Probing of unusual DNA structures in topologically constrained form V DNA: use of restriction enzymes as structural probe

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ABSTRACT

The ability of DNA sequences to adopt unusual structures under the superhelical torsional stress has been studied. Sequences that are forced to adopt unusual conformation in topologically constrained pBR322 form V DNA (Lk=0) were mapped using restriction enzymes as probes. Restriction enzymes such as BamHI, PstI, AvaI and HindIII could not cleave their recognition sequences. The removal of topological constraint relieved this inhibition. The influence of neighbouring sequences on the ability of a given sequence to adopt unusual DNA structure, presumably left handed Z conformation, was studied through single hit analysis. Using multiple cut restriction enzymes such as NarI and FspI, it could be shown that under identical topological strain, the extent of structural alteration is greatly influenced by the neighbouring sequences. In the light of the variety of sequences and locations that could be mapped to adopt non-B conformation in pBR322 form V DNA, restriction enzymes appear as potential structural probes for natural DNA sequences.

INTRODUCTION

Over the last decade our understanding of DNA structure as a rigid uniform double helix has significantly changed to that of a sequence-dependent dynamic conformation (1,2). Although several crystal structures of oligonucleotides have shown that the DNA conformation is indeed sequence-dependent (3), paucity of data does not enable us to arrive at a generalised rule. However, the influence of ionic environment and topological strain (resulting from supercoiling) in altering DNA secondary structure has provided valuable information with regard to structural transition from B to Z form (4–8). Enzymes such as DNase I and S1 nuclease have been extensively used to probe DNA conformation in solution (2,9–11). Although restriction endonucleases are believed to be only sequence-specific and not structure sensitive, recent studies have shown that some restriction enzymes fail to recognize and cleave DNA in left handed Z conformation (12–14).

Sequences consisting of alternating purine-pyrimidine repeats have been shown to undergo structural transitions involving change in handedness under the influence of supercoiling (2,6,15,16). Few deviations from regular purine-pyrimidine repeats could also adopt left handed Z conformation depending on the length and sequence characteristics of the repeat (16,17). Since long repeats of alternating purine-pyrimidine sequences are uncommon, the formation of alternate DNA structures (like Z form) in natural sequences solely depends on the stabilization of such a structure in short stretches. What sequences in natural DNA favour the formation of these structures and what kind of structures are adopted under the influence of supercoiling. Topologicaly unlinked form V DNA, obtained by reannealing two single stranded complementary circles of pBR322, forms a suitable model system for such a study (16,18,19). Since the two strands are not linked (Lk=0), every right handed helical turn in form V DNA molecule has to be compensated by a left handed helical turn or negative supercoil. Due to topological constraints, 35–40% of the sequences in form V DNA adopt altered left-handed helical conformation (20,21). We have shown earlier that some of the restriction enzymes could not cleave pBR322 form V DNA even when the specific recognition sequence is present (16). In this paper we have studied the potential of different sequences present in natural DNA to undergo structural alteration under the influence of supercoiling. Also we report the effect of topological unwinding on several restriction enzymes accessibility for cleavage and demonstrate that many restriction enzymes are not only sequence specific but also structure sensitive. Alternating purine-pyrimidine hexanucleotide sequence (TGCGCA) is not sufficient to form Z-conformation in highly supercoiled form V DNA. Stretches of pyrimidine or purine were also found to adopt unusual conformations.

MATERIALS AND METHODS

Restriction enzymes, nuclease free BSA were from New England Biolabs or Boehringer. Topoisomerase I was isolated from Wheat germ following the procedure reported earlier (22,23). pBR322 form V DNA was prepared as described earlier (19). Plasmid pBR322 form I and form V was used throughout the analysis.

Restriction enzyme digestions were carried out as per vendor's specifications. Electrophoresis were carried out in 1% agarose

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in Tris-acetate (TAE) buffer. For double digestion reactions with Ava I + EcoRI and Bam HI + EcoRI, 0.4μg DNA was digested in a 40μl reaction buffer with AvaI or Bam HI. After incubation at 37°C for 1 hr, a 20 μl aliquot was taken to which EcoRI was added. Incubation was continued for a further period of 1 hr. Then the reaction was arrested by the addition of stop mix and samples were analysed by electrophoresis on a 2% agarose gel in TAE buffer run at 4 V/cm for 8−10 hrs. Hind III − EcoRI double digest of λ wt was used as molecular weight marker. Gels were stained with ethidium bromide and visualised on U.V. transilluminator.

Form V DNA was relaxed by topoisomerase I in a reaction buffer containing 50 mM Tris HCl (pH 7.9), 1mM EDTA, 1 mM DTT, 100 μg/ml BSA, 50 mM NaCl at 37°C for 1 hr. Following inactivation of the enzyme, subsequent restriction enzyme digestions were carried out in the same buffer after adjusting the $MgCl_2$ concentration to 10mM.

For the time course analysis of Nru I digestion, 1 μg of DNA was digested in a 100 μl reaction buffer. 10 units of enzyme was added and the reaction was carried out at 37°C. 20 μl aliquots were taken at different time intervals and the reaction was arrested by the addition of stop mix.

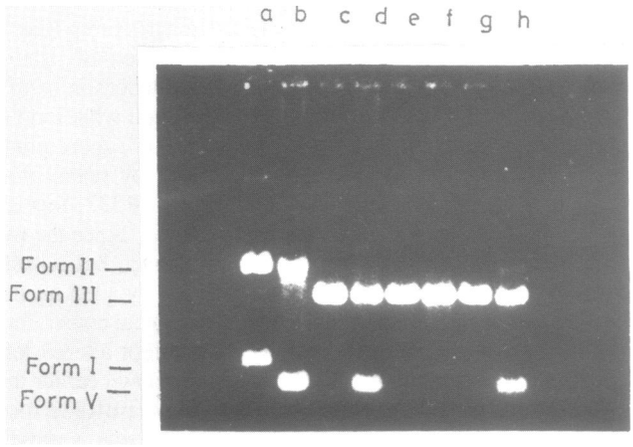

Fig 1. BamHI, EcoRI and PstI digestion of form I and form V pBR322 DNA. Form I: lane (a) control, (c) BamHI , (e) EcoRI , (g) PstI. Form V: lane (b) control, (d) BamHI, (f) EcoRI, (h) PstI .

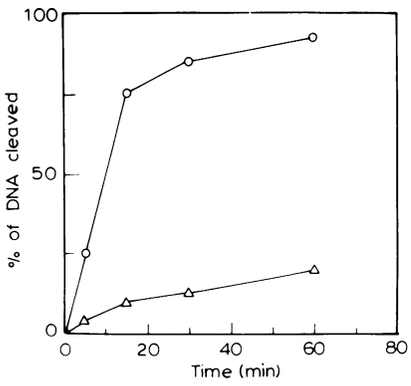

Fig 2. Cleavage of form I (○———○) and form V (△———△) pBR322 DNA by NruI as a function of time. This data was collected by calculating the area of the peak representing supercoiled band after gel scanning. The rate of cleavage was plotted as the extent of the reduction of intensity of the supercoiled band with respect to time.

For single hit analysis, 0.1 μg of form I or form V DNA was digested in 50 μl of medium salt reaction buffer. 4 units of NarI and 2 units of FspI were added and the reaction was carried out at 37°C. 5 μl Aliquots were taken at 0, 2, 5,10 and 20 min intervals and the enzyme was inactivated by heating at 65°C for 10 min. To each of the aliquots, high salt buffer containing EcoRI was added and the reaction was allowed to proceed to completion at 37°C after which the EcoRI site was labelled with α-^{32}P-dATP using DNA polymerase large fragment. Electrophoresis was carried out in a 20 cm gel of 1.5% agarose at 2.5 V/cm for 16 hrs, in TAE buffer. After electrophoresis, the gels were dried under vacuum and autoradiographed at −70°C. Autoradiograms were scanned on a Joyce Loebl micro-densitometer and quantitated.

RESULTS

Restriction enzyme accessibility on form V DNA

Structural alteration in pBR322 form V DNA was monitored by studying its susceptibility to various restriction enzymes. Table 1 shows the enzymes used in this analysis and their recognition sequences along with their neighbouring sequences. We find that SalI, SphI, EcoRI, PvuII, EcoRV and ClaI cleave form V DNA completely and as efficiently as form I DNA. AvaI, BamHI, PstI and HindIII fail to digest form V DNA even after the addition of excess enzyme and prolonged incubation(Fig 1). The open circular DNA present, in form V DNA preparations, was completely digested and it served as an internal control for monitoring the enzyme activity.

It was observed that form V DNA was partially cleaved at sites of PvuI and NruI under conditions where form I DNA was completely digested. Fig. 2 shows the extent of cleavage of form I DNA and form V DNA by NruI. Form I is completely cleaved within 30 minutes whereas a large proportion of form V DNA

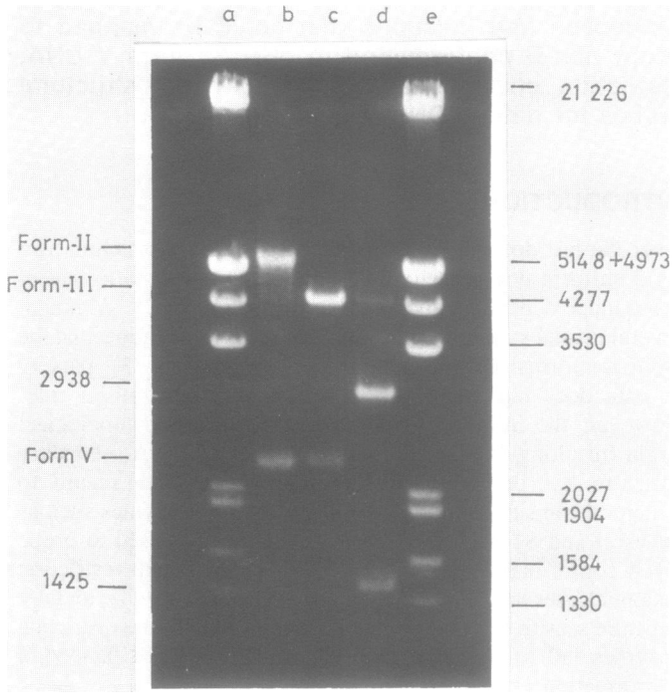

Fig 3. EcoRI-AvaI double digestion of pBR322 form V DNA. Lanes (b) control, (c) AvaI, (d) AvaI + EcoRI, (a) and (e) Hind III−EcoRI fragments of λ wt.

remained undigested (30% digestion) even after 60 min. Complete cleavage was observed with NruI and PvuI even with ten fold excess enzyme only after prolonged incubation.

Supercoil induced structural alteration in form V DNA

The inability of several restriction enzymes to cleave their recognition sequences in form V DNA was further investigated to determine the reasons for such resistance. If structural alteration induced by negative supercoiling is responsible for such inhibition, then the removal of torsional stress should lead to cleavage at these sites. We have adopted two methods to remove the torsional stress.

i) Torsional stress in form V DNA was relieved by cleavage with restriction enzymes to which it was susceptible. Double digestion of form V DNA with AvaI followed by EcoRI produced two bands corresponding to 2938 and 1425 bp. Digestion was not observed in the aliquot which contained only AvaI (Fig 3). This suggests that on removal of the topological constraint by EcoRI digestion, AvaI site became accessible for cleavage. Similarly BamHI site was cleaved only when torsional stress was removed by EcoRI digestion (data not shown).

ii) pBR322 form V DNA was relaxed by topoisomerase I and was then digested with restriction enzymes which failed to cleave form V DNA. Both BamHI and HindIII cleaved the relaxed form V DNA molecule (data not shown).

Thus the inability of the enzymes AvaI, BamHI, HindIII and PstI to cleave form V DNA reflects the inaccessibility of these sites due to structural alterations at their respective recognition sequence induced by torsional stress.

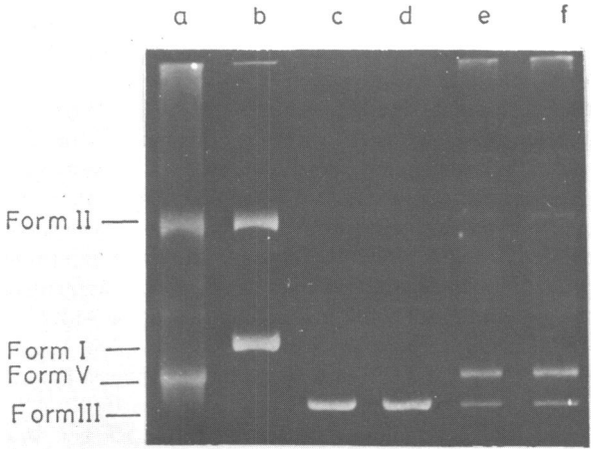

Fig 4. BamHI and Hind III digestion of dimeric pBR322 form I and form V DNA. Form I : lane (a)control, (c) BamHI , (d)Hind III . form V dimer: lane (b)control, (e) BamHI , (f)Hind III .

Influence of neighbouring sequences

(a) Form V of pBR322 dimer:

Form V prepared from pBR322 homologous dimer (8726bp) was used for restriction digestion. Even in the dimer, the two EcoRI sites were cleaved whereas both of the BamHI and Hind III sites

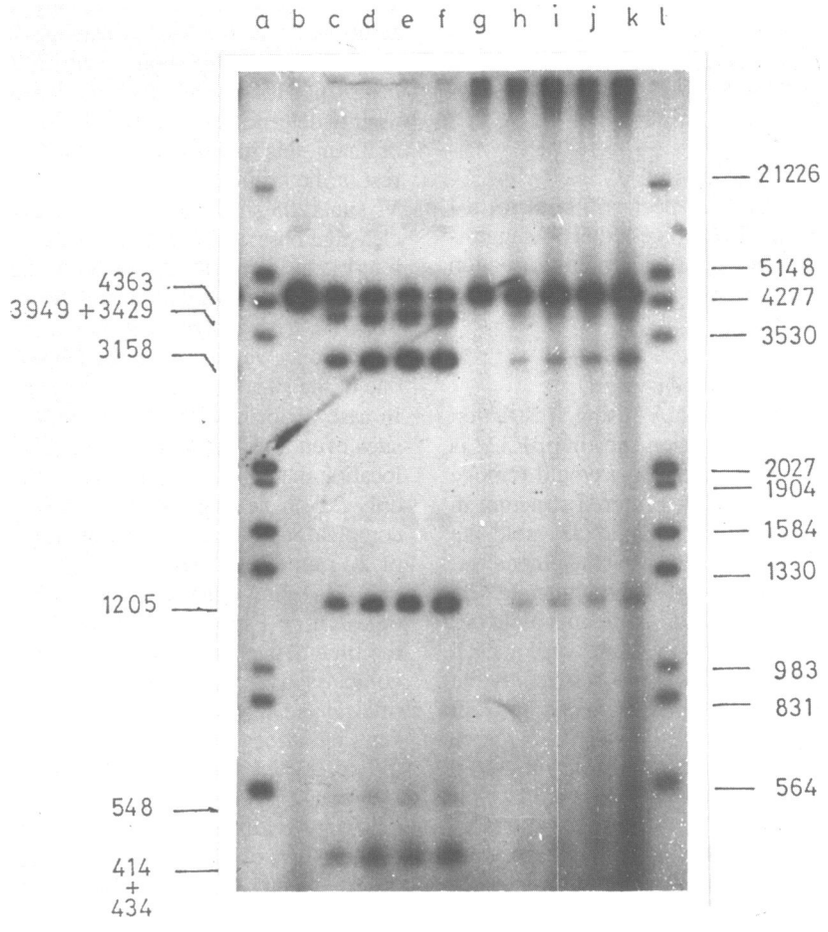

Fig 5. Single hit analysis of form I and form V pBR322 with NarI. Lanes a & l are marker Hind III-EcoRI double digest of λ wt, lanes b to f are 0,2,5,10 & 20 min aliquots respectively of pBR322 form I and lanes g to k are 0,2,5,10 & 20 min aliquots respectively of pBR322 form V.

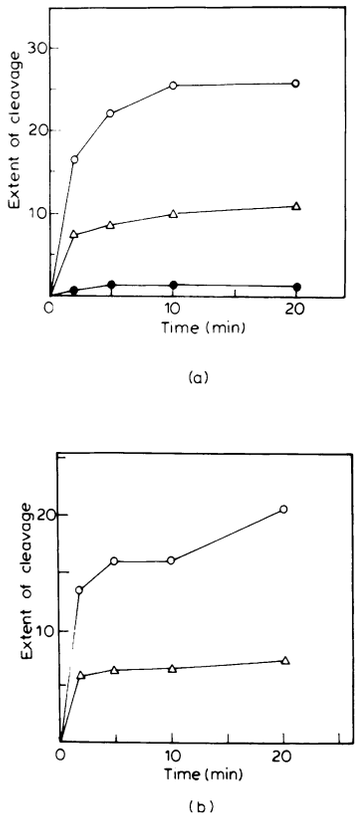

Fig 6. (a) Rates of cleavage of NarI sites 548 (●———●), 414 + 434 (△———△) and 3158 (○———○) in pBR322 form I DNA. This data was obtained by calculating the intensities of bands in Fig 5. (b) Comparison of the rate of cleavage of site 3158 in form I (○———○) and form V (△———△) pBR322.

were resistant (Fig 4). The open circle species in both form I and form V preparations were completely digested. This suggests that a given sequence of DNA adopts an altered conformation at different locations provided it has similar neighbouring sequences and topological constraint.

b) Probing with multiple cut restriction enzymes:
Probing of structures in form V DNA using restriction endonucleases that have multiple recognition sites in pBR322 is complicated by the fact that cleavage at one site would remove the torsional stress responsible for inducing altered structure in an identical sequence at other locations. A single cleavable site therefore could mask the nature of the structure at the other sites. This difficulty was circumvented by using a single hit condition where an enzyme was allowed to make only one nick per molecule and then the position and frequency of cleavage at each site was determined by labelling the DNA at a restriction site such as EcoRI site. By this labelling, the fragments arising from more than one nick in the molecule were eliminated . Relative resistance to cleavage at each site reflects the extent of altered conformation at that site in form V DNA. Since identical sequences are being probed at different locations, the extent of alteration will indicate the influence of neighbouring sequence in directing a given sequence to adopt unusual structure. Restriction enzymes NarI(GGCGCC) & FspI (TGCGCA) were used in such studies since their recognition sites have the potential to adopt altered conformation.

Digestion of form I and form V DNA were followed as a function of time in order to achieve 14−20% cleavage of form V DNA. Fig. 5 shows an autoradiogram after the partial digestion of form I and form V DNA with NarI. It is evident that under identical conditions, form V DNA shows resistance to cleavage compared to form I DNA. At 5 min interval 70% of form I DNA was cleaved, whereas only 13% of form V is cleaved. This made it difficult to carry out single hit analysis for both under identical conditions. Since our main interest was to look for the accessibility of various sites in form V DNA, experimental conditions were adjusted to achieve single hit condition for form V DNA, allowing overdigestion of form I DNA.

End labelling of EcoRI site results in labelling of the complementary fragments which were retained for further analysis. If a fast site exists between a moderately slow site and a reference site, analysing only one fragment will result in misinterpretation of the moderately slow site as a very slow site unless ideal single hit condition is achieved. Such an erroneous interpretation can be avoided by analysing the complementary fragments. In pBR322, NarI recognition sites are at 414,434,548 and 1205. End labelling after NarI digestion gave four fragments of expected length and four complementary fragments. Fragments of 414 and 434 bp moved as a single band and those of 3949, 3929 and 3815 bp could not be resolved in 1.5% gel. In case of the 1205 bp fragment, the complementary fragment of 3158 bp was resolved.

Comparison of partial digestion data reveals that site 1205 is most sensitive to NarI in form I DNA (Fig 5,lanes c to f, Fig. 6). The 1205 bp fragment extending from EcoRI site has two additional sites for NarI. Cleavage at these sites will affect the measurement of digestion at site 1205. Since the complementary fragment 3158 has no internal cleavage site, this fragment was used to determine the extent of digestion at site 1205. At the end of 2 min, this fragment constituted 14% of the total DNA whereas rest of the fragments contributed less than 10%. In case of form V, site 1205 shows significant cleavage, although the extent of cleavage observed is much less than that in form I (Fig 5, lanes h to k). In form I DNA, the sum of the intensities of fragments 414 and 434 is less than that of the complementary fragment of 1205 (Fig. 6). This suggests that either both the sites show intermediate resistance to the enzyme or even in form I DNA, one of the sites is completely resistant and the other is sensitive. In case of form V DNA very little cleavage was seen at these sites even at the end of 20 min (Fig 5, lane k). Site 548 is the location that is least reactive in form I (Fig 5, lane f) and shows only 2.5% cleavage at the end of 20 min. This site showed complete resistance to cleavage in form V DNA even at the end of 20 min (Fig 5, lane k).

Fig. 7 shows the autoradiogram of the products of partial digestion by FspI at different time intervals. Fig. 8 a and b show the time dependent change in intensity of various fragments in comparison to form I DNA. All the sites showed a lower rate of cleavage in form V DNA in comparison to form I DNA. FspI has sites at locations 260, 1356, 1454, and 3588 bp with respect to EcoRI sites. End labelling at EcoRI site gave complementary fragments of 4103, 3007, 2909 and 775 bp in addition to the expected fragments. The 260bp fragment moved as a diffused band in front of all other bands whereas the complementary 4103 bp fragment moved with uncleaved linear DNA. Therefore accessibility of site 260 could not be analysed in this experiment. From fig. 7 it can be seen that site 1454 is completely resistant in form V DNA and is cleaved partially in form I DNA.

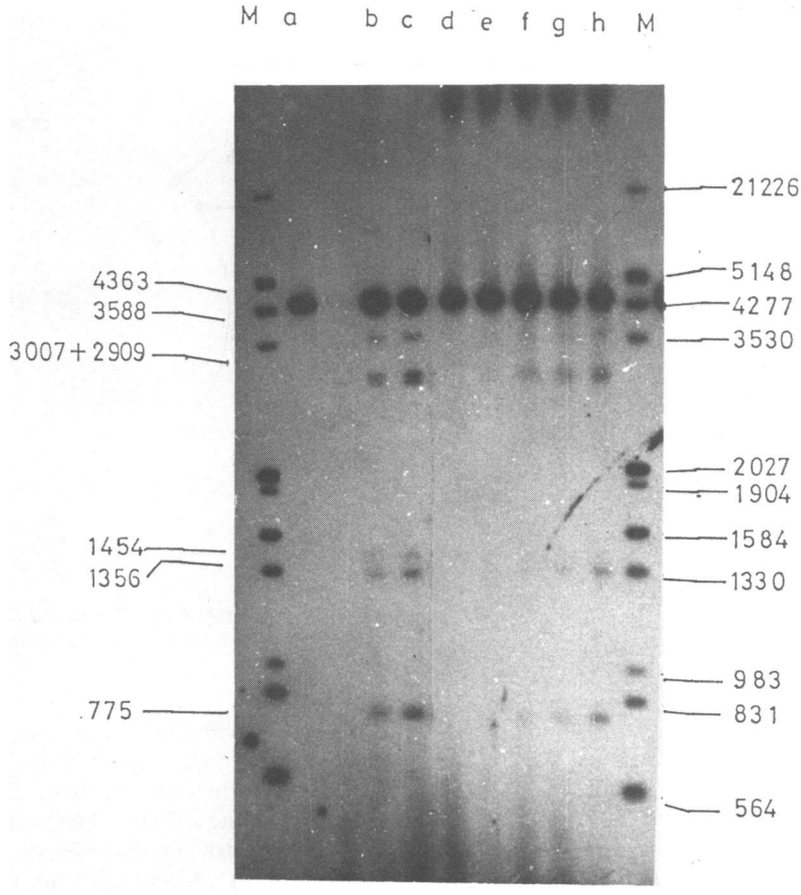

Fig 7. Single hit analysis of form I and form V pBR322 with FspI. M: Hind III-EcoRI double digest of λ wt. Lanes a to c are 0,10 and 20 min aliquots respectively of pBR322 form I. Lanes d to h are 0,2,5,10 and 20 min aliquots respectively of pBR322 form V.

DISCUSSION

Altered structure in Form V DNA : Probed by Restriction enzymes

Since the two strands of form V molecule are not topologically linked (ie, Lk=0), writhe must be equal to twist.

$$Lk = 0 = Tw + Wr \text{ or } Tw = -Wr$$

For a form V molecule with 5000bp, in order to have all the sequences in the right-handed double helical conformation, it must have 500 negative supercoils. Since such a high level of supercoiling cannot be achieved, a net reduction in the twist of the molecule is warranted to have maximum possible hydrogen bonding. The twist of the form V DNA molecule may be resolved into two components,

$$Tw = Tw_R + (-Tw_L)$$

Single stranded melted regions, cruciform and left-handed helical structures, including Z-form will either contribute to zero or negative twist, therefore,

$$Lk = (N-x)/n_1 - (x-\Delta x)/n_2 + Wr = 0$$

N = Total number of base pairs.
N−x = Number of base pairs contributing to positive twist with average n_1 bp/helical turn.
x −Δx = Number of base pairs contributing to negative twist (e.g. left handed helices including Z-form) with average n_2 bp/helical turn. Where Δx is the number of bp which do not contribute to twist (melted regions and cruciform).

It has been shown earlier that 35−40% of the sequences in form V molecule indeed adopt non-B conformation including Z-form (16,20). Eventually, writhe in form V molecule is decided by the potential of sequences to adopt left-handed helical conformations and other hydrogen bonded structures. Thus the molecule adopts highest possible writhe to accomodate maximum number of sequences in the right handed B-DNA conformation. In this paper we have probed the sequences that adopt altered structures in pBR322 form V DNA using restriction enzymes. In form V DNA (Fig 9) BamHI, AvaI, PstI, and HindIII sites showed complete resistance to cleavage whereas ClaI, EcoRI, EcoRV, SalI, SphI, and PvuII sites were cleaved with an efficiency equal to that in form I DNA. Interestingly, NruI and PvuI showed partial digestion indicating that the DNA conformation at these sites are altered and in dynamic equillibrium with cleavable conformation. The probability of static or dynamic equilibrium seems to be low at the sites which showed complete resistance to cleavage since even after prolonged incubation no cleavage was observed at these sites in form V DNA. Complete cleavage was observed at PvuI site after prolonged incubation, indicating a dynamic equilibrium between the cleavable and non-cleavable structure at this site. Similar dynamic nature of B to Z transition under the influence of supercoiling was observed in $(CG)_{16}$ inserts in pLP332 when probed with BssHII restriction endonuclease (14). Distribution of the altered sites showed that

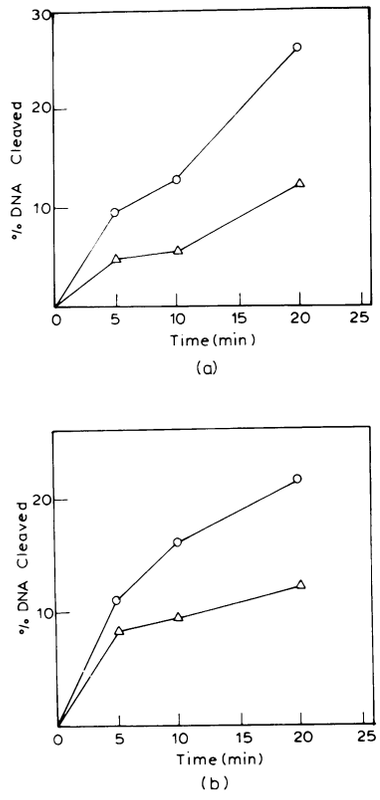

Fig 8. Comparison of the cleavage of FspI sites in form I (○———○) & form V (△———△) pBR322. Sites 3588 (a) and 1356 (b) were chosen for comparison. This data was obtained by calculating intensities of the bands in Fig 7.

the conformational alteration in form V DNA is spread all over the molecule (Fig 9). Since only 5% of the sequences in pBR322 are Z potential having a stretch of 6 or more alternating purine-pyrimidine bases, topological constraint requires that several other sequences should adopt left handed (non-B) conformation in form V DNA.

The restriction enzyme sites which were resistant to cleavage but became sensitive after removal of the topological strain, must exist in an altered conformation away from the optimal structure for cleavage in form V DNA. On the other hand restriction enzyme like EcoRI, EcoRV, ClaI etc, whose sites are efficiently cleaved, are either structure insensitive or their recognition sites remain in an unaltered optimal-cut DNA conformation in form V DNA. We have classified these sites as B-like conformation for convenience although it seems quite possible that the optimally-cut DNA structure may vary (from the ideal B-helix) depending upon the recognition sequence of the enzyme tested. This method of probing allows the restriction enzyme recognition sites to be divided into two groups: one that adopts cleavable or B-like conformation and the other that are uncleavable by restriction endonucleases in form V DNA. The latter includes Z DNA, single stranded DNA and other altered structures. The conformation of each assayed sequence can be placed into either cleavable B DNA like or non-B DNA category, since the exact nature of the non-B DNA conformation cannot be ascertained by this analysis although it is evident that all these unusual structures must contribute to negative twist or zero twist. From the UV melting, electron microscopy and VUV CD studies on form V DNA, less than 5−10% of the sequences were shown to exist in the form of single strand(18−21).

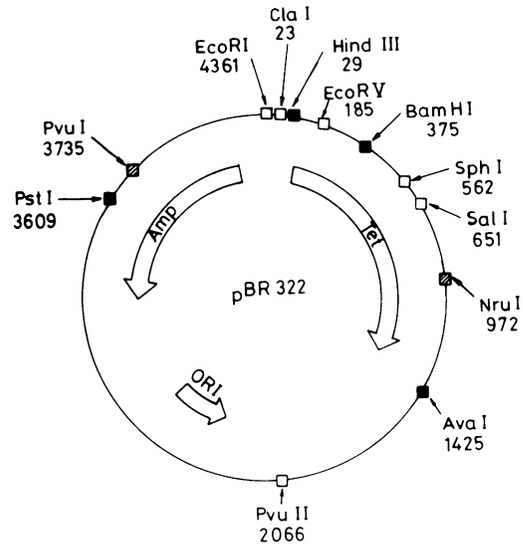

Fig 9. Distribution of the restriction sites studied in pBR322 DNA with their cleavage potentials in form V DNA. □—complete cleavage, ▨—partial cleavage, ■no cleavage.

Sequences with high AT content could adopt single stranded conformation under high torsional stress. However efficient cleavage of form V by EcoRI, EcoRV and ClaI, whose sites are AT rich, indicate a B-DNA like conformation at their recognition sites since restriction enzymes do not normally cleave single stranded DNA. Additionally the EcoRI recognition sequence (GAATTC) has been shown to adopt B-DNA like conformation in single crystal even when flanked by CG sequences (3). The possibility that BamHI, AvaI, PstI, PvuI, NruI, sites, which show resistance to cleavage, adopt single stranded conformation may be ruled out as these sites are neither AT rich nor are they flanked by AT rich regions.

It is evident that in pBR322 form V DNA extensive non-B conformation with negative twist (like Z-DNA) would manifest at all those sequences which have the potential. This is possible due to the presence of large negative supercoiling in this molecule which favours left handed helical conformation. The upstream flanking sequence of AvaI site (Table 1) which has a 14bp long track of Z-potential alternating pu-py sequence (CGCACGCGGCGCAT) with a GG break is likely to undergo B to Z transition under the influence of the high degree of supercoiling in form V DNA. AvaI site may thus adopt a Z−B junction structure leading to the non-cleavage of the site. B−Z junction structure has been shown to be resistant to cleavage by BamHI (13). There are no obvious Z-forming alternating pu-py sequences including or around BamHI site, but a hexanucleotide poly pyrimidine stretch (TCCTCT) is present. The sequence around BamHI site in pBR322 is highly G-C rich (Table 1) and therefore it could adopt double helical non-B conformation that is not cleaved. Several such poly pu-poly py sequences have been shown to adopt altered conformation under supercoiling force in form V DNA (16), and may take up novel non-B left handed helical structures proposed earlier (1,24,25).

The resistance of Pst I site may be correlated to a 8bp stretch of Z-potential alternating pu-py sequence (TGCAGGCA) with one G out of alternation. Alternating pu-py sequence with one G out of alternation has been shown to adopt Z-conformation in synthetic oligonucleotides (26). The most interesting and striking observation is that the SphI site (GCATGC) exists in

Table 1 Sensitivity of Form V pBR322 DNA to restriction enzymes

Position relative to EcoRI site in pBR322	Restriction Endonuclease	Sequence			Remarks
4361	EcoRI	TTTCGTCTTC	AA(G↓AATTC)TC	ATGTTTGACA	complete cleavage
23	ClaI	TGACAGCTTA	TC(AT↓CGAT)AA	GCTTTAATGC	"
185	EcoRV	GGCCTCTTGC	GG(GAT↓ATC)GT	CCATTCCGAC	"
562	SphI	CGCCATCTCC	TT(GCATG↓C)AC	CATTCCTTGC	"
651	SalI	ATAAGGGAGA	GC(G↓TCGAC)CG	ATGCCCTTGA	"
2066	PvuII	TGAGCTTTAC	CG(CAG↓CTG)CC	TCGCGCGTTT	"
972	NruI	TCTTGCTGGC	GT(TCG↓CGA)CG	CGAGGCTGGA	partial cleavage
3735	PvuI	CCTTCGGTCC	TC(CGAT↓CG)TT	GTCAGAAGTA	"
29	HindIII	CTTATCATCG	AT(A↓AGCTT)TA	ATGCGGTAGT	No cleavage
1425	AvaI	CACGCGGCGC	AT(C↓TCGGG)CA	GCGTTGGGTC	"
375	BamHI	CACCCGTCCT	GT(G↓GATCC)TC	TACGCCGGAC	"
3609	PstI	TTGTTGCCAT	TG(CTGCA↓G)GC	ATCGTGGTGT	"

Bracket includes the recognition sequences. The cutting site is indicated by arrow.

Table 2 Probing of altered structures in pBR322 form V DNA with NarI

Position relative to EcoRI site	Cleavage site and flanking sequences			Remarks
414	GCCGGCATCA	CC GGCGCC AC	AGGTGCGGTT	CR
434	GGTGCGGTTG	CT GGCGCC TA	TATCGCCGAC	CR
548	GGGGGACTGT	TG GGCGCC AT	CTCCTTGCAT	CR
1205	GCATGGATTG	TA GGCGCC GC	CCTATACCTT	PR

CR = Completely resistant, PR = Partially resistant.

a B-DNA like conformation whereas PvuI site (CGATCG) adopts an altered conformation. Although the percentage of AT is identical in SphI and PvuI sites, the nature of the conformation adopted is dictated by the potential of the sequences to undergo structural transition. SphI site has an alternating pu -py sequence (GCATGC), but it is known that sequence starting with (GC) has a lower Z-potential than the one starting with (CG) (27–29). The hexanucleotide (CGATCG) which is the PvuI recognition sequence, although not alternating pu-py, has been shown to adopt Z-conformation in the crystal (30). Further the 5′flanking sequences of both these sites have long stretches of polypurine sequences, TCTCCTT for SphI and TCCTCC for PvuI. Moreover the PvuI site contains a part of TCCTCC segment whereas the TCTCCTT tract does not overlap with the SphI site. Hence it is probable that PvuI site adopts non-B conformation whereas SphI site remains unaltered.

HindIII and ClaI sites are adjacent to each other in pBR322. In this 12 bp sequence (ATCGATAAGCTT) in form V DNA, in spite of having equal number of AT bp, the 6 bp Hind III sequence (AAGCTT) undergoes structural change whereas the 6 bp ClaI sequence (ATCGAT) remains unaltered with TA sequence to adopt the structural junction (Fig 9). This observation is well corroborated by the resistance shown by the AluI sequence within HindIII site towards AluI methylase (16) suggesting an altered conformation at this site. ClaI site is certainly double stranded since it is efficiently cleaved by the enzyme. HindIII site (pu-pu-pu-py-py-py) is unlikely to be single stranded because pu-pu stacking interactions are known to have higher thermodynamic stability than pu-py stacking interactions which are found in ClaI site (31). It is interesting to note that from the EcoRI site (considering downstream) the B-like conformation alternates with the non-B conformation 4 times within a span of 40 bp.

Effect of neighbouring sequences

In form V of pBR322 dimer, the two sites of each single cut restriction enzyme (of monomer pBR322) are flanked by similar sequences and found to adopt similar structure. To study the effect of different neighbouring sequences on the structure adopted by identical recognition sequences, we probed the structure of restriction sites which occur more than once in monomer pBR322 using single hit approach.

Out of the four NarI sites, three showed resistance to cleavage. Site 414 has a downstream 10bp alternating purine-pyrimidine sequence with one G out of alternation (Table 2) which can undergo B to Z transition in form V DNA, thus leading to cleavage resistance at site 414 (32). Site 548 has a (TCTCCTT) polypurine tract as 5′ flanking sequence (Table 2) which can also adopt non-B conformation (presumably left handed) in form V DNA. Even under physiological superhelical density this site shows significant resistance to cleavage and is likely to exist in an altered conformation. M.HhaI recognition sequence (GCGC)

Table 3 Probing of altered structures in pBR322 form V DNA with FspI

Position relative to EcoRI site	Cleavage site and flanking sequences			Remarks
260	ATGCAATTTC	TA TGCGCA CC	CGTTCTCGGA	CR
1356	GAGAACTGTG	AA TGCGCA AA	CCAACCCTTG	PR
1454	CCTGGCCACG	GG TGCGCA TG	ATCGTGCTCC	CR
3588	CAGTTAATAG	TT TGCGCA AC	GTTGTTGCCA	PR

CR = Completely resistant, PR = Partially resistant.

forms a subset of NarI recognition sequence (GGCGCC). Earlier we have used sequence specific methylases as probes for altered conformations in form V DNA (16). Sites 414 and 434 were found to be undermethylated by HhaI methylase indicating altered conformation at these sites. Structural alteration as probed by restriction enzymes correlated well with the methylation studies.

In case of Fsp I sites, site 1454 which is resistant in form V DNA, is within a large stretch of alternating pu-py sequence (CACGGGTGCGCATG) with one G out of alternation (Table 3). This sequence is located in the HaeIII fragment that binds to anti Z DNA antibody in supercoiled pBR322 DNA (17) and is within sites 1448 to 1478 that has been shown to react with diethyl pyrocarbonate a chemical reagent that prefentially reacts with Z DNA (33,34). Thus, this stretch of sequence shows a propensity to fold into Z conformation both in natural supercoiled pBR322 and form V pBR322 DNA with zero link. Sites 1356 and 3589 were cleaved efficiently and hence presumed to remain in the B-like conformation.

M.HhaI recognition sequence (GCGC) is also a subset of FspI recognition sequence (TGCGCA). Results of methylation studies indicated the presence of an altered conformation (presumably Z form) at sites 1454 and 260 in form V DNA (16). Recent results from our laboratory show that, even unconstrained synthetic oligonucleotides like CGTGCGCACG and CGCGCGGCGCGC (with a GG break) adopt Z-conformation under stringent ionic conditions in solution (26). Out of the four TGCGCA sites probed by FspI, two adopt B-DNA like (cleavable) and other two adopt non-B-DNA (uncleavable) conformation. Thus a perfectly alternating purine-pyrimidine hexanucleotide sequence is not sufficient to guarantee Z-conformation when flanked by non-Z favouring sequences, even under very high superhelical density. In the case of NarI, four GGCGCC sites probed show different accessibility in form I DNA itself. Moreover, the sites that show lower accessibility in form I DNA, become uncleavable under high superhelical strain in form V DNA. Both FspI and NarI single hit data show that near neighbour sequences have profound influence on a given sequence to undergo structural transition involving change in handedness under high negative supercoiling.

In summary, sequences that have adopted unusual structures in form V DNA under topological constraint were mapped using restriction enzymes as probes. Some of the restriction enzyme recognition sites which occur only once in pBR322 were found to adopt non-B conformation in pBR322 form V DNA. Removal of topological constraint made these sites accessible to cleavage. Our results indicate that folding interactions in form V DNA are not highly cooperative so that a single B or Z like conformation is not propagated for many helical turns. Further, similar hexanucleotide sequences under identical topological strain were found to adopt varied conformations in the presence of different neighbouring sequences. The present study shows that restriction enzymes could be employed as important probes in studying potential sites for structural alteration in natural DNA sequences which is not amenable to spectroscopic study. Inspite of the limitations that the study would yield no information with regard to the exact non-B conformation adopted by a given sequence, it precisely localizes the sequence that adopts unusual structure. With the availability of a large number of restriction enzymes, their use as a structural probe would provide a means for detecting sites of unusual DNA structure in natural DNA sequences inaccessible to other methods. The use of powerful techniques like pulse field gel electrophoresis in conjunction with restriction enzymes as structural probes, could enable us to map potential sites of structural alteration in the supercoiled domains of chromosomal DNA.

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