Osmium Tetroxide Probing of Local DNA Structure in Linear and Supercoiled Plasmids Containing Curvature-Inducing Sequences

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Abstract. Recombinant plasmids pK1A108, pK3A108, pK4A108 and pK5/6T217 containing 80 ± 1 base pair inserts with different curvature-inducing sequences were studied using the DNA structure probe osmium tetroxide in the presence of pyridine (Os, py). The insertion sequences of the plasmids pK1A108, pK3A108, and pK4A108 are strongly related while the degree of curvature increases from pK1A108 (no curvature) < pK3A108 < pK4A108 < pK5/6T217. The Os, py probe reacts selectively with single-stranded and distorted double-stranded regions in the DNA double helix. Nuclease S1 was used to recognize and cleave regions made permanently single-stranded due to osmium modification. In linearized plasmids treatment with Os, py produced no S1-detectable site-specific modification. This result is in agreement with models suggested for DNA curvature; in general, continuous base pairing and base stacking is considered through different sequence blocks as well as through structural junctions. Os, py-probing of the plasmids in the supercoiled state also resulted in no S1-detectable site-specific modification within the inserts of pK1A108, pK3A108, and pK4A108 plasmids (while the regions containing inverted repeat nucleotide sequences in these plasmids were site-specifically modified). In contrast, supercoiled pK5/6T217 DNA was site-specifically modified within the curvature-inducing insert sequence. The nucleotide sequence of the insert of this plasmid strongly differs from the insertion sequences of the other three plasmids; it is extremely AT-rich and contains regularly arranged dAGAGA and dATATA sequences. The structural distortion observed in supercoiled pK5/6T217 is most probably due to the presence of these sequences in a particular arrangement in the insertion sequence.

Key words: DNA curvature — Chemical probing of DNA structure — DNA structural distortions — Osmium modification of DNA — Recombinant supercoiled plasmids

Introduction

The conception of polymorphy of the DNA double helix was discussed quite early (Paleček 1976) and is now widely accepted (Rich et al. 1984). Recently, special attention has been paid to the properties of curved DNA. A common feature of the sequences inducing DNA curvature is the periodic presence of $d(A)_{2-9}$ runs with a period of 10 to 11 base pairs (for reviews see Trifonov 1985; Diekmann 1987a).

Poly(dA) · poly(dT) has a B′-form DNA structure slightly different from standard B-form, as detected by various experimental techniques (Peck and Wang 1981; Arnott et al. 1983; Alexeev et al. 1985; Wartell and Harrell 1986; Taillandier et al. 1987; Diekmann and Zarling 1987). The different structure of this sequence is also recognized by proteins (Kunkel and Martinson 1981; Prunell et al. 1984; Diekmann and Zarling 1987). Not only long but also rather short stretches of $d(A)_n \cdot d(T)_n$, with n as small as 4 to 6, already seem to adopt this B′-form structure (Diekmann and Wang 1985; Burkhoff and Tullius 1987; Diekmann and Zarling 1987). When the $d(A)_n$ tract is followed directly by a $d(T)_n$ block, the sequence $d(A)_n \cdot d(T)_n$ is already curved for $n = 2$ (Hagerman 1985; Diekmann 1987b; Diekmann et al. 1988). A sufficient condition for the helix axis curvature is the alternation of dA tracts in phase with the turn of the DNA helix (Marini et al. 1982; Wu and Crothers 1984; Hagerman 1985; Diekmann and Wang 1985; Hagerman 1986; Diekmann 1986; Koo et al. 1986; Ulanovsky et al. 1986; Griffith et al. 1986).

Recently, Kitzing and Diekmann (1987) suggested that poly(dA) · poly(dT) might adopt a B′-form structure due to the influence of the exocyclic substituents at the 2- and 6-positions of the adenines. This hypothesis was experimentally tested (Diekmann 1987b; Diekmann et al. 1988). The experimental findings support the hypothesis of Kitzing and Diekmann (1987) but no other hypothesis like the stabilizing influence of a spine of hydration in the minor groove of the helix (Chuprina 1987) or the hydrophobic interaction of the 5-methyl groups at the pyrimidines (Jernigan et al. 1986, 1987).

The structural features of poly(dA) · poly(dT) tracts might have an influence on biological processes (Bossi and Smith 1984). $d(A)_n \cdot d(T)_n$ tracts are found in biologically important sequences like e.g. the upstream scaffold-attached regions of several *Drosophila* genes (Gasser and Laemmli 1986), yeast centromers, and the replication origin of bacteriophage lambda (Zahn and Blattner 1985).

The linkage of the B′-form structure of the $d(A)_n \cdot d(T)_n$ tracts to the standard B-DNA is of specific interest. Models discussed for DNA curvature assume perfect base-stacking through this linkage (Selsing et al. 1979; Wu and Crothers 1984; Diekmann 1986; Ulanovsky and Trifonov 1987; Kitzing and

Diekmann 1987). Thus, in general no structure distortion is assumed to occur at the B-B′- and B′-B-junctions.

Two enzymatic probes sensitive to even small local structure variations in DNA (S1 and mung bean nuclease) were applied to a curved DNA sequence of kinetoplast DNA origin. In both cases no specific reaction was observed (Marini et al. 1984). In addition, no site-specific bromoacetaldehyde modification was found when S1 nuclease was used for the detection of chemically modified nucleotides (Kitchin et al. 1986). Minicircle kinetoplast DNA containing curved DNA sequences was linearized by mung bean nuclease in the presence of 50 % formamide. Cleavage in the curved sequence range was not detected (Muhich and Simpson 1986).

We could show previously (Paleček et al. 1981; Lukášová et al. 1982; Lukášová et al. 1984; Glikin et al. 1984; Paleček 1986) that osmium tetroxide in the presence of pyridine (Os, py), may serve as a probe for DNA structure. This probe binds preferentially to single-stranded and distorted double-stranded regions in DNA. It was applied to study cruciform structures (Lilley and Paleček 1984), B—Z junctions (Nejedlý et al. 1985; Galazka et al. 1986; Paleček et al. 1987a, b, c), alternating purine-pyrimidine (Galazka et al. 1986; McClellan et al. 1986), and homopurine · homopyrimidine (Vojtíšková and Paleček 1987) sequences in supercoiled and linear DNAs. Os, py reacts with the bases and not with the backbone of the DNA helix. As it can react with structurally modified base pairs (Lukášová et al. 1984), it might be able to detect base stacking distortions in the double helix. Therefore, in this work we applied this probe to curved DNA to look for subtle structural distortions (not necessarily involving base unpairing) in the curvature-inducing sequences and especially at the B-B′- and B′-B-junctions.

We used Os, py to study four recombinant plasmids containing inserts of similar length (Table 1): one which does not induce curvature and three with different curvature-inducing sequences (Diekmann 1986; Diekmann and Pőrschke 1987). The insertion sequences of the plasmids pK1A108, pK3A108, and pK4A108 are strongly related to one another (see Table 1). The degree of curvature as measured by the migration anomaly in polyacrylamide gels increases according to pK1A108 (no curvature) < pK3A108 < pK4A108 < pK5/6T217. The nuclease S1 was used to detect the osmium-modified sequence regions. All four linearized plasmids did not show any site-selective osmium modification. Also in the negatively supercoiled state, the insertion sequences of three of the four plasmids remained undigested by S1 nuclease after treatment with Os, py. However, for one of the supercoiled plasmids strong modification within the insert was observed, suggesting the presence of structural distortion in the insert. The data suggest that this distortion is not merely due to the presence of $d(A)_n \cdot d(T)_n$ tracts.

Materials and Methods

Recombinant plasmids pK1A108, pK3A108, pK4A108 and pK5/6T217 (Table 1, Fig. 1) were prepared as described elsewhere (Diekmann 1986). Restriction endonucleases PstI, EcoRI and HindIII were purchased from the Institute of Sera and Vaccines, Prague. Nuclease S1 was isolated from "Takadiastase" (Vogt 1973).

Unless stated otherwise chemical modification was performed in 5 mmol/l Tris-HCl buffer (pH 7.8), 0.5 mmol/l EDTA, 1 mmol/l OsO_4, 3 % pyridine at 25 °C for 60 min. The reaction was terminated by ethanol precipitation. Other conditions were as described elsewhere (Paleček et al. 1987c).

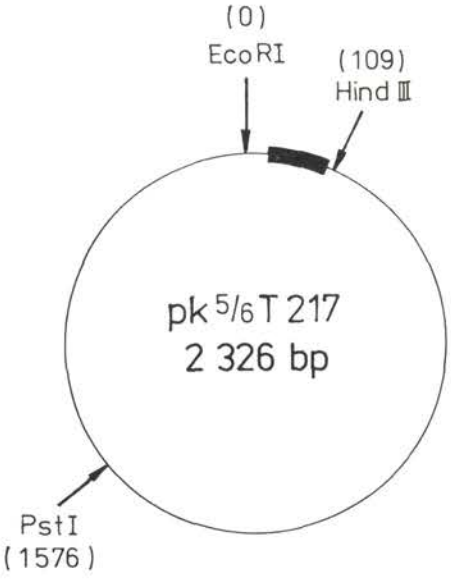

Fig. 1. Map of the plasmid pK5/6T217 showing the relative positions of cleavage sites of restriction endonucleases used in this study. pK5/6T217 is a recombinant 2326 bp plasmid derived from pJW200 which contains 82-bp insert at the ClaI site (Diekmann 1987a); pJW200 is derived from pBR322 by the deletion of the sequence in between 2 HaeII sites at bp 236 and 2356). Other plasmids used in this paper differ from pK5/6T217 only in their inserts (see Table 1).

Results

We used three plasmids pK3A108, pK4A108 and pK5/6T217 containing the inserts (Table 1) which have been previously shown (Diekmann 1987a) to introduce curvature into linear DNA fragments. As a control we used pK1A108 DNA with an insert of the same length but not containing the curvature-inducing sequence. To test for possible site-selectivity of chemical modification in the region of the curvature-inducing inserts we used the procedure as previously applied for the detection of B—Z junctions (Nejedlý et al.1985) and cruciforms (Lilley and Paleček 1984). The essence of this approch is the use of a restriction endonuclease to provide sequence-specific cleavage and the single-strand selective nuclease S1 to digest regions made permanently single-stranded due to chemical modification of bases.

Table 1. Recombinant plasmids and nucleotide sequences of their inserts

Plasmid	Insert repeat sequence	Insert length (bp)	*k* factor[a]	Insertion sequence
pK1A108	GACAGGACTC (10 bp)	80	1.00	atc GACAGGACTC · GACAGGACTC · GACAGGACTC · GACAGGACTC · GACAGGACTC · GACAGGACTC · GACAGGACTC · GACAGGACTC · gata(agct)
pK3A108	GACAAAGCTC (10 bp)	79	1.03	atc GACAAAGCTC · GACAAAGCTC · GACAAAGCTC · GACAAAGCTC · GACAAAGCTC · GACAAAGCTC · GACAAAGCTC · GACAAAGCTC · gata(agct)
pK4A108	GACAAAACTC (10 bp)	80	1.27	atc GACAAAACTC · GACAAAACTC · GACAAAACTC · GACAAAACTC · GACAAAACTC · GACAAAACTC · GACAAAACTC · GACAAAACTC · gata (agct)
pK5/6T217	ATATATTTTTT AGAGATTTTT (21 bp)	81	1.51	atc GATATATTTTTTAGAGATTTTTATATATTTTTTAG AGATTTTTATATATTTTTTAGAGATTTTTATATAT TTTTTAGAGA gata (agct)

[a] *k* factor, i. e. apparent length in a 10 % polyacrylamide gel divided by sequence length; HaeIII fragment containing the insert (Diekmann 1987a).

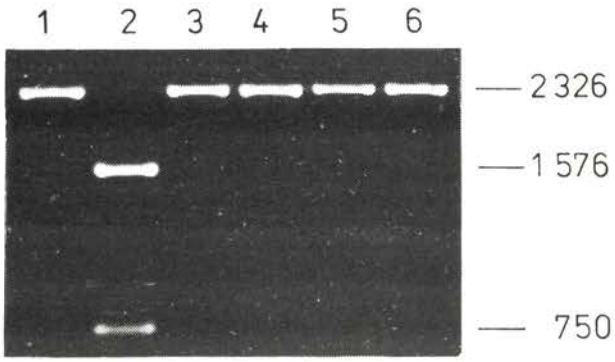

Fig. 2. Osmium tetroxide modification of linearised plasmids. DNAs were linearised with PstI and treated with 1 mmol/l OsO_4, 3 % pyridine, 60 min at 26 °C. After modification, pK1A108 (lane 3), pK3A108 (lane 4), pK4A108 (lane 5), pK5/6T217 (lane 6) were treated with nuclease S1. Unmodified pK1A108 cleaved by PstI (lane 1) and PstI followed by EcoRI (lane 2). Figures on the right show bp numbers.

Absence of site-specific osmium-modification detectable by nuclease S1 in linearized plasmids

Several models have been suggested to explain the structural nature of the DNA curvature (Diekmann 1987b). It can be assumed that the sequences responsible for the DNA curvature (short runs of dAs repeated with a period of 10 to 11 bp) have a non-B structure which might be detectable by Os, py. To test this possibility we linearized four plasmids with PstI, treated them with 1 mmol/l OsO_4, 3 % pyridine 60 min at 26 °C, purified and treated with nuclease S1 (30 min at 37 °C). Agarose gel electrophoresis showed no sign of any site-specific modification (Fig. 2) as a result of this treatment. This result thus suggests that no single-stranded or greatly distorted double stranded regions are involved in DNA curvature of these sequences.

Osmium modification site within the insert of negatively supercoiled pK5/6T217 DNA

Previously it has been suggested by Diekmann and Wang (1985) that the curvature in the kinetoplast DNA sequence can easily be distorted due to torsional stress in supercoiled DNA molecules. Such a distortion might result in structural changes detectable by a chemical probe. To test for distortion we modified the supercoiled plasmids (at native superhelical density) with Os, py under the conditions used for linearized plasmids (Fig. 2), then linearized them with PstI and digested with nuclease S1. As a result of this treatment pK1A108,

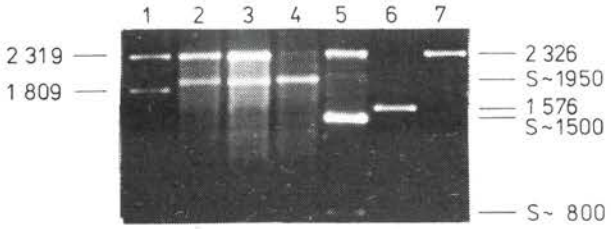

Fig. 3. Osmium tetroxide modification of supercoiled plasmids. pK1A108 (lane 2), pK3A108 (lane 3), pK4A108 (lane 4), pK5/6T217 (lane 5); supercoiled plasmids were treated with 1 mmol/l OsO_4, 3 % pyridine and cleaved by PstI followed by nuclease S1. Unmodified pBR322 cleaved with BglI (lane 1) and unmodified pK1A108 cleaved with PstI and EcoRI (lane 6) and with PstI (lane 7) were used as markers. Figures on the sides show bp numbers.

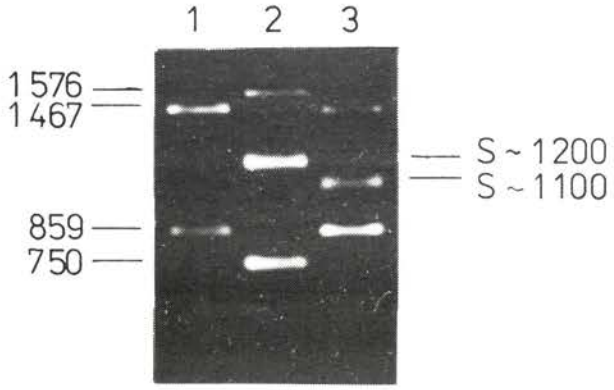

Fig. 4. Mapping of osmium stabilized single-stranded region in pK4A108. Unmodified pK4A108 cut by PstI and HindIII (lane 1), supercoiled pK4A108 modified with 1 mmol/l OsO_4, 3 % pyridine was cleaved by PstI and EcoRI followed by S1 (lane 2) and PstI and HindIII followed by S1 (lane 3). Figures on the sides show bp numbers.

pK3A108 and pK4A108 produced strong bands corresponding to fragments of 1950 bp (Fig. 3, lanes 2—4). This is consistent with site selective modification within the region of the three inverted repeats of pBR322 sequences which are known to extrude the cruciform structure (Lilley 1983). To remove the ambiguity of the restriction mapping the osmium treated pK4A108 DNA was cleaved with PstI followed by EcoRI or HindIII and then with nuclease S1. PstI followed by EcoRI yielded 1576, 1200 and 750 bp fragments (Fig. 4, lane 2), PstI followed by HindIII produced 1467, 1100 and 859 bp fragments (Fig. 4, lane 3). Thus the osmium binding sites are located in the region containing inverted repeat nucleotide sequences of pBR322 (Sutcliffe 1978).

Plasmid pK5/6T217 produced a much weaker band of about 1950 bp (see

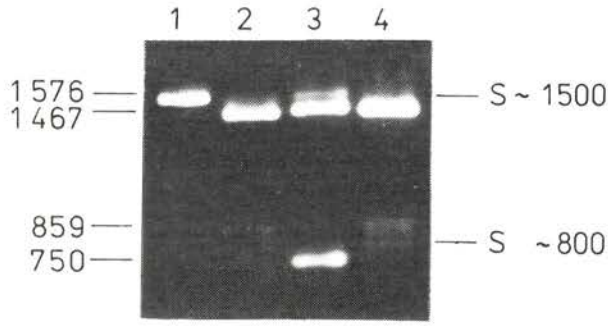

Fig. 5. Mapping of osmium-stabilized single-stranded region in supercoiled pK5/6T217. pK5/6T217 was modified with 1 mmol/l OsO_4, 3% pyridine and cleaved by PstI and EcoRI followed by S1 (lane 3), PstI and HindIII followed by S1 (lane 4). As controls pK1A108 cleaved by PstI and EcoRI (lane 1), PstI and HindIII were used (lane 2). Figures on the sides show bp numbers.

above) and two additional strong bands corresponding to 800 bp and 1500 bp (Fig. 3, lane 5). These two bands are consistent with the selective modification within the insert. To remove the ambiguity of the restriction mapping osmium-reacted samples of pK5/6T217 DNA were (a) cleaved with EcoRI or (b) with PstI followed by EcoRI or (c) with PstI followed by HindIII; all samples were then digested with nuclease S1. EcoRI produced a full-length linear and slightly shorter DNA fragment (not shown). PstI followed by EcoRI produced in addition to 1576 bp band and (a very weak) 750 bp band (Fig. 5, lane 1) and 1500 bp band (Fig. 5, lane 3). PstI followed by HindIII gave 1467 bp and 859 bp bands and an additional 800 bp band (Fig. 5, lane 4). The results thus show that site-selective modification occurs within the insert of pK5/6T217 DNA roughly 50 bp from the EcoRI site. Unmodified supercoiled DNA was not site-selectively cleaved with nuclease S1 in the insert.

Site-specific osmium modification of negatively supercoiled pK5/6T217 depends on NaCl concentration

It has been shown (Diekmann 1987a) that curving of DNA (Diekmann and Wang 1985) as well as other structures (Diekmann and Lilley 1987) and structural distortions depend on the salt concentration. The abnormal electrophoretic mobility of the DNA fragment from pK5/6T216 (very similar to pK5/6T217) is decreased with increasing NaCl concentration in the range 0—60 mmol/l, however only by about 20%. Thus, DNA curvature remains strong even at elevated NaCl concentrations like 60 mmol/l. We tested the dependence of the

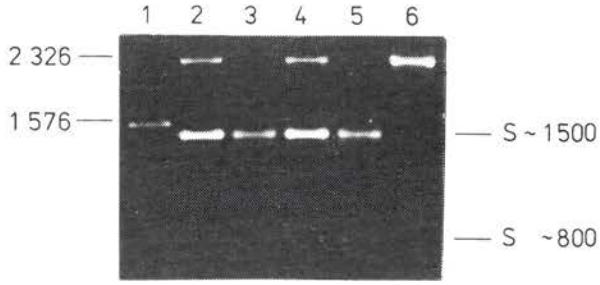

Fig. 6. Dependence of modification of supercoiled pK5/6T217 on NaCl concentration. pK5/6T217 was treated with 1 mmol/l OsO_4, 3 % pyridine in 5 mmol/l Tris-HCl, 0.5 mmol/l EDTA with no NaCl (lane 2), 5 mmol/l NaCl (lane 3), 10 mmol/l NaCl (lane 4), 20 mmol/l NaCl (lane 5), 50 mmol/l NaCl (lane 6); as a control unmodified pK1A108 cleaved by EcoRI and PstI (lane 1). Figures on the sides show bp numbers.

site-specific osmium-modification of the supercoiled pK5/6T217 DNA on NaCl concentration in the range from 0 to 100 mmol/l NaCl. We observed the site-specific modification in the range of 0—20 mmol/l NaCl (Fig 6, lanes 2—5). At 50 mmol/l (Fig. 6, lane 6) and higher NaCl concentrations (not shown) the bands characteristic of the site-specific modification within the insert disappeared.

Discussion

Chemical probing of the DNA structure in linearized plasmids

The plasmids pK1A108, pK3A108, pK4A108, and pK5/6T217 (see Table 1) contain inserts of nearly identical length which induce curvature of different degrees (Diekmann 1986; 1987a). We have found no S1-detectable site-specific chemical modification in the region of these curvature-inducing sequences due to the treatment of linearized plasmids with Os, py (Fig. 1). This finding agrees well with the results obtained with bromoacetaldehyde treatment of other linearized plasmids containing curvature-inducing sequences of kinetoplast origin (Kitchin et al. 1986). For these sequences no site-specific chemical modification was detected either.

Bromoacetaldehyde attacks adenine and cytosine at sites involved in Watson-Crick base pairing. Thus, only unpaired or non-Watson-Crick base pairs can be modified. In contrast, Os, py adds across the 5, 6 double bond of thymine and cytosine (Chang et al. 1977; Lukášová et al. 1982; 1984), i. e. to the sites which are outside the Watson-Crick hydrogen bonding system. Thus, Os, py is also able to detect structural distortions other than unpaired bases or non-Watson-Crick base pairs.

To detect the site-specific chemical modification, nuclease S1 was used both for bromoacetaldehyde (Kitchin et al. 1986) and Os, py-treated plasmids (Figs. 2—6). This enzyme was shown to detect site-specific chemical modifications of cruciform structures (e. g. Lilley and Paleček 1984), B—Z junctions (Nejedlý et al. 1985), structural changes in homopurine homopyrimidine tracts (Vojtíšková and Paleček 1987), and other unusual structures (Glikin et al. 1984; McClellan et al. 1986). Recently, however, we have found (Nejedlý et al., unpublished) that nuclease S1 is not able to recognize single osmium-modified nucleotides (while the single modified nucleotides are detectable by nucleotide sequencing techniques). The chemical modification induced by chloroacetaldehyde, bromoacetaldehyde, glyoxal and Os, py may not be recognized by nuclease S1 if the chemical modification is limited to one or few nucleotides in one DNA strand (Galazka et al. 1986; Paleček et al. 1987a, c). Thus, the absence of site specific cleavage of bromoacetaldehyde- and Os, py-treated linear plasmids observed by Kitchin et al. (1986) and by us (Fig. 2), respectively, indicates only the absence of *larger* chemically modified regions in the curvature-inducing sequences. To exclude also the possibility of osmium modification of isolated nucleotides, nucleotide sequencing techniques have to be applied (Johnston and Rich 1985); Galazka et al. 1986; McClellan et al. 1986). Experiments of this type are in progress.

Structural changes due to supercoiling

Properties of supercoiled DNA containing curvature-inducing sequences are of particular interest when the biological importance of curved DNA is considered. However, so far little is known about the behaviour of curved DNA in the supercoiled state. The helical repeat of curved sequences was measured by the band shift method by Diekmann and Wang (1985). For small topoisomer numbers the influence of the curved DNA in the plasmid on the writhing of the molecule has already been detected; the studied plamids were, however, not identical with the plasmids used in this paper. Probing of the structural distortions in supercoiled DNA containing curvature-inducing sequences with Os, py may improve knowledge on the influence of supercoiling on the properties of these sequences.

a. Plasmids pK1A108, pK3A108, and pK4A108. No site-specific modification was detected within the inserts of the three plasmids pK1A108, pK3A108, and pK4A108 (Table 1). All four plasmids contain inserts of nearly identical length (80 ± 1 bp). Plasmid pK4A108 contains considerable curvature in the insert, pK3A108 is only slightly curved, while the pK1A108 insertion is uncurved and

serves as a control (Diekmann and Pőrschke 1987; Diekmann 1986). Thus, the structure of the curvature-inducing sequences is not strongly distorted by superhelical stress. Although no modification is detected in the curvature-inducing sequence, the supercoiled plasmids are modified by Os, py in a region containing an inverted repeat sequence (Fig. 3) in agreement with the results obtained earlier (Lilley and Paleček 1984) with DNA not containing strong curvature-inducing sequences.

b. Plasmid pK5/6T217. Treatment of the plasmid pK5/6T217 at native superhelical density with Os, py resulted in site-specific modification (Fig. 3—6) in the region of the insert which contains curvature-inducing sequences (Fig. 1). This structural distortion might either be connected with the influence of supercoiling on the strong curvature of DNA observed in linear fragments containing the insert of pK5/6T217 (Diekmann 1987a), or it might be due to the response of the given nucleotide sequence to supercoiling with little or no relation to the DNA curvature. The nucleotide sequence of the insert of pK5/6T217 contains the motif

dATATATTTTTTAGAGATTTT

which is repeated about four times. This repeated motif clearly differs from that of the other three plasmids (Table 1). The pK5/6T217 insert contains only two G · C base pairs per 21 base pairs (about 10 % G · C content), while in the other three plasmids about four times more G · C base pairs are present. Thus, one possible explanation for the structural distortion in the insertion sequence in pK5/6T217 driven by torsional stress might be the instability of the double helix due to its A · T richness. Under close to physiological conditions, $d(AG)_n \cdot d(CT)_n$ segments in supercoiled plasmids can assume an unusual protonated H structure (Lyamichev et al. 1985; 1986) in which the pyrimidine bases are hypersensitive to Os, py (Vojtíšková and Paleček 1987). According to these studies, however, in pK5/6T217 DNA the dAGAGA sequence is too short to adopt the H structure. Alternatively, the observed local conformational change might be due to the presence of the dATATAT sequences. $d(AT)_n$ sequences are known to form a cruciform structure (Greaves et al. 1985; Panyutin et al. 1985; Haniford and Pulleyblank 1985) at less negative superhelical densities that other inverted repeat sequences. In linear DNA, $d(AT)_n$ sequences may adopt an unusual structure which is hypersensitive to Os, py (McClellan et al. 1986). However, the length of the $d(AT)_n$ sequence has to be substantially longer than $n = 3$ (as in pK5/6T217) both for the cruciform formation in supercoiled DNA and for the unusual structure in linear DNA. In the latter case a strong hypersensitivity towards Os, py was observed for $d(AT)_{16}$ and longer segments but not for $d(AT)_8$. Nevertheless, it cannot be excluded that

even shorter $d(AT)_n$ segments (even $n = 3$) may assume unusual structures under topological stress provided they are flanked by suitable nucleotide sequences. The possibility that dATATA sequences are involved in the formation of the local structural distortion detected by Os, py is supported by the observed dependence on NaCl concentration (Fig. 6); a similar dependence of the hypersensitivity to Os, py was observed with plasmids containing $d(A—T)_n$ inserts (Paleček, unpublished). The observed NaCl dependence is not in agreement with the properties of the migration anomaly of curved DNA in polyacrylamide gels.

To decide between the above mentioned possible alternative explanations for the structural modification of the insertion sequences in the supercoiled pK5/6T217, additional experiments are required which include the detection of the chemical modification at single-nucleotide resolution. Such experiments are under way.

Recently we have shown (Paleček et al. 1987b) that the osmium probe can also be applied for DNA structure studies in the bacterial cell. Thus, the structural distortions of pK5/6T217 and the behaviour of the other plasmids can be studied directly in the cells; the possible role of the detected structure variations can be analyzed *in situ*.

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