Sequence rearrangements may alter the in vivo superhelicity of recombinant plasmids

O. Amster+ and A. Zamir*

Biochemistry Department, Weizmann Institute of Science, Rehovot 76100, Israel

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Electrophoretic resolution of topoisomers was used to compare the in vivo superhelicity of recombinant plasmids containing a fragment of cDNA for an immunoglobulin light chain, cloned in the two possible orientations into the *Bam*HI site of pBR313 or pBR322. Previously, frequent transpositions of IS1 or IS5 were observed into the sequence upstream to the cloned fragment in recombinants in one orientation [(+) plasmids] but not in recombinants in the opposite, (−) orientation [(1982) Nucleic Acids Res. 10, 4525–4542]. The results of the present analyses show that, on average, (−) plasmids are less negatively supercoiled than (+) plasmids, or pBR322. These results suggest that primary sequence rearrangements in plasmids could affect their in vivo topological state, and consequently, perhaps, their effectiveness as recipients of transposable elements.

DNA sequence rearrangement *Transposition* (E. coli) *DNA supercoiling*

1. INTRODUCTION

We have previously observed frequent transpositions of IS1 or IS5 into the vector part of a recombinant plasmid containing a 109 bp fragment of the cDNA for L-321, the immunoglobulin light chain from MOPC321 myeloma [1]. The transpositions appeared to depend on the relative orientation of the cloned and vector sequences; IS elements were only detected in recombinants in one orientation [(+) plasmids] and not in those in the opposite orientation [(−) plasmids]. Indirect evidence suggested that the orientation of the cDNA fragment within the recombinant plasmids actually affected their ability to serve as recipients of IS elements.

Although the structural features that determine the effectiveness of DNA as a recipient of transposable elements in bacteria are not fully understood [2], a correlation between the superhelical density of the target DNA and the frequency of transposition has been indicated for the Tn5 element [3]. Accordingly, we considered the possibility that (+) and (−) plasmids could differ in their topological state in vivo.

Here, electrophoretic resolution of topoisomers was used to compare the superhelical densities of (+) and (−) plasmids and of the cloning vector pBR322. The results showed that, on average, (+) plasmids were more negatively supercoiled than (−) plasmids, but about as supercoiled as pBR322. These results point to the possibility that primary sequence rearrangements in plasmids could alter their topological state in vivo, and suggest that differences in supercoiling might be involved in the different effectiveness of the two recombinant plasmids as targets for transposition.

2. MATERIALS AND METHODS

2.1. *Bacterial strains, plasmids and growth conditions*

Escherichia coli HB101 [4] was used throughout. Recombinant plasmids containing V_{BH1}, a 109 bp

+ Present address: Genetics Department, Weizmann Institute of Science, Rehovot 76100, Israel
* To whom correspondence should be addressed

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*Bam*HI fragment of cDNA for L-321, in either one of the two possible orientations with respect to the pBR322 vector, were constructed as described ([1] and fig.1).

2.2. *Isolation of form I plasmid DNA for topoisomer analysis*

Procedure 1: Plasmid DNA was purified from transformed cells essentially as described [5], but with several modifications: cells from cultures kept under growth conditions for 12–24 h after reaching stationary phase were harvested, washed once with fresh LB, resuspended in a minimal volume of the same medium and kept at 4°C for 30–60 min. Cells were lysed by the rapid addition of an equal volume of 2.5% SDS, 0.2 M EDTA, pH 7.4. After 1 h incubation at 4°C, the lysate was incubated at 65°C for 5 min, CsCl added to a final concentration of 1 M and the precipitate, containing the high-M_r DNA as well as SDS, removed by centrifugation at 30 000 rpm for 1 h at 4°C in a Beckman Ti60 rotor. The supernatant was dialyzed against 10 mM Tris-HCl, pH 7.8, 1 mM EDTA, at 4°C for 2–3 h. Proteinase K (Boehringer) was added to the dialysis bag to a final concentration of approx. 100 μg/ml, and the mixture further dialyzed overnight, at room temperature, against the Tris-EDTA buffer as above, and subsequently for 1–2 h against the same buffer containing 25% (w/v) polyethylene glycol 6000. The dialyzate was then centrifuged to equilibrium in ethidum bromide-CsCl gradients [6] and form I plasmid DNA isolated.

Procedure 2: Plasmid DNA was extracted by the clear lysate method [6] from stationary cultures similar to those used in procedure 1, with the following modifications: the lysis mixture contained 0.2% Triton X-100 and 125 μg/ml heparin (both from Sigma).

2.3. *Topoisomer resolution*

DNA samples were dissolved in 40 mM Tris-acetate, pH 7.8, 5 mM Na acetate, 1 mM EDTA (electrophoresis buffer) containing 9% sucrose. Topoisomers were resolved by electrophoresis on 1.2% agarose gels in electrophoresis buffer containing ethidium bromide or chloroquine phosphate at the concentrations indicated in each experiment. Electrophoresis was carried out at a constant voltage of 3 V/cm for 23 h, at room temperature, in the dark. The electrophoresis buffer was circulated between the electrode compartments at a rate of 1.5–2.5 ml/min. Gels were stained as described [5], photographed on Polaroid film type 665 (P-N) and negatives traced with a Beckman DU-8 microdensitometer at 500 nm.

3. RESULTS

V_{BHI}, a 109 bp *Bam*HI fragment of L-321 cDNA, was cloned in pBR313 or pBR322 (fig.1). Initially, IS insertions were identified and mapped in recombinants of pBR313, but similar observations were subsequently made in constructions in pBR322. With either vector, target sites for IS insertions were confined to the sequence within the tetracycline-resistance region delimited by the *Eco*RI and *Bam*HI sites.

Topoisomer analysis was performed with recombinants of pBR322. Plasmid DNAs were isolated from bacteria by two different procedures designed to minimize topoisomerase activity during isolation. The procedure of Shure et al. [5] avoids the formation of spheroplasts, a step shown to lead to partial relaxation of supercoiled DNA. In the second procedure, spheroplasts are formed, but heparin, demonstrated to inhibit nicking-closing

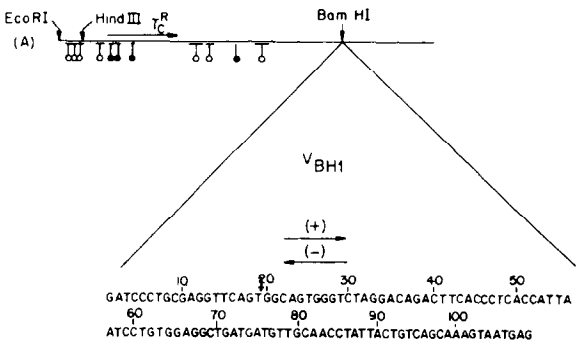

Fig.1. Recombinant plasmids. The map shows part of the tetracycline-resistance (Tc^R) region common to pBR313 [7] and pBR322 [8], the two vectors used to construct the recombinant plasmids studies. The (+) orientation: sequences coding for the immunoglobulin light chain (nucleotide sequence determined by O. Bernard, personal communication) and for tetracycline resistance are present on the same DNA strand. (↓) Sites of cleavage by the restriction endonucleases indicated, (δ) ISI insertions, (↓) IS5 insertions. Horitontal lines above IS symbols denote the regions in the plasmids within which the insertions have been localized.

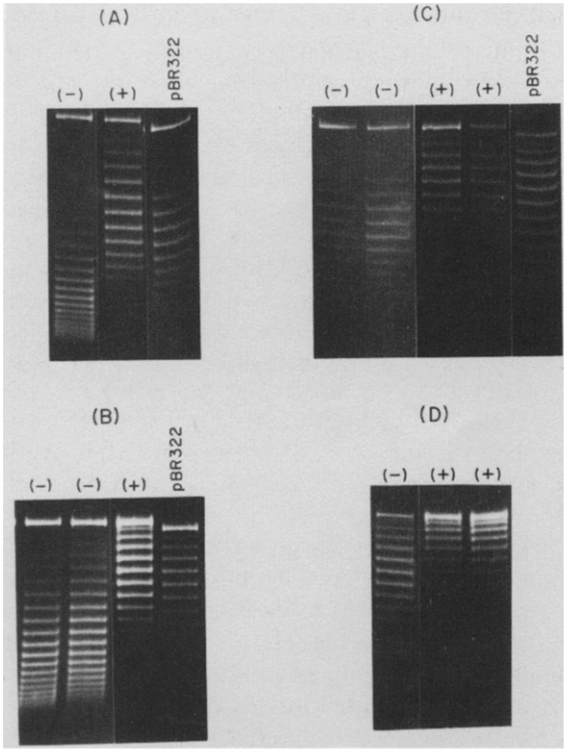

Fig.2. Comparison of plasmid topoisomer profiles in the presence of chloroquine phosphate. Form I DNAs of (+) and (−) plasmids and pBR322 were isolated from cells by procedure 2 (A,B) or procedure 1 (C,D), as described in section 2. The topoisomers were separated on 1.2% agarose gels in the presence of chloroquine phosphate at: (A) 125 μg/ml, (B) 70 μg/ml, (C) 125 μg/ml, (D) 75 μg/ml. Independently isolated DNAs from different transformant clones with (+) or (−) plasmids were analyzed in (B–D).

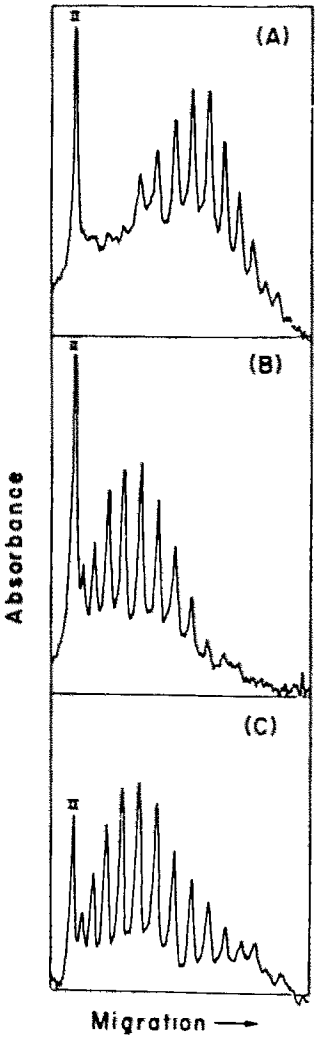

Fig.3. Densitometer tracing of topoisomer profiles. Scans were made of photographic negatives of the second, third and fifth lanes (from the left) in gel C shown in fig.2. A, (−) plasmids; B, (+) plasmids; C, pBR322.

activity [9], is added. Gel electrophoretic resolution of topoisomers was performed in the presence of the intercalators chloroquine phosphate or ethidium bromide.

A representative analysis with chloroquine phosphate is shown in fig.2. As is evident, the mobility of the topoisomers is increased by raising the chloroquine phosphate concentration, and consequently the majority of the species present under these conditions are positively superoiled. In this system, the originally least negatively supercoiled topoisomer becomes the most positively supercoiled and shows the highest electrophoretic mobility. Evidently, the topoisomer profile is somewhat different for the two DNA preparations analyzed. In agreement with [5], the DNA prepared from spheroplasts (fig.2A,B) appears to be more relaxed than plasmid DNAs extracted directly from whole cells (fig.2C,D). However, reproducible patterns were observed with plasmid DNAs prepared by the same method from cultures grown from several randomly selected transformant clones.

Comparison of the topoisomer profiles of pBR322 and of the recombinant (+) and (−) plasmids (all prepared by the same method) shows that (−) plasmids are, on average, significantly

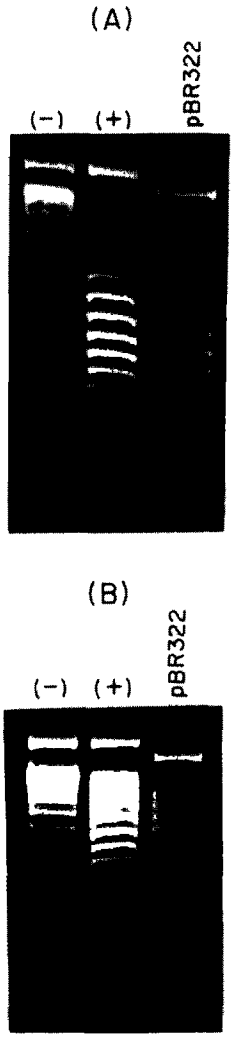

Fig.4. Comparison of plasmid topoisomer profiles in the presence of ethidium bromide. Form I DNAs of (+) and (−) plasmids and pBR322 were isolated from cells by procedure 2 (section 2). Topoisomers were separated on a 1.2% agarose gel with ethidium bromide at: (A) 33 ng/ml, (B) 43 ng/ml.

less negatively supercoiled (i.e. more positively supercoiled under the assay conditions) than (+) plasmids or pBR322. The latter two plasmids display similar topoisomer distributions. A more accurate comparison is provided by densitometer tracing (fig.3) of the photographic negatives of the gels shown in fig.2C. Considering the most abundant species as reference, it appears that (−) plasmids contain, on average, 4 negative superhelical turns less than (+) plasmids, or pBR322.

Some of the plasmid preparations electrophoresed with chloroquine phosphate were also analyzed in the presence of ethidium bromide (fig.4). In this instance, the majority of the topoisomers were negatively supercoiled, since an increase in intercalator concentration caused a decrease in electrophoretic mobility. In this system, the originally most negatively supercoiled topoisomer shows the highest electrophoretic mobility. The results demonstrate again the lower average superhelical density of (−) plasmids relative to (+) plasmids, or pBR322.

4. DISCUSSION

The topoisomer distributions of the 3 types of plasmid tested are affected by the method used to prepare form I DNA. Nevertheless, the differences in the average superhelical density between the different plasmids analyzed persist irrespective of the preparation method used. Moreover, cultures grown from randomly selected transformant clones yield essentially identical topoisomer populations, indicating that the distributions are not significantly affected by clonal or growth variations. It is therefore reasonable to conclude that the observed shifts in topoisomer distribution reflect genuine differences in the in vivo topological state of the plasmids.

The analyses with the two types of intercalator molecules indicate that under the assay conditions (−) plasmids are, on average, less supercoiled by approx. 4 negative superhelical turns than (+) plasmids or pBR322. (Since the cloned fragment is only 109 bp long, the size difference between the recombinant plasmids and the cloning vector should have, in itself, only a small effect on the electrophoretic behavior of the topoisomers.)

Our analyses do not provide sufficient information to estimate the actual superhelical density of the plasmids. A population average of 27 ± 2 negative turns (average superhelical density 0.064 ± 0.005) was previously estimated for pBR322 extracted from exponentially growing bacteria [10]. Assuming a similar superhelical density for the plasmids under study, the difference in the degree of supercoiling between (+) and (−) plasmids is in the order of 15%.

The intracellular topological state of DNA in prokaryotes is considered to be determined mainly by an interplay of DNA relaxing and supercoiling activities catalyzed by type I and II topoisomerases, respectively [11]. To differ in their topoisomer distribution, (+) and (−) plasmids may interact differently with topoisomerases, or with other factors that affect the superhelical characteristics of DNA. For example, some structural feature in (−) plasmids could make them better substrates for type I topoisomerases, or poorer substrates for type II topoisomerases, as compared to (+) plasmids or pBR322. Alternatively, the potential of specific DNA sequences to form secondary structures may in itself play a role in the twisting into a supercoiled structure. The differences in primary structure between (+) and (−) plasmids include the vector-insert junctions as well as the relative orientation of more distantly located cDNA and vector sequences. In searching for such possible distal sequences we found that a stretch of 54 bp in the cDNA shows close to 70% homology to a sequence in the pBR322 vector (fig.5). These sequences are aligned as direct or inverted repeats in (−) or (+) plasmids, respectively. These sequences are unlikely to interact by intra-strand base-pairing as they are separated by 1.2 kb. Another possibility is that the repeats could form a recognition signal for a factor(s) involved is supercoiling. In this respect it may be worth noting that Tn3 resolvase, a site-specific type I topoisomerase, senses the relative orientation of two distant recognition sites in the substrate DNA [12,13].

Previously, we showed that bacterial hosts of both (+) and (−) plasmids grew more slowly than transformants with the cloning vector alone. But, when IS1 or IS5 transposed into (+) plasmids, host growth inhibition was relieved, thus raising the possibility that selective growth advantage could be responsible for the high frequency of transposition events detected in these plasmids. However, since the introduction, by in vitro recombination, of IS1 into (−) plasmids also provided a similar relief from inhibition, selective advantage alone could not, in this case, account for the absence of observed transpositions [1]. It thus appeared that (+) and (−) plasmids actually differed in their effectiveness as recipients of transposable elements. Can this difference be correlated with the superhelical densities of (+) and (−) plasmids, demonstrated in the present analysis? While insufficient to answer this question definitively, our results suggest the following interpretation. Transpositions into (+) plasmids are not necessarily more frequent than into the similarly supercoiled pBR322, but because of selective advantage, are more easily detected. On the other hand, the reduced superhelical density of (−) plasmids can actually result in a lower frequency of IS transposition, that even selective advantage fails to reveal. This interpretation differs from the one we proposed previously [1] in assuming that suppression, rather than enhancement, of transposition frequency is responsible for the distinction between (+) and (−) plasmids as targets for IS insertions.

Recently, topoisomerase I mutations and DNA

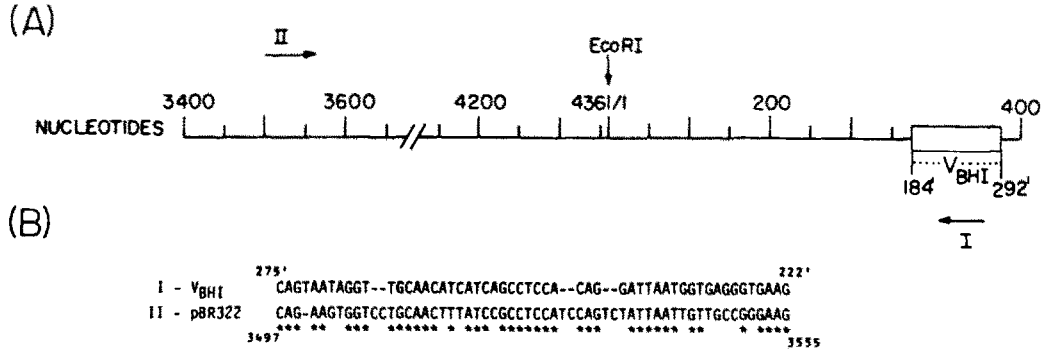

Fig.5. Homologous sequences of V_{BHI} and pBR322. (A) The region in (+) plasmids containing the homologous sequences as inverted repeats (arrows). Line, pBR322 sequence (numbered as in [8]); box, V_{BHI} sequence (primed numbers define position with respect to L-321 coding sequence). (B) Nucleotide sequence of repeats I and II aligned to maximize homology. Dashes indicate gaps; stars indicate identical nucleotides.

gyrase mutations were shown to affect differently the supercoiling of different related plasmids [15]. These results support the notion that hitherto uncharacterized primary sequence features could affect the in vivo topological state of DNA.

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