid knotting were not related to molecular sizes and copy umbers (qualitative estimates in plasmid preparations) of pBR322 derivatives.

The above results suggest that the region from the *Eco*RI site to the *Ava*I site is responsible for high levels of plasmid knotting. This region contains the *tet* promoter, the *tet* coding region, and second promoter of the *bla* gene (figure 2). If the entire *tet* region remains, deleting the *bla* gene outside of its second promoter does not affect the level of knotting. When the entire *tet* region is present, the level of knotting is highest. Introducing various deletions within the *tet* region reduces the level of knotting depending on the site and length of deletion (figure 3, lanes 2−8). The deleting the *Eco*RI-*Eco*RV fragment eliminates most

of the knotted DNA from the plasmid preparation (figure 3, lane 4). The deletion plasmid which lacks the *tet* region outside of the *Eco*RI-*Eco*RV fragment displays lowest level of plasmid knotting (figure 3, lane 3). These may imply that transcription of the *tet* gene sequence (or certain segments of it) is responsible for high levels of plasmid knotting.

Levels of plasmid DNA knotting of other pBR322 derivatives

If transcription of the pBR322-*tet* gene sequence is responsible for high levels of plasmid knotting, inactivation of the *tet* promoter should affect knotting. pBR322-*tet* promoter was inactivated by insertion of two base pairs at the *Cla*I site (14,15). This mutation (figure 2, sample pBR-C) reduced the population of knotted DNA species, but it did not completely abolish the formation of knotted DNA (figure 3, lane 16; compare with lane 15). This shows that transcription of the *tet* gene sequence is truly one of the determinants of the level of plasmid knotting. But as for the middle level of plasmid knotting, it does occur even if the *tet* gene sequence is not transcribed.

In the pBR322 genome the *tet* and *bla* transcription units are oriented in opposite directions (16). The *rom* transcription unit, which is located between the *ori* (origin of replication) and the *tet* gene, is transcribed in the opposite direction to the *bla* gene (17) (see figure 2). Mutant plasmid of which both the *tet* transcription unit and the major part of the *rom* transcription unit are inverted by inverting the *Eco*RI-*Pvu*II fragment was constructed (figure 2, sample pBRinv) and used to examine the level of knotting. The population of knotted molecules in the plasmid preparation was reduced, but the middle level of plasmid knotting was still observed (figure 3, lane 14; compare with lane 9). This shows that the formation of knotted DNA is also affected by the direction of *tet* transcription.

What if part of the *tet* coding region is relocated to another site of plasmid DNA? The *Eco*RV-*Sal*I fragment and the *Nae*I-*Nae*I (nucleotides 929−1283 in figure 2) fragment were inserted at the *Pvu*II site (within the *rom* gene) of the deletion plasmid lacking the *Eco*RV-*Ava*I fragment which shows lowest level of plasmid knotting (figure 2); besides the supply of parts of the tet *coding* region, the insertion of *tet* fragments should result in an extension of the size of transcript initiated by the *rom* promoter. The two sequence-rearranged pBR322 derivatives exhibited middle level and low level of plasmid knotting (figure 3, lanes 17 and 18), showing a positive effect of the fragments of *tet* region on plasmid knotting.

DISCUSSION

This paper describes correlations between DNA sequences present in mutant pBR322 derivatives and the level of plasmid knotting produced in *E. coli* topoisomerase I deletion mutant DM800. The results, summarized in figure 2, show that the presence of the region on pBR322 that encodes resistance to tetracycline is responsible for high levels of plasmid knotting. However, plasmid knotting produced in DM800 is not so simple and seems likely to be controlled by plural factors. Inactivating the *tet* promoter by insertion of the two base pairs at the *Cla*I site (sample pBR-C in figure 2) and inverting the *tet* (and major part of the *rom*) region (sample pBRinv in figure 2) clearly reduced the population of the knotted DNA species in the plasmid preparations, but they did not completely abolish the formation of knotted DNA. These show that transcription of the *tet* gene sequence and its direction in the pBR322 genome are the factors which determine the level of plasmid knotting, but these are not solely responsible for plasmid knotting; the presence of nontranscribed *tet* gene sequence is actually associated with the middle level of plasmid knotting. Insertion of part of the *tet* coding region at the internal site of *rom* gene of the

deletion plasmid lacking the *Eco*RV-*Ava*I fragment is manifested by elevation of the level of plasmid knotting (see samples [EV-S]-ins[EV-A] and [Na-Na]-ins[EV-A] in figure 2), suggesting that supply of the *tet* sequence and its transcription initiated by another promoter are also effective for plasmid knotting.

The knotted DNA molecules of pBR322 found in the topoisomerase I deletion mutant DM800 are considered to be formed by the DNA gyrase reaction (7,11). This work shows that the knotted pBR322 molecules in DM800 are not the product of an intramolecular recombination of DNA, implying a relatively simple gyrase reaction. pBR322 was isolated from DM800 grown in LB medium containing a low concentration of coumermycin, a gyrase inhibitor, and analyzed it for plasmid knotting. It was found that the level of knotting rather increases by the coumermycin treatment. The production of knotted pBR322 DNA was not observed in DM800 treated with a sufficient amount of rifampicin, an inhibitor of initiation of RNA synthesis, 15 min before harvesting the cells. These data seem to support a model in which gyrase is also responsible for unknotting DNA knotted by transcription; the unknotting reaction is inhibited by coumermycin binding, and in the absence of transcription, the unknotting reaction exclusively occurs.

pBR322 DNA isolated from DM800 has been shown to be extremely heterogeneous in linking number and highly negatively supercoiled (14)(see figure 4, lane 1). The high levels of negative supercoiling are attributed to the generation of twin supercoiled domains during transcription: in the absence of the topoisomerase I, only the positively supercoiled domain is effectively relaxed by gyrase, resulting in a net accumulation of negative supercoils (19,20). As transcription proceeds, DNA in front of the transcription ensemble (the RNA polymerase with its associated RNA and proteins) becomes positively supercoiled, and DNA behind the ensemble becomes negatively supercoiled (18, 20). In pBR322, diffusion of positively and negatively supercoiled regions is considered to be prevented by both anchoring of the N-terminal region of Tet protein in the inner membrane, coupled to the plasmid DNA by transcription and translation of the *tet*, and forming a barrier possibly at replication origin (21), and/or by the divergent transcription of the *bla* and *tet* (and *rom*) genes on the plasmid genome (19). If the knotted pBR322 molecules originate in the gyrase-catalyzed reaction in the positively supercoiled domain, the topological sign of all the knots with an odd number of nodes should be positive according to the accepted convention (22). All the knots with an even number of nodes should have both negative and positive nodes in the same knot. Our previous paper (11) describes that predominantly knots with an odd number of nodes are produced and almost all the trefoils and 5-noded knots tested have a negative topological sign. This predicts that the knotted DNA is predominantly produced in the actions of the gyrase in negatively supercoiled domain. If a duplex breakage-passage reaction of the gyrase occurs in the negatively supercoiled domain by the manner of positive sign of crossover of two duplex segments, knots with an odd number of negative nodes should be exclusively produced. On the other hand, if this gyrase reaction occurs by the negative sign of crossover, knots with an even number of nodes should be exclusively generated, all of which have both negative and positive nodes in the same knot. Our previous paper seems to show that the knotted plasmid molecules are produced predominantly by the former manner. The number of nodes in the knots seems likely to be determined by how many superhelical turns are there between the two sites at which the duplex breakage-passage reaction by the gyrase is to occur. The plasmid knotting of pBR322 in DM800 is a minor gyrase reaction, because only about 10% of the plasmid molecules is estimated to be the knotted forms (11). Probably, a small portion of the gyrase molecules displays

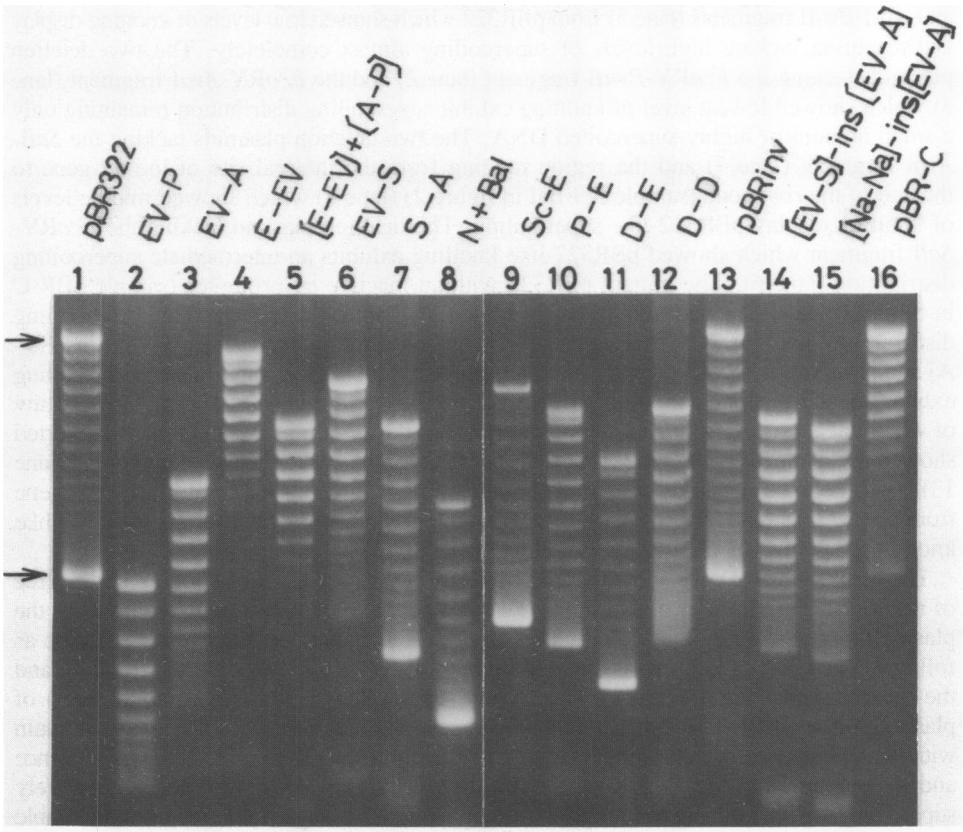


Figure 4. Chloroquine-agarose gel electrophoretic analysis of the degree of plasmid DNA supercoiling found in various derivatives of pBR322. Samples (2 μg each) of pBR322 derivatives isolated from DM800 were electrophoresed in a 1% agarose gel containing chloroquine (12 μg/ml) under the conditions described by Pruss (13). Derivatives of pBR322 are designated as in figure 2. The lowest pBR322 band (marked with an arrow) is the most supercoiled, the intermediate bands are less supercoiled, and the top band (marked with an arrow) is nicked circular DNA. The distribution of topoisomers shifts upwards with less supercoiling.

the above actions in negatively supercoiled domain. If the gyrase actually functions in the negative domain, the knotting level of pBR322 should reduce in TopA$^+$ cells. Our previous paper (11) describes that pBR322 DNA isolated from SD108, a TopA$^+$ transductant of DM800, contains the decreased amount (6%) of the knotted forms, supporting the above idea.

To interpret our experimental results of plasmid knotting presented here, we have assessed the state of negative supercoiling in the mutant pBR322 derivatives by a chloroquine-agarose gel electrophoresis. The results obtained are shown in figure 4. In the negative supercoiling distribution of pBR322 DNA isolated from DM800 (lane 1), the bottom band contains the most negatively supercoiled DNA, the intermediate bands contain DNA with fewer supercoils, and the top band contains nicked circular DNA. The two deletion plasmids constructed by deleting the *Eco*RI-*Eco*RV fragment (lane 4) and both the *Eco*RI-*Eco*RV

and *Ava*I-*Pvu*II fragments (lane 5) from pBR322 which showed low levels of knotting display a distribution lacking high levels of supercoiling almost completely. The two deletion plasmids lacking the *Eco*RV-*Pvu*II fragment (lane 2) and the *Eco*RV-*Ava*I fragment (lane 3) which showed lowest level of knotting exhibit supercoiling distribution remaining only a small amount of highly supercoiled DNA. The two deletion plasmids lacking the *Sal*I-*Ava*I fragment (lane 7) and the region ranging from the internal site of the *tet* gene to the end of the *rom* gene (sample N+Bal in figure 2) (lane 8) which showed middle levels of knotting display pBR322-like supercoiling. The deletion plasmid lacking the *Eco*RV-*Sal*I fragment which showed pBR322-like knotting exhibits an intermediate supercoiling distribution (lane 6). The mutant pBR322 with an inactive *tet* promoter (sample pBR-C in figure 2) which showed middle level of knotting displays an intermediate supercoiling distribution (lane 16). The two sequence-rearranged deletion derivatives [EV-S]-ins[EV-A] and [Na-Na]-ins[EV-A] (figure 2) which showed middle level and low level of knotting exhibit an intermediate supercoiling distribution (lanes 14 and 15). The mutant pBRinv of which the *tet* transcription unit and major part of the *rom* transcription unit are inverted showed middle level of knotting (figure 2), but this displays pBR322-like supercoiling (lane 13). Four deletion plasmids constructed by deletion of the portions or all of the *bla* gene from pBR322 (samples Sc-E, P-E, D-E, and D-D in figure 2) all which showed pBR322-like knotting display pBR322-like supercoiling (lanes 9−12).

The results stated above show that the correlation between level of knotting and degree of negative supercoiling is not straightforward; a negative supercoiling is required for the plasmid knotting, but not sufficient. The simplest interpretation of the results may be as follows. The plasmid knotting is affected by both the degree of negative supercoiling and the overall sequence (structure) of negatively supercoiled region (negative domain) of plasmid DNA. It is a critical matter what sequence is neighbored in a negative domain with the *tet* sequence which is thought to be essential for knotting. In pBRinv the *tet* sequence and the region from the *Pvu*II to the replication origin are considered to be negatively supercoiled together during transcription (see figure 2). This negative domain is unfavorable for the knotting reaction of gyrase when compared with those formed in pBR322 and the *bla* deletion derivatives of it. In pBR-C, on the other hand, the overall sequence of negative domain is preferable to the gyrase reaction in itself, but the degree of supercoiling in the negative domain formed during divergent transcriptions of the *bla* and *rom* genes is not high. To verify these idea, a more detailed analysis is necessary.

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