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Structural interconversion of alternating purine–pyrimidine inverted repeats cloned in supercoiled plasmids

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ABSTRACT

Two self complementary oligonucleotides, $T(GC)_4AT(GC)_4ACATG$ and $C(GC)_2(AT)_5(GC)_3ATG$, were synthesized and cloned into plasmids. Negative supercoiling causes a structural transition in the primary helix of both inserts. The first sequence converts into the left-handed helix, whereas the second sequence undergoes a transition into a cruciform or a Z-type structure depending on the experimental conditions employed. This has been deduced from the mapping of S1 nuclease sensitive sites, OsO_4-sensitive sites, DEP modification pattern and relaxation studies. In addition, the differential effect of 5-cytosine methylation and binding of the AT-specific drug distamycin on these transitions further supports this interpretation. Thus, it is demonstrated, that the same sequence which is both inverted repeat and alternating purine-pyrimidine type may adopt either the left-handed conformation or the cruciform structure in response to the superhelical stress. Formation of the Z-type helix can be transmitted through the $d(AT)_n$ region which is 10 bp in length.

INTRODUCTION

The sequence dependent structural microheterogeneity of duplex DNA has been well established in recent years. Among others, transitions from right-handed B-DNA to left-handed Z-DNA (reviewed in 1,2) as well as cruciform extrusions (reviewed in 3) belong to the dramatic and extensively characterized intramolecular conversions.

Alternating purine-pyrimidine sequences such as $d(GC)_n$ (4-9) or $d(AC)_n$ (10-14) most readily adopt left-handed conformations. However, the sequence requirements that govern the B-Z transition are complex and only partially understood. For instance, $d(CATG)_{10}$ (15) and long alternating $d(AT)_n$ sequences (16-18) cloned in recombinant plasmids apparently are not able to adopt a Z-type helix and convert into cruciform structures. The inability of $d(AT)_n$ to form a left-handed helix may be related to the lack of an amino group at position 2 of adenines (19) since $d(T\text{-}2\text{-amino A})_3$ readily forms a left-handed structure in high salt solution (20). On the other hand, short tracts of alternating adenines and thymines flanked by "Z-genic" $d(GC)_n$ on both sides will adopt left handed helices in negatively supercoiled plasmids (14,21,22).

If a given sequence is both an inverted repeat and an alternating purine-pyrimidine type, at least two intramolecular conversions may be considered: a) transition from a B- to a Z-helix or b) transition from B-helix to a cruciform structure. In this paper we describe the behavior of two such sequences as a function of negative supercoiling. Both inserts are 32 bp long and are perfect inverted repeats in which purines alternate with pyrimidines.

The first molecule contains two $d(GC)_4$ sequences interrupted in the center by $d(AT)_1$. The second molecule contains $d(AT)_5$ in center flanked by $d(CG)_3$ on both sides. All assays used in these studies indicate that the first molecule adopts a left-handed conformation whereas second sequence undergoes conversion into a cruciform-type or a left-handed-type structures. These results further evaluate the sequence requirements of supercoil-induced structural transitions in a double stranded DNA in recombinant plasmids.

MATERIALS AND METHODS

Chemicals and Enzymes

Diethylpyrocarbonate, OsO_4, Distamycin A and chloroquine diphosphate were obtained from Sigma Chemical Company. Topoisomerase I was purified from calf thymus according to a published procedure (54). S1 nuclease and SstI were from Bethesda Research Labs, HhaI methylase from New England Biolabs. Restriction and other enzymes were from Boehringer Mannheim or New England Biolabs and were used according to the recommendations of the suppliers.

Synthetic fragments

The self-complementary fragments $T(GC)_4AT(GC)_4ACATG$ (a) and $C(GC)_2(AT)_5(GC)_3ATG$ (b) were synthesized on solid support by the phosphoramidite triester approach (23) using methyl N,N'-diisopropylphosphoramidite dimeric synthons (24) [GC] and [AT] in addition to base-protected nucleoside-3'-O-methyl-N,N'-diisopropylphosphoramidite units. The controlled pore glass long chain alkylamine (ABN) was used as a solid support. The synthesis was performed manually (25) on a 1 micromole scale using Applied Biosystems microcolumns. The crude 5'-dimethoxytrityl-protected oligodeoxyribonucleotides a and b were purified by reverse phase HPLC (μ Bondapack C18 semi-preparative column, Waters) followed by preparative electrophoresis (20% polyacrylamide gel) of detritylated fragments.

Plasmid constructions

After kinasing and annealing, the self-complementary fragments a and b were cloned into the SphI site of pBR322 resulting in the derivatives pSpHI and pKJ2, respectively. The sequences of the inserts were verified by Maxam-Gilbert approach (26). Plasmids pUCSpHI and pUCKJ2 were obtained by recloning the 88 bp long HaeIII fragments containing the cloned sequences from pSpHI and pKJ2 into the HincII site of pUC19 (27). This was done in order to facilitate sequencing experiments of chemically modified plasmids since number of polylinker restriction sites are available to the left and to the right of the recloned fragments. pBR322 sequences surrounding the synthetic fragments are the same as in pSpHI and pKJ2.

Although the lengths and sequences of both recombinant plasmids pUCSpHI and pUCKJ2 are identical (except for the synthetic insert), it is worth noting that the orientation of the 64 bp pBR322 sequence recloned together with the synthetic fragments a and b is different. Due to the pBR322 vector sequence flanking the synthetic inserts, the perfect alternating purine-pyrimidine blocks are 32 bp long in all constructs.

Chemical modification

Reaction of DNA with 2 mM OsO_4 was performed as described previously (28,29,30). Modification with diethyl pyrocarbonate (DEP) was performed according to Herr (31) and Johnston and Rich (32).

Other methods

Preparation of topoisomeric samples of the plasmids (33), S1 nuclease reactions (8,13,14,22), detection of chemical modifications by S1 nuclease cleavage (28-30) or by sequencing (29-32), *in vitro* methylation of plasmids with HhaI methylase (36,53) and 2D gel electrophoresis (13,14,34-36) were performed as described earlier.

RESULTS

Mapping of S1 cleavage sites and OsO_4 modification sites.

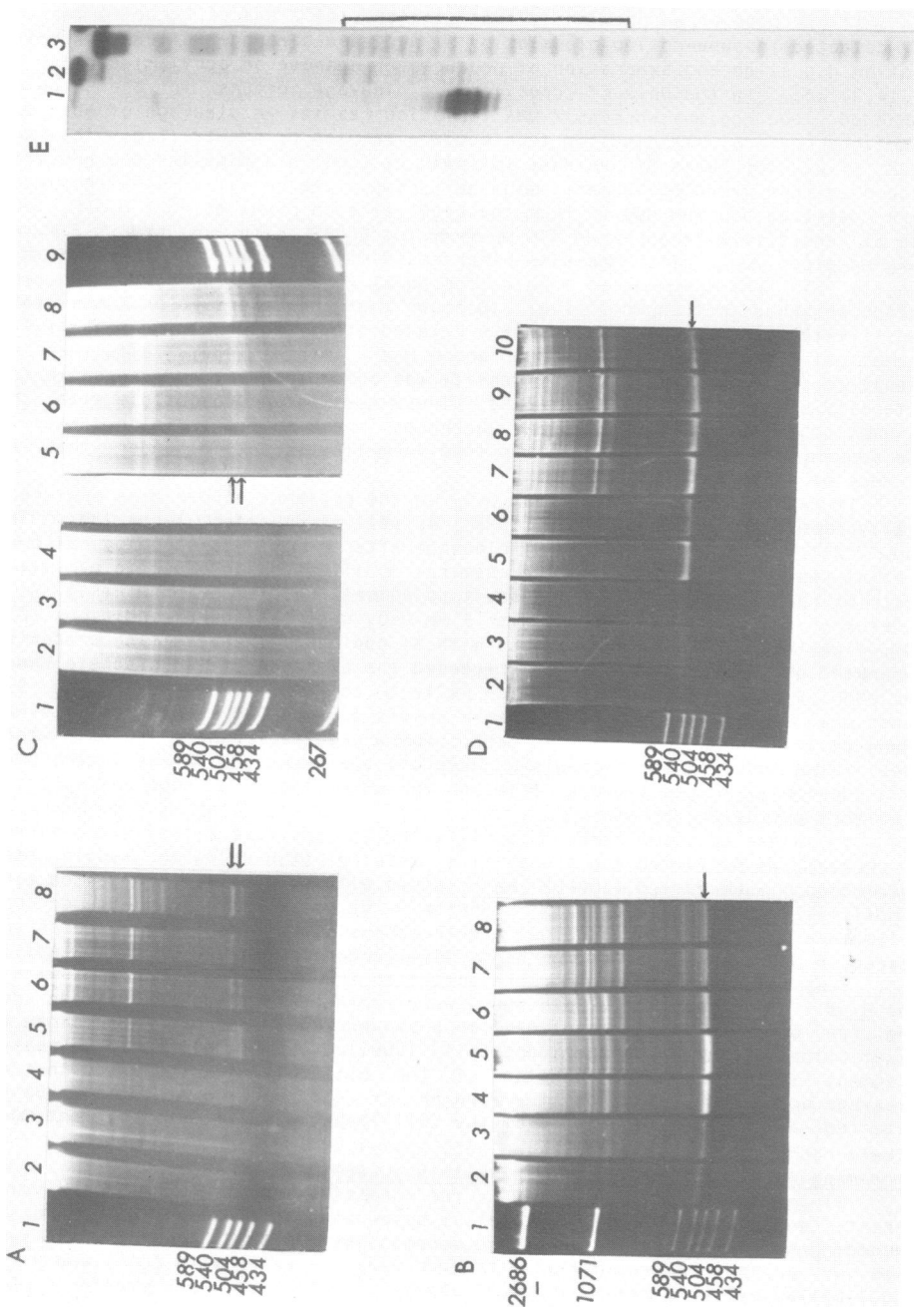
It has been shown previously that stretches of Z-DNA or cruciforms can be detected in supercoiled plasmids by S1 nuclease cleavage followed by restriction digestion and separation of products on agarose or acrylamide gels (8,14,37,38). In the case of cruciforms, cleavage occurs in the single-stranded loop regions whereas Z-DNA formation results in cleavage of both B/Z junctions regions. We digested topoisomeric samples of pSpHI (Fig. 1A) and pKJ2 (Fig. 1B) with S1 nuclease followed by EcoRI cleavage and the products were electrophoresed on 4% acrylamide gel. Two sites sensitive to S1 nuclease were detected 560 and 580 bp from the EcoRI site in pSpHI at $-\bar{\sigma} \geq 0.042$, and one S1 sensitive site appeared 570 bp from the EcoRI site in pKJ2. Similar mapping experiments performed with other restriction enzymes localized unambiguously S1 sensitive sites within (for pKJ2) or around (for pSpHI) the synthetic sequence (not shown). Fig. 1B shows that the insert in pKJ2 has some sensitivity to S1 nuclease even in the relaxed state, but that the sensitivity dramatically increases at $-\bar{\sigma} = 0.035$ and 0.042. Interestingly, a further increase in the negative superhelix density was accompanied by a decrease in the intensity of the 570 bp S1-EcoRI band. These results are compatible with the assumption that the insert of pSpHI undergoes the B to Z transition, whereas the sequence cloned in pKJ2 adopts a cruciform-like structure under the influence of negative supercoiling.

It has been demonstrated that OsO_4 in the presence of pyridine preferentially modifies B/Z junctions (28-30) as well as cruciform loops (39). The modification can be detected by S1 cleavage after removing of topological constraint that was necessary for the chemical modification to occur (e.g. after restriction cleavage). In this assay topoisomeric samples of pSpHI (Fig. 1C) or pKJ2 (Fig. 1D) were modified with 2 mM OsO_4 under standard conditions (29, 30), digested with EcoRI and cleaved with S1 nuclease. The resulting products, separated on a 4% acrylamide gel, revealed the presence of two discrete bands for pSpHI (indicated by arrows) which reflect the modification of the B/Z junctions around the cloned synthetic insert a. One OsO_4-sensitive site was found at the cloned sequence b in pKJ2. These results further indicate that the structures of the cloned sequences are different and can be interpreted as a supercoil-induced Z-block formation for pSpHI, and a supercoil-induced cruciform extrusion for pKJ2.

In order to unambiguously localize the S1-sensitive sites in the insert of pUCKJ2, we performed additional fine mapping studies with single base resolution. Supercoiled pUCKJ2 was cleaved with S1 nuclease, digested with HindIII+SstI and labelled with $\alpha[^{32}P]$dATP using Klenow fragment of DNA polymerase. After acrylamide gel electrophoresis, two radioactive bands appeared in addition to the vector band. One band corresponded to the HindIII-SstI fragment and the other corresponded to the S1 cleavage product within the HindIII-SstI fragment. Both bands were isolated from the acrylamide gel and run on a sequencing gel next to a sequencing ladder of A+G obtained by Maxam-Gilbert reaction of the identically labelled but S1 nuclease untreated fragment (Fig. 1E). The lengths of the half molecules indicated that S1 cleavage occurred exactly at the center of the sequence studied. Bands detected after electrophoresis of the full length HindIII-SstI fragment most likely represent unspecific nicking activity of S1 nuclease.

Fine mapping of OsO_4-modified bases within pUCSpHI and pUCKJ2.

Since the structures adopted by the inserts in pSpHI and pKJ2 are different, we determined the OsO_4 reactive bases at single nucleotide resolution according to the previously developed methodology (30,32). Topoisomeric samples of pUCSpHI were modified with 2 mM OsO_4, cleaved with EcoRI and PstI, end-labelled by using Klenow fragment and $\alpha[^{32}P]$dATP (40), and smaller EcoRI-


E
1 2 3
C
1 2 3 4 5 6 7 8 9
589 540 504 458 434 267
D
1 2 3 4 5 6 7 8 9 10
589 540 504 458 434
A
1 2 3 4 5 6 7 8
589 540 504 458 434
B
1 2 3 4 5 6 7 8
2686
1071
589 540 504 458 434

PstI fragments were isolated from acrylamide gels, treated with hot piperidine and loaded on the gel next to Maxam-Gilbert sequencing reactions of the same, but OsO_4 unmodified, fragment (Fig. 2A, lanes 1-6). The same procedure was employed for the mapping of OsO_4-modified bases on the opposite strand, except that topoisomeric samples of the plasmid were cut with HindIII+KpnI after chemical modification (Fig. 2A, lanes 7-12).

Sequencing gel electrophoresis of the pUCSpHI and pUCKJ2 inserts revealed the compression of bands above the middle of the palindromic sequence of the inserts. Thus, in order to complete the full map of OsO_4-modified bases (Fig. 2C), we sequenced both strands after labelling 5' ends by $\gamma[^{32}P]ATP$ and T4 polynucleotide kinase (data not shown).

An interpretation of the collected data indicates that the supercoil-inducible OsO_4 modification at the pUCSpHI insert (Fig. 2C) covers both B/Z junction regions. There are, however, some unexpected features in the modification pattern detected. Apart from T residues, which are the main target points (29,32), we detected modifications of G, A and C residues at the B/Z interphases. The finding of osmium-modified thymines and cytosines is not unexpected since it is well known that this agent is reactive towards both pyrimidines (41-46). The chemistry responsible for modification and detection of adenine and guanine residue is much less understood. It can be concluded, however, that OsO_4 modification of purines reflects a peculiar conformation of

Fig. 1. Detection of S1 nuclease and OsO_4 sensitive sites in the inserts of pSpHI, pKJ2 and pUCKJ2.

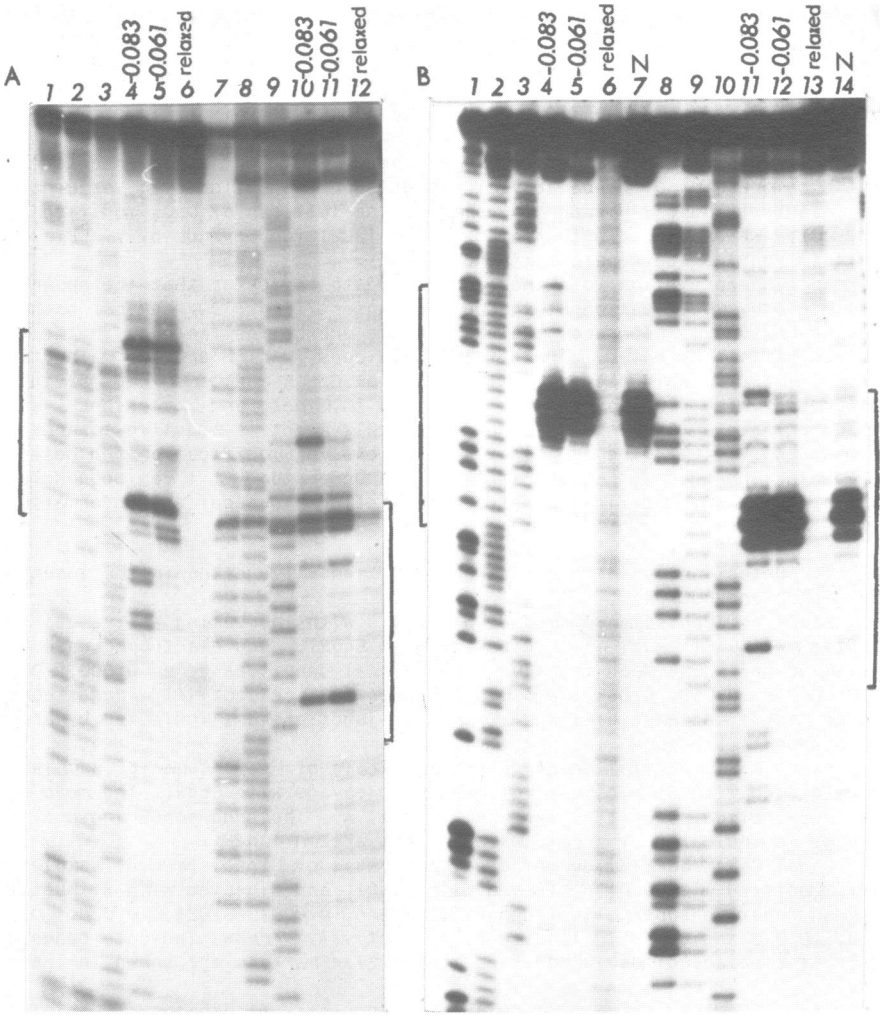
Panel A: Topoisomeric smaples of pSpHI with increasing $-\bar{\sigma}$ values were digested with S1 nuclease, cleaved with EcoRI, and electrophoresed on 4% acrylamide gel. The mean negative superhelical densities were 0.000, 0.021, 0.030, 0.042, 0.052, 0.063 and 0.075 in lanes 2-8, respectively. Bands corresponding to S1 nuclease cleavage at the B/Z junctions are indicated by arrows. Size standarts are shown in lane 1.

Panel B: 4% acrylamide gel electrophoresis of topoisomeric samples of pKJ2 digested with S1 nuclease followed by cleavage with EcoRI. The mean negative superhelical densities were as in panel A. The band appearing due to S1 cleavage is indicated by arrow. Size markers are in lane 1.

Panel C: 4% acrylamide gel electrophoresis of topoisomeric samples of pSpHI modified with OsO_4, cleaved with EcoRI, and digested with S1 nuclease. The mean negative superhelical densities were 0.000, 0.021, 0.030, 0.052, 0.063, 0.075 and 0.082 in lanes 2-8, respectively. Arrows indicate bands caused by S1 cleavage of OsO_4 modified loci. Size markers are shown in lanes 1 and 9.

Panel D: 4% acrylamide gel electrophoresis of topoisomeric samples of pKJ2 modified with OsO_4, linearized with EcoRI, and digested with S1 nuclease. The mean negative superhelical densities were 0.000, 0.021, 0.035, 0.042 0.052, 0.063, 0.075 and 0.082 in lanes 3-10, respectively. The sample in lane 2 was first digested with EcoRI, then modified with OsO_4 and cleaved with S1 nuclease. Size markers are shown in lanes 1. The arrow indicates the S1-EcoRI fragment appearing due to OsO_4 modification.

Panel E: Fine mapping of S1 nuclease cleavage sites within the pUCKJ2 insert. Native plasmid was cleaved with S1 nuclease, digested with HindIII+SstI, labelled by "filling in" reaction, and the full-length HindIII+SstI as well as the radioactive half-molecules were extracted from an acrylamide gel after electrophoresis. After denaturation in formamide the samples were loaded on a sequencing gel. Lane 1, Half-molecules; lane 2, HindIII+SstI full-length fragment; lane 3, sequencing ladder of purines of HindIII+KpnI fragment labelled in the same way. Identical amount of radioactivity was loaded on each channel.


A
1 2 3 4 5 6 7 8 9 10 11 12
-0.083
-0.061
relaxed
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14
-0.083
-0.061
relaxed
N
C
←Hind III
Eco RI→
pUCSpHI
CTGTTGGGCG CCATCTCCTT GCATGTGCGCGCGCATGCGCGCGCACATGC ACCATTCCTT GCGGCG
GACAACCCGC GGTAGAGGAA CGTACACGCGCGCGTACGCGCGCGTGTACG TGGTAAGGAA CGCCGC
←EcoRI
Hind III→
pUCKJ2
CTGTTGGGCG CCATCTCCTT GCATGCGCGCATATATATATGCGCGCATGC ACCATTCCTT GCGGCG
GACAACCCGC GGTAGAGGAA CGTACGCGCGTATATATATACGCGCGTACG TGGTAAGGAA CGCCGC

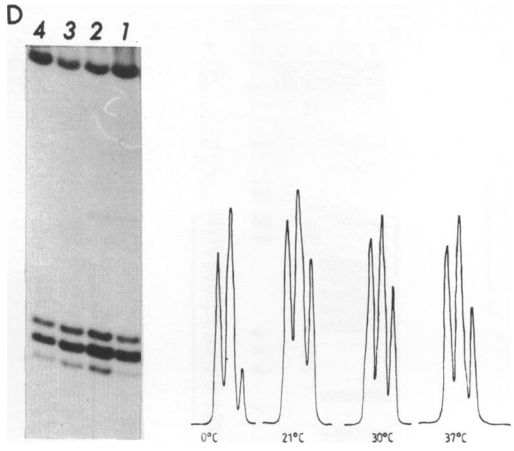


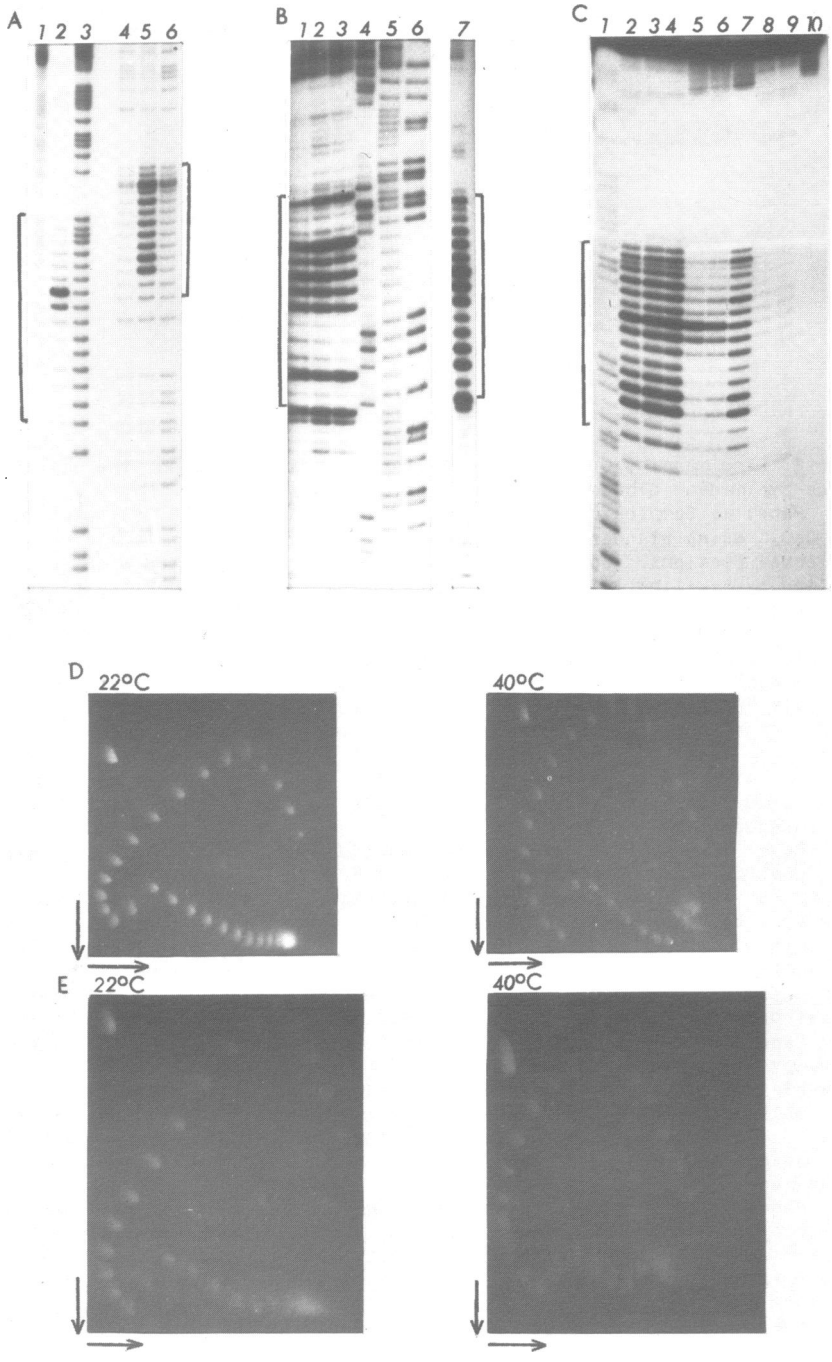
Fig. 2. Fine mapping of OsO_4 hyperreactive bases around the alternating purine-pyrimidine blocks of pUCSpHI and pUCKJ2

Panel A: Samples of pUCSpHI ($-\bar{\sigma}$ = 0.000, 0.061 and 0.083) were modified with OsO_4 using standart conditions (30) and then each sample was split into two equal portions. One portion of each sample was digested with EcoRI+PstI, labelled by "filling in" reaction, and the radioactive EcoRI+PstI fragments were extracted from an acrylamide gel. After incubation with piperidine at 90°C, the material was loaded on a sequencing gel (lanes 4-6) next to Maxam-Gilbert sequencing reactions of the same strand (lanes 1-3). The second portion of each OsO_4 modified sample was treated identically, except for digestion with EcoRI+PstI which was replaced by digestion with HindIII+KpnI (lanes 10-12). The samples were loaded on the sequencing gel next to Maxam-Gilbert sequencing reactions of the HindIII+KpnI fragment (lanes 7-9) labelled by "filling in" reaction at the HindIII site. The 32 bp alternating purine-pyrimidine blocks are indicated by brackets. The same amount of radioactivity was loaded on each channel.

Panel B: Mapping of OsO_4-hyprreactive bases in the pUCKJ2 insert was done identically as described in panel A for pUCSpHI. Samples with EcoRI sites "filled in" after OsO_4 modification are shown in lanes 4-7, and should be compared with the sequencing pattern of the same strand (lanes 1-3). Modifications in opposite strand were mapped by labelling the HindIII ends (lanes 11-14) of OsO_4 treated samples and comparing the pattern with the sequencing reactions (lanes 8-10). The $-\bar{\sigma}$ values used are indicated. N = native superhelical density. The same number of counts was loaded on each channel.

Panel C: Physical map of OsO_4-hyperreactive bases around the alternating purine-pyrimidine blocks (underlined) of pUCSpHI and pUCKJ2 at $-\bar{\sigma}$ = 0.061. Experimental data obtained for each strand of pUCSpHI after labelling 5' ends with polynucleotide kinase (not shown) are combined with the results shown in panel A. Double sequencing of each OsO_4-modified strand was necessary since compression of bands occured on sequencing gels above the middle of the inverted repeat sequence.

Panel D: Temperature dependent OsO_4 modification of T residues within the cruciform loop of pUCKJ2. Samples of pUCKJ2 ($-\bar{\sigma}$ = 0.083) were modified with 2mM OsO_4 at standart conditions at 0°C (lane 1), 21°C (lane 2), 30°C (lane 3) and 37°C (lane 4), cleaved with EcoRI+PstI, labelled by "filling in" reaction and the EcoRI-PstI fragment, after incubation with hot piperidine, was loaded on a sequencing gel. Each lane contains the same amount of counts loaded. Densitometric tracing of the autoradiogram is shown near by.


A
1 2 3 4 5 6
B
1 2 3 4 5 6 7
C
1 2 3 4 5 6 7 8 9 10
D
22°C
40°C
E
22°C
40°C

the double helix at the B/Z junctions.

Another unexpected feature of the sequence cloned in pUCSpHI is that a weak OsO_4 modification was always detectable at T residues located centrally and within Z-block. It is also shown that the elevation of $-\bar{\sigma}$ value from 0.062 to 0.092 creates new hyperreactive sites located further away from the Z-block. This phenomenon has been observed before (30,32).

The supercoil-dependent OsO_4 hyperreactivity of the pUCKJ2 insert is presented in Fig. 2B and 2C. The modification of T residues occurred mainly at the center of the inverted repeat sequence. Modification of residues other than T was not detected. Other sites not specified in Fig. 2C were detectable occasionally at higher superhelix densities (e.g. 0.083), but the relative intensities of the corresponding bands were irreproducible. It suggests that other type of the structural transition, in addition to that observed at $-\bar{\sigma}$=0.061, may occur. In general, the OsO_4 modification pattern obtained for pUCSpHI strongly indicates a Z-type helix formation within cloned sequence, whereas the modification pattern obtained for the pUCKJ2 insert indicates a cruciform formation under the topological stress of negative supercoiling.

Fig. 3. Mapping of DEP-modified purines in pUCSpHI and pUCKJ2 inserts and 2D agarose gel electrophoresis.

Panel A: 3µg of DNA were suspended in 100µl o 25 mM Tris-HCl, pH 7.6, 0.2 M NaCl and 2.5 mM EDTA. After adding 10 µl of DEP and vortexing, modification was continued at room temperature for 30 min. and stopped by ethanol precipitation. A sample of relaxed pUCKJ2 was cleaved with HindIII+KpnI, labelled at the HindIII site using $\alpha[^{32}P]$dATP and Klenow fragment of DNA polymerase, and the HindIII-KpnI was recovered from an acrylamide gel after electrophoresis. It was then cleaved with hot piperidine and loaded on a sequencing gel (lane 1). Supercoiled pUCKJ2 (native) was treated in the same way except that HindIII+SstI were used for digestion before labelling (lane 2). A sequencing ladder of purines was prepared from HindIII-KpnI fragment of pUCKJ2 labelled by "filling in" reaction at the HindIII site (lane 3). DEP-modified samples of relaxed (lane 4) and supercoiled (lane 5) pUCSpHI were treated in a similar way except the EcoRI+PstI didestion was employed before labelling of the EcoRI site. The same fragment was used for preparing the sequencing ladder of purines (lane 6). The 32 bp alternating purine-pyrimidine sequences are indicated by brackets.

Panel B: Experimental procedure was as described in panel A, except that modification of pUCKJ2 was performed in the presence of 100 mM (lane 1), 50 mM (lane 2) and 10 mM (lane 3) $NiCl_2$. Sample shown in lane 7 was equilibrated in TBE buffer for 16 hours at 22°C prior to DEP treatment. The modified plasmid was cleaved with EcoRI+PstI and labelled at the EcoRI site. Sequencing reactions C, A+G and G are shown in lanes 4-6, respectively. The 32 bp inverted repeat sequence is indicated by brackets.

Panel C: Native pUCKJ2 was equilibrated in TBE buffer supplemented with 0, 0.002, 0.02, 0.2, 1 and 4 M NaCl (lanes 2-7, respectively) before DEP modification. Modification pattern of the insert of the linear plasmid suspended in TBE containing 0, 0.2 and 4 M NaCl is shown in lanes 8-10, respectively. Sequencing reaction C+T is shown in lane 1. Modified plasmid was cleaved with HindIII+KpnI and labelled at the HindIII site.

Panels D and E: 2D agarose gel electrophoresis of topoisomeric samples of pUCSpHI (panel D) and pUCKJ2 (panel E). Electrophoresis in the first dimension was performed at the temperature indicated using a gel box with a circulating water plate (International Biotechnologies, Inc. model HRH) as described previously (52). Electrophoresis in the second dimension was performed in the presence of chloroquine (1µg/ml) at room temperature. Arrows indicate the first and the second dimensions.

Temperature dependent OsO_4 modification of pUCKJ2

The optimal length of the single-stranded loop of a cruciform is believed to be 4-6 bp (55). If a cruciform is formed within an inverted repeat sequence, the length of the loop may be sensitive to certain physico-chemical parameters, e.g. temperature. Thus, we performed OsO_4 modification studies on pUCKJ2 at different temperatures. Fig. 2D shows the relative intensities of modified T-residues in the center of the pUCKJ2 insert obtained after OsO_4 modification at 0°C, 21°C, 30°C, and 37°C. Reaction at 0°C resulted in a clear modification inhibition at one out of 3 modifiable T's. An identical result was obtained for the other strand (data not shown). Thus, we conclude that the length of the loop in the cruciform formed in the pUCKJ2 insert is temperature sensitive, and OsO_4 used here as a probe is sensitive enough to detect this behavior.

Diethyl pyrocarbonate (DEP) modification of pUCSpHI and pUCKJ2.

DEP has been described as a sensitive probe for the detection of Z-segments (31,32) and cruciform structures (47,48) within supercoiled plasmids. We used this chemical in order to confirm the conformations of the pUCSpHI and pUCKJ2 inserts induced by negative supercoiling. As expected, the pUCKJ2 insert revealed a supercoil-dependent modification of A residues located in the center of the inverted repeat sequence (cruciform loop), whereas supercoiled pUCSpHI revealed DEP-hypersensitive purines at distances corresponding to the length of the Z-segment (Fig. 3A, lanes 2 and 5). The above conclusion is consistent with all of the experimental data presented thus far.

Recently, Bourtayre et al. (56) have shown that $d(AT)_n$ polymer adopt left-handed conformation in the 5M NaCl and 95mM $NiCl_2$ solution. Since the synthetic insert of pUCKJ2 contains $d(AT)_5$ in the center of the 32 bp long alternating purine-pyrimidine tract it was of our interest to determine whether $NiCl_2$ may facilitate the conversion of the entire inverted repeat block into the left-handed helix. Fig. 3B shows DEP modification pattern of pUCKJ2 insert obtained in the presence of 100, 50 and 10mM $NiCl_2$ (lanes 1-3). Strong modification of A residues and slightly weaker modification of G residues detected within the entire alternating purine-pyrimidine block clearly indicates the left-handed helix formation. As low as 10mM concentration of $NiCl_2$ in combination with native supercoiling is sufficient to cause this effect.

Another modification experiment was done by placing native pUCKJ2 plasmid in TBE-buffer (which was used for two dimensional agarose gel electrophoresis described in the next section) and equilibrating the sample for 16 hours at 22°C prior to DEP treatment. Equilibration time was similar to the time of electrophoresis in the first dimension. Fig. 3B, lane 7 shows that the modification pattern of 32 bp inverted repeat sequence is as expected for the left-handed helix formation. G residues within the purine-pyrimidine sequence are more accessible for DEP modification in case of TBE-induced Z-like structure as compared with that induced by $NiCl_2$. Apparently both conformations are slightly different. Control DEP-modification experiments performed with native pUCSpHI in TBE buffer resulted in modification pattern of purines similar to that, presented on Fig. 3A, lane 5. No hypersensitivity to DEP was detected neither on linear pUCSpHI nor linear pUCKJ2 placed in TBE buffer (data not shown).

It is clear, that the insert of supercoiled pUCKJ2 adopts left-handed conformation when $NiCl_2$ is present in the otherwise cruciform favoring mixture (Fig. 3B, lane 1-3). We tried, however, to identify the salt component which causes, that this sequence behaves as a cruciform when placed in 25 mM Tris-HCl, pH 7.6, 0.2 M NaCl and 2.5 mM EDTA (Fig. 3A, lane 2), or as a left-handed when suspended in TBE buffer (Fig. 3B, lane 7). Fig. 3C shows the DEP modification pattern of the insert of supercoiled pUCKJ2 obtained in TBE buffer supplemented with increasing concentration of NaCl. Note, that opposite strand to that shown on Fig. 3B is investigated. Hypersensitivity of all purines within

the 32 bp inverted-repeat sequence was detected at 0, 2 and 20 mM NaCl indicating the presence of the left-handed helix (Fig. 3C, lane 2-4). Further increase in the NaCl concentration to 0.2 or 1 M (lane 5 and 6) resulted in the strong modification of A's in the center of the inverted-repeat sequence. This we interpret in terms of the cruciform structure formation. At the highest salt concentration tested (4 M NaCl) Z-type DEP-modification pattern appears again (lane 7). Results presented above indicate, that at the low and high concentration of NaCl the Z-DNA formation within the pUCKJ2 insert is favored, whereas at the moderate salt concentration apparently the cruciform structure is more stable.

Thus, we have demonstrated that the same chemical probe can detect the sequence $TGCAT(GC)_3(AT)_5(GC)_3ATGCA$ in the cruciform state (Fig. 3A, lane 2) or in the left-handed state (Fig. 3B, lane 1-3 and lane 7) depending on the environmental conditions employed. We can also predict, that under the conditions of 2D-gel electrophoresis (TBE buffer) the pUCKJ2 should behave as having the insert in Z-type configuration.

Two Dimensional (2D) agarose gel electrophoresis.

2D agarose gel electrophoresis is a widely used method for the detection of structural transitions within the double helix caused by negative supercoiling (10,13-18,21,34,36,50,51).

In negatively supercoiled plasmids the linking number (α) is lower than the linking number of the same but relaxed molecule (α_0). A change in the linking number creates a change in the twist of the duplex (Tw) and axial writhe (Wr) (34,49), according to the equation

$$(\alpha - \alpha_0) = \Delta Tw + \Delta Wr$$

Topoisomers with different $\alpha - \alpha_0$ values possess different hydrodynamic properties since they differ in their ΔWr values and can thus be separated by one-dimensional or two-dimensional agarose gel electrophoresis. If a segment in the negatively supercoiled plasmid undergoes a transition from a right-handed duplex form to a cruciform or to a left-handed helix, the decrease of the duplex twist is accompanied by an increase of ΔWr, which causes slower migration of the topoisomer relative to the topoisomer with the same $\alpha - \alpha_0$ value in which the transition has not occurred. The extent of retardation corresponds to the amount of unwinding due to the structural transition and may be indicative of the type of transition that has occurred. Fig. 3D shows 2D agarose gels of topoisomeric samples of pUCSpHI obtained at different temperatures. At 22°C the first topoisomer that undergoes relaxation is topoisomer -11. If we assume that topoisomer -13 has completed the transition, the extent of relaxation which is observed due to the structural conversion corresponds to the loss of about 5 superhelical turns. Thus, we can calculate the length of Z-segment which is:

$$\frac{5 \times 10.4}{1.86} = 28 \text{ bp}$$

Although all other assays used here are consistent with these relaxation studies and indicate a left-handed helix formation, it is worth noting that the entire length of the alternating purine/pyrimidine sequence is 32 bp rather than 28 bp.

2D gel electrophoresis performed at 40°C revealed no apparent influence on the B/Z conversion except for the presence of another transition at the more highly supercoiled topoisomers which may correspond to the effects described previously by Lee and Bauer (50).

Results of similar studies performed with pUCKJ2 are presented in Fig. 3E. At 22°C the transition starts at topoisomer -12. If we assume that the transition has been completed with the topoisomer -14, the observed relaxation of 5 superhelical turns is exactly the same as observed for pUCSpHI indicating left-handed helix formation within the 32 bp long inverted repeat sequence. Cruciform formation under the 2D-electrophoresis conditions apparently does

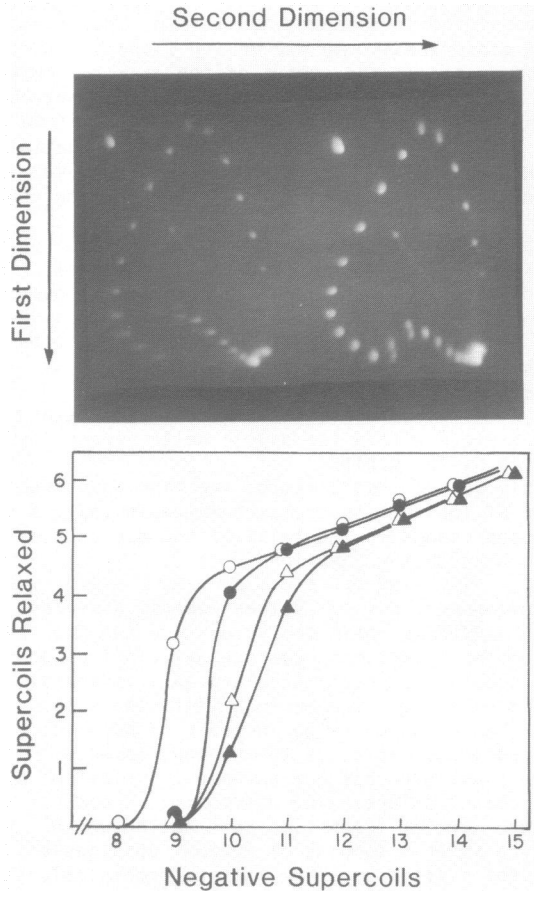


Fig. 4. Effects of 5-cytosine methylation on the supercoil-induced structural transition in pUCSpHI and pUCKJ2. Topoisomer populations of plasmid DNA fully methylated *in vitro* by HhaI methylase were mixed with population of unmethylated parent plasmid and separated on a 1.5% agarose 2D-gel, with 0.8 μM chloroquine present in the second dimension.

Top panel: Distribution of topoisomers (mixture of methylated and unmethylated pUCSpHI on the left, mixture of methylated and unmethylated pUCKJ2 on the right) after 2D-gel electrophoresis.

Bottom panel: B-Z transition curves determined from the above gel by measuring the amounts of supercoil relaxation in individual topoisomes as a function of their number of supercoils. Closed circles: pUCSpHI unmethylated; open circles: pUCSpHI methylated; closed triangles: pUCKJ2 unmethylated; open triangles: pUCKJ2 methylated.

not occur since its formation should result in the relaxation of

$$-\frac{32}{10.4} = 3.07 \text{ superhelical turns.}$$

Thus, we conclude that the conversion of the synthetic sequence of pUCKJ2 into

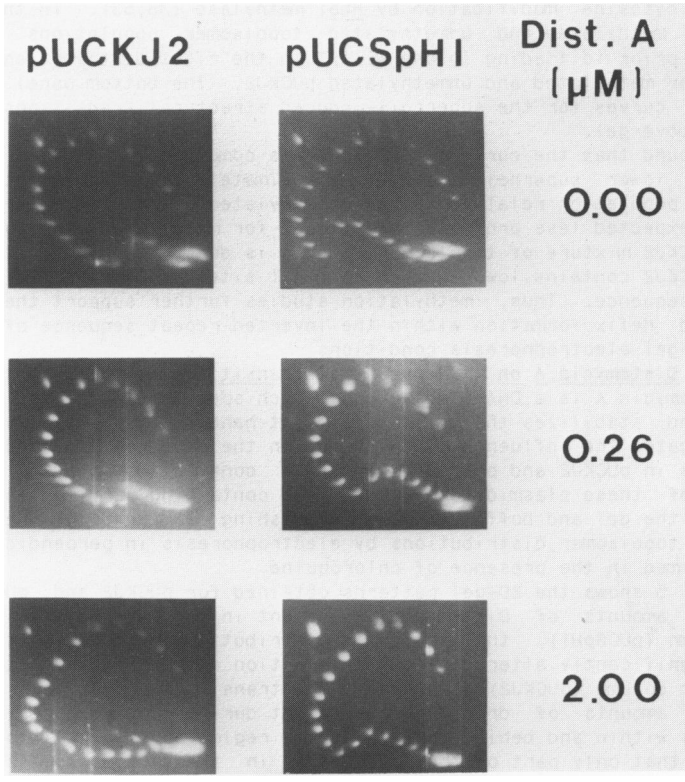


Fig. 5. Effects of Distamycin A on the supercoil-induced structural transitions in pUCKJ2 and pUCSpHI. Topoisomer populations of pUCKJ2 (left panel) or pUCSpHI (right panel) were run on 1.5% agarose gels in 1xTBE buffer with Distamycin A present in gel and buffer at the concentrations indicated on the right. After soaking each first dimension (top to bottom) gel strip 3x in 1 M NaCl, 1x TBE bufer and 3x in 1x TBE to wash out the bound Distamycin, the topoisomer distributions were resolved in the second dimension (left to right) on 1.5% agarose in 1xTBE plus 0.8 μM chloroquine.

the left-handed helix does not require the presence of $NiCl_2$ and depends on the experimental conditions employed.

As in the case of pUCSpHI, 2D-gel electrophoresis of topoisomers of pUCKJ2 performed at 40°C revealed no apparent effect on the B-Z transition. The effect on other types of transitions occurring at highly supercoiled topoisomers is evident. It is interesting that an additional transition (clearly resolved at 40°C) starts at topoisomer -18 in pUCSpHI, but begins at topoisomer -16 in pUCKJ2.

Effects of 5-cytosine methylation on the structural transitions in the inserts

We determined by 2D-gel electrophoresis the influence of 5-cytosine methylation on the thermodynamic stabilities of the secondary structures in the inserts of pUCKJ2 and pUCSpHI. Fig. 4 (top panel) compares the 2D-gel patterns obtained with topoisomer families of the two plasmids with and without

preceding cytosine modification by HhaI methylase (36,53). In the left distribution, methylated and unmethylated topoisomer populations of pUCSpHI were mixed prior to loading in one well; in the right distribution, the same was done for methylated and unmethylated pUCKJ2. The bottom panel shows the transition curves for the supercoil-induced structural transitions as deduced from the above gel.

We found that the curve for pUCSpHI is completely shifted to the left (i.e., to lower superhelicity) by approximately one supercoil turn for the methylated population relative to the unmethylated plasmid. Similar shift, although as expected less profound, was found for methylated and unmethylated pair of pUCKJ2 mixture of topoisomers. This is due to the fact, that the insert of pUCKJ2 contains lower number of MHhaI sites as compared to the pUCSpHI synthetic sequence. Thus, methylation studies further support the concept of left-handed helix formation within the inverted-repeat sequence of pUCKJ2 under the 2D-gel electrophoresis conditions.

Effects of Distamycin A on the structural transitions in the inserts

Distamycin A is a DNA-binding drug which specifically binds to AT-rich regions and stabilizes the helix in a right-handed B-type structure (57,58). We investigated the influence of this drug on the supercoil-induced structural transitions in pUCKJ2 and pUCSpHI. This was done by separating topoisomer families of these plasmids on agarose gels containing varying concentrations of drug in the gel and buffer and, after washing out the drug, resolving the resulting topoisomer distributions by electrophoresis in perpendicular dimension performed in the presence of chloroquine.

Fig. 5 shows the 2D-gel patterns obtained for pUCKJ2 and pUCSpHI with increasing amounts of Distamycin A present in the first dimension. In the right column (pUCSpHI), the topoisomer distribution in the transition region is not significantly altered by the interaction of the drug with the plasmid. In the left column (pUCKJ2), however, the transition region is altered with increasing amounts of drug being present during electrophoresis. Certain topoisomers within and behind the transition region have split into two spots, indicating that only part of the molecules in these topoisomers have experienced a relaxation and part has migrated according to increasing superhelicity without obvious relaxation. In addition, the transition region is gradually shifted to higher topoisomer numbers with increasing concentration of Distamycin A. An interpretation of this drug-induced change of a supercoil-dependent structural transition is presented in the Discussion section.

DISCUSSION

In this paper we compare the structural behavior of two otherwise identical plasmid molecules which differ only in the sequences of their synthetic inserts (Fig. 2C). Both sequences are perfect alternating purine-pyrimidine inverted repeats 32 bp in length.

The insert of pSpHI (or pUCSpHI) contains in the center a "Z-genic" sequence $(GC)_4AT(GC)_4$, which by all assays and in all experimental conditions used here shows the formation of left-handed helix in the negative supercoiled plasmid. S1 nuclease cleaves the B/Z junctions (Fig. 1A), which are also modifiable by OsO_4 and subsequently cleavable by S1 nuclease due to the chemical modification (Fig. 1C). Fine mapping of OsO_4 hyperreactive bases also revealed the modification of the B/Z junctions. Interestingly, we observed modification of all four types of bases (Fig. 2A and 2C) with this chemical. In accordance with the above studies, fine mapping of DEP-hyperreactive purines (Fig. 3A) and 2D-gel electrophoresis of topoisomers of pUCSpHI (Fig. 3D and Fig. 4) confirmed the formation of a left-handed helix in the cloned segment at all experimental conditions employed.

Cruciform formation within the pKJ2 and pUCKJ2 inserts

Studies on the left-handed helix formation within the inserts of pSpHI or pUCSpHI were performed in parallel with studies on pKJ2 or pUCKJ2 containing the 32 bp inverted repeat-type sequence $(GC)_3(AT)_5(GC)_3$. The supercoil-inducible transition in pKJ2 resulted in S1 sensitivity located at the cloned sequence (Fig. 1B). In order to localize unambiguously the S1 cleavage sites, we performed additional fine mapping studies. The cleavage pattern obtained in this way indicated that S1 cleavage (Fig. 1E) occurred exactly at the center of the sequence studied. Fine mapping of OsO_4 and DEP modified bases at the nucleotide resolution strongly indicated the cruciform formation within the pUCKJ2 insert (Fig. 2B and 2C; Fig. 3A). We shall stress, that conformation of the insert of pUCKJ2 plasmid is apparently very sensitive to changes in the salt composition/concentration of the environmental mixture (discussed below). Therefore, experiments mentioned above should be viewed as a set of experimental conditions under which the cruciform structure is detectable. We have also demonstrated that the cruciform loop size is temperature sensitive as shown on Fig.4.

32 bp inverted repeat sequence of pUCKJ2 adopts left-handed conformation

Z-type helix formation within the supercoiled pUCKJ2 insert was detected in two independent assays:

1. DEP treatment under the condition where cruciform-like structure is formed (25mM Tris-HCl, pH 7.6, 0.2M NaCl and 2.5mM EDTA)(Fig. 3A) but in the presence of $NiCl_2$ (10, 50 or 100 mM) resulted in modification pattern exactly as expected for the left-handed helix formation within the synthetic sequence (Fig. 3B). Hypermodification of all purines within the 32 bp inverted repeat sequence (A residues beeing more reactive) indicated their syn-type conformation which is belived to be responsible for elevated reactivity towards DEP (31,32). This is consistent with the notion that $NiCl_2$ is a powerful chemical which is able to convert even $d(AT)_n$ polymer (under the high NaCl concentration conditions) into the left-handed structure (56). Our preliminary results indicate, that DEP-modification pattern suggesting the Z-type helix formation within the $d(AT)_{16}$ insert of the plasmid can be obtained as a result of the combined action of supercoiling, high NaCl and $NiCl_2$ concentrations (K.Nejedly and J.Klysik - unpublished results). We wish to emphasize, that the conversion of pUCKJ2 insert into the left-handed conformation can be also detected by DEP at the 2D-gel electrophoresis condition (TBE-buffer) in absence of $NiCl_2$ (Fig. 3B, lane 7 and Fig. 3C, lane 2).

2. 2D-gel electrophoresis revealed that the pUCKJ2 insert undergoes the structural transition that give rise to the relaxation extent which can be explained only by the left-handed helix formation (Fig. 3 and 4). Indeed, the extent of relaxation determined for pUCKJ2 and pUCSpHI was almost identical at least at 22°C. Discrepancy exists between the length of the alternating purine-pyrimidine tracts (32 bp) and the length of the Z-segments detected by 2-D gels (28 bp). We believe, however, that pUCSpHI insert is unambiguously converting into the left-handed helix in the supercoiled plasmid, since its detection was possible also by all other probes and independently of the experimental conditions used. This molecule can be regarded as a reference molecule for pUCKJ2 behavior. Not only the vector sequences are identical in these two plasmids but also the ends of the inverted repeats are similar. On this basis the same extent of relaxation obtained on 2-D gels in TBE buffer we interpret as indicative of the same structure induced. Thus, it is demonstrated that depending on the environmental conditions the inverted repeat type sequence composed of alternating purines and pyrimidines may adopt either cruciform-like or Z-like conformations. It is also clear that transition to Z-form can be transmitted through $d(AT)_5$.

The existence of the pUCKJ2 insert in the Z-type structure is unex-

pected since:

a) Studies performed on similar but not identical molecule containing sequences $G(CG)_6(TA)_4(CG)_6C$ have shown that alternating $d(TA)_4$ sequence placed between two strong Z-forming blocks can not adopt the left-handed structure (51). However, the cruciform formation in long $d(AT)_n$ sequences have been described (16-18).

b) The Z-forming sequences composed of perfectly alternating dG-dC surrounding the $(AT)_5$ block of the pKJ2 and the pUCKJ2 inserts on both sides are 6 bp in length. The shortest $(GC)_n$ sequence shown to adopt a left-handed helix is 8 bp long (20).

Recently, interconversion of the certain sequences into the cruciform or Z-like structures, depending on experimental conditions employed was postulated on the basis of data obtained with different model molecules (60).

Contradictory effect of NaCl on structural interconversion within the pUCKJ2 insert.

What are the conditions that favor the cruciform over the left-handed DNA formation in supercoiled pUCKJ2?. Cruciform can be detected in the S1 digestion mixture (Fig. 1B and Fig. 1E), OsO_4 modification (Fig. 2B) and DEP modification buffers (Fig. 3A, lane 2). Both DEP and OsO_4 treatments detecting the cruciform structure were done in the presence of 0.2 M NaCl. However, when this buffer (which favored the cruciform) is supplemented with $NiCl_2$ (10-100 mM), Z-type helix was obtained (Fig. 3B, lane 1-3). Z-type helix formation was also observed in TBE buffer by DEP treatment (Fig. 3B, lane 7) or 2D-gel electrophoresis (Fig. 3E, Fig. 4 and 5). Close inspection of the composition of buffers employed lead us to the assumption, that NaCl might be responsible for the dual behavior of the pUCKJ2 insert.

As shown on Fig. 3C, DEP-modification pattern indicates Z-type helix formation in TBE buffer without or with low concentration of NaCl (lane 2-4). At 0.2 or 1 M NaCl the cruciform-type DEP-modification pattern was obtained (lanes 5 and 6). Further increase in NaCl concentration resulted in conversion of the cruciform back to the left-handed state. This contradictory effect of salt on Z-type helix formation is not surprising, since it has been observed before in another experimental system (59). Apparently, at the moderate NaCl concentration the left-handed helix formation is inhibited and therefore, cruciform extrusion becomes possible, whereas, at the low and very high salt conditions Z-helix is favored. The above studies demonstrate, that even a small change in the concentration of the buffer component(s) within the physiological range might exert dramatical influence on the type of the structural interconversion appearing within the alternating purine-pyrimidine inverted repeats.

Recently, very similar effect of NaCl was observed for supercoiled plasmids containing $d(AT)_n$ tracts. At moderate salt concentrations cruciform-type structure was detected, whereas at the absence of NaCl another type of perturbed helical conformation arises within these sequences (61).

Different concentrations of other components present in buffers used in our studies on pUCKJ2 behavior have not been tested. However, it is reasonable to assume, that they may be also important and contribute substantially to the cruciform or left-handed helix formation within the insert. Salt dependent Z-DNA/cruciform/Z-DNA interconversion is most likely not limited to the sequence of pUCKJ2 insert. It might be a more general phenomenon associated with other types of alternating purine-pyrimidine inverted repeats as well. More work is necessary to support this hypothesis.

Effects of 5-cytosine methylation and Distamycin A on the structural transitions in the inserts

The replacement of cytosine by 5-methylcytosine in blocks of alternating dC-dG strongly supports Z-DNA formation in supercoiled plasmids (53,59). The effects of cytosine methylation on cruciform extrusion has not been inves-

tigated. However, we believe that cruciform formation should not be influenced by this base modification in inverted-repeat sequences containing dC-dG segments within the stem region, since both in the extruded and unextruded form these regions are believed to be in a right-handed B-type structure.

We determined that for methylated pUCSpHI the entire structural transition is shifted to lower topoisomer numbers relative to unmethylated pUCSpHI (Fig. 4). This behavior is characteristic for Z-DNA formation in alternating dC-dG sequences. Methylation of pUCKJ2 also results in facilitating the transition. The observed effect is not as profound as in the case of pUCSpHI but less MHhaI sites are present on pUCKJ2 insert. This analysis provides a further indication of Z-DNA formation in both plasmids in TBE buffer.

Our results on the differential effects of Distamycin A on pUCKJ2 and pUCSpHI (Fig. 5) also support this interpretation for the following reason: for both inserts, Distamycin A binds to the central AT-rich regions. This region is longer in pUCKJ2 insert where more efficient binding may occur. The drug is believed to occupy the major groove of B-helix. Thus, the inhibition of the transition from the right to the left-handed conformation by Distamycin A is an expected effect for pUCKJ2 plasmid.

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REFERENCES

1. Rich,A., Nordheim,A. and Wang,A.H.J. (1984) Annu. Rev. Biochem. 53, 791 -846.
2. Wells,R.D. (1988) J.Biol.Chem., in press
3. Lilley,D.M.J. (1984) Biochem.Soc.trans. 12, 127-140.
4. Pohl,F.M. and Jovin,T.M. (1972) J.Mol.Biol. 67, 375-396.
5. Klysik,J., Stirdivant,S.M., Larson,J.E., Hart,P.A. and Wells,R.D. (1981) Nature (London) 290, 672-677.
6. Wells,R.D., Brennan,R., Chapman,K.A., Goodman,T.C., Hart,P.A., Hillen,W., Kellogg,D.R., Kilpatrick,M.W., Klein,R.D., Klysik,J., Lambert,P.F., Larson,J.E., Miglietta,J.J., Neuendorf,S.K., O'Connor,T.R., Singleton, C.K., Stirdivant,S.M., Veneziale, C.M., Wartell,R.M. and Zacharias,W. (1983) Cold Spring Harbor Symp. 47, 77-84.
7. Zacharias,W., Larson,J.E., Klysik,J. Stirdivant,S.M. and Wells,R.D. (1982) J.Biol.Chem. 257, 2775-2782.
8. Singleton,C.K., Klysik,J. Stirdivant,S.M. and Wells,R.D. (1982) Nature (London) 299, 312-316.
9. Peck,L.J., Nordheim,A., Rich,A. and Wang,J.C. (1982) Proc.Natl.Acad.Sci. USA 79, 4560-4564.
10. Haniford,D.D. and Pulleyblank,D.E. (1983) Nature (London) 302, 632-634.
11. Wells,R.D., Miglietta,J.J., Klysik,J., Larson,J.E., Stirdivant,S.M. and Zacharias,W. (1982) J.Biol.Chem. 257, 10166-10171.
12. Zimmer,C., Tymen,S.,March,C. and Guschlbauer,W. (1982) Nucleic Acids Res. 10, 1081-1091.

13. Singleton,C.K., Kilpatrick,M.W. and Wells,R.D. (1984) J.Biol.Chem. 259, 1963-1967.
14. Kilpatrick,M.W., Klysik,J., Singleton,C.K., Zarling,D., Jovin,T.M., Hanau, L.M., Erlanger,B.F. and Wells,R.D. (1984) J.Biol.Chem. 259, 7268-7274.
15. Naylor,L.H., Lilley,D.M.J. and van de Sande,J.H. (1986) EMBO J. 5, 2407-2413.
16. Greaves,D.R., Patient,R.K. and Lilley,D.M.J. (1985) J.Mol.Biol. 185, 461-478.
17. Haniford,D.B. and Pulleyblank,D.E. (1985) Nucleic Acids Res. 13, 4343-4363.
18. Panyutin,I., Lyamichev,V. and Mirkin,S. (1985) J.Biomol.Struct.Dyn. 2, 1221-1223.
19. Callahan,L. Han,Fu-Son., Watt,W., Duchamp,D. Kezdy,F.J. and Agarval,K. (1986) Proc.Natl.Acad.Sci.USA 83, 1617-1621.
20. Gaffney,B.L., Marky,L.A. and Jones,R.A. (1982) Nucleic Acids Res. 10, 4351-4361.
21. McLean,M.J., Blaho,J.A., Kilpatrick,M.W. and Wells,R.D. (1986) Proc.Natl. Acad.Sci.USA 83, 5884-5888.
22. Singleton,C.K., Klysik,J. and Wells,R.D. (1983) Proc.Natl.Acad.Sci.USA 80, 2447-2451.
23. Atkinson,T. and Smith,M. (1984) in Gait,M.J.(ed.), Oligonucleotide Synthesis, a Practical Approach, IRL Press, Ltd. Oxford, pp. 35-81.
24. Kumar,G. and Poonian,M.S. (1984) J.Org.Chem. 49, 4905.
25. Uznanski,B., Koziolkiewicz,M., Stec,W.J., Zon,G., Shinozuka,K. and Marzilli,L.G. (1986) Chemica Scripta 26, 221.
26. Maxam,A.M. and Gilbert,T. (1980) Methods Enzymol. 65, 499-560.
27. Yanish-Perron,C., Vieira,J. and Messing,J. (1985) Gene 33, 103-119.
28. Nejedly,K., Kwinkowski,M., Galazka,G., Klysik,J. and Palecek,E. (1985) J.Biomol.Struct.Dyn. 3, 467-478.
29. Galazka,G., Palecek,E., Wells,R.D. and Klysik,J. (1986) J.Biol.Chem. 261, 7093-7098.
30. Galazka,G., Klysik,J., Kwinkowski,M., Szala,S. and Palecek,E. (1987) in Bruzik,K.S. and Stec,W.J (eds), Biophosphates and Their Analogues, Synthesis, Structure, Metabolism and Activity, Elsevier, Amsterdam, pp. 515-524.
31. Herr,W. (1985) Proc.Natl.Acad.Sci.USA 82,8009-8013.
32. Johnston,B.H. and Rich,A. (1985) Cell 42, 713-724.
33. Singleton,C.K. and Wells,R.D. (1982) Anal.Biochem. 122, 253-257.
34. Wang,J.C.,Peck,L.J. and Becherer,K. Cold Spring Harbor Symp. Quant. Biol. 47, 85-91.
35. Kang,D.S. and Wells,R.D. (1985) J.Biol.Chem. 260, 7783-7790.
36. Zacharias,W., Larson,J.E., Kilpatrick,M.W. and Wells,R.D. (1984) Nucleic Acids Res. 12,7677-7692.
37. Panayotatos,N. and Wells,R.D. (1981) Nature 289, 466-470.
38. Singleton,C.K. and Wells,R.D. (1982) J.Biol.Chem. 257, 6292-6295.
39. Lilley,D.M.J. and Palecek,E. (1984) EMBO J. 3, 1187-1192.
40. Maniatis,T., Fritch,E.F. and Sambrook,J. (1982) Molecular Cloning, Cold Spring Harbor Laboratory, Cold Spring Harbor,NY.
41. Beer,M., Stern,S., Carmalt,D. and Mohlenrich,K.M. (1966) Biochemistry 5, 2283-2288.
42. Burton,K. and Riley,W.T. (1966) Biochem.J. 98, 70-77.
43. Subbaraman,L.R., Subbaraman,J. and Behrman,L.J. (1971) Bioinorg.Chem. 1, 35-55.
44. Chang,C.-M., Beer,M. and Marzilli,L.G. (1977) Biochemistry 16, 33-38.
45. Marzilli,L.G. (1977) Prog.Inorg.Chem. 23, 255-378.
46. Barton,J.K. and Lippard,S. (1980) in Thomas,T.G. (ed.), Nucleic Acids - Metal Ion Interactions, John Willey and Sons, New York,pp.32-113.

47. Furlong,J.C and Lilley,D.M.J. (1986) Nucleic Acids Res. **14**, 3995-4007.
48. Sholten,P.M. and Nordheim,A. (1986) Nucleic Acids Res. **14**, 3981-3993.
49. Fuller,F.B. (1971) Proc Natl.Acad.Sci.USA **68**,815-819.
50. Lee,F.S. and Bauer,W.R. (1985) Nucleic Acids Res. **13**, 1665-1682.
51. Ellison,M.J., Feigon,J., Kelleher III,R.J., Wang,A.M.-J.,Habener,J.F. and Rich,A. (1986) Biochemistry **25**, 3648-3655.
52. O'Connor,T.R., Kang,D.S. and Wells R.D. (1986) J.Biol.Chem. **261**, 13302-13308.
53. Zacharias,W., O'Connor,T.R. and Larson,J.E. (1988) Biochemistry in press.
54. Ferro,A.M. and Olivera,B.M. (1984) J.Biol.Chem **259**, 547-554.
55. Gough,G.W, Sullivan,K.M. and Lilley,D.M.J. (1986) EMBO J. **5**, 191-196.
56. Bourtayre,P., Liquier,J., Pizzorini,L. and Taillandier,E. (1987) J.Biomol.Struct.Dyn. **5**, 97-104.
57. Van Dyke,M.W, Hertzberg,R.P. and Dervan,P.B. (1982) Proc.Natl.Acad.Sci. USA **79**, 5470-5474.
58. Klevit,R.E., Wemmer,D.E. and Reid,B.R (1986) Biochemistry **25**, 3296-3303.
59. Klysik,J., Stirdivant,S.M., Singleton,C.K., Zacharias,W. and Wells,R.D. (1983) J.Mol.Biol. **168**, 51-71.
60. McLean,M.J. and Wells,R.D. (1988) J.Biol.Chem. in press.
61. McClellan,J.A. and Lilley,D.M.J. (1987) J.Mol.Biol. **197**, 707-721.