Gen. Physiol. Biophys. (1987), **6**, 327—341

Inhibition of Restriction Endonuclease Cleavage due to Site-specific Chemical Modification of the B-Z Junction in Supercoiled DNA

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Abstract. Structural distortions on the boundary between right-handed B and left-handed Z DNA segments in plasmid pRW751 (a derivative of pBR322 containing $(dC\text{-}dG)_{13}$ and $(dC\text{-}dG)_{16}$ segments) were studied by means of chemical probes. Samples of supercoiled DNA were treated with the respective chemical probe, linearized with EcoRI and inhibition of BamHI (whose recognition sequence GGATCC lies on the boundary between the (dC-dG)*n* segments and the pBR322 nucleotide sequence) cleavage was tested. Treatment with osmium tetroxide in the presence of pyridine or 2,2′-bipyridine, respectively, resulted in a strong inhibition of the BamHI cleavage at both restriction sites, provided the (dC-dG)*n* segments were in the left-handed form. In the presence of 2,2′-bipyridine submillimolar concentrations of OsO_4 (at 26 °C) were sufficient to induce the inhibition of BamHI. Chloroacetaldehyde was used as a probe reacting selectively with atoms involved in the Watson-Crick hydrogen bonding. Similarly as in the case of osmium tetroxide treatment of pRW751 with this agent resulted in the inhibiton of BamHI cleavage. It was concluded that the B-Z junction regions in pRW751 contain few solitary bases with disturbed hydrogen bonding or non-Watson-Crick base pairs.

Key words: Recombinant supercoiled plasmid — DNA chemical probes — DNA structural distortions — Left- and right-handed DNA boundary

Introduction

Supercoiling of DNA is known to have a dramatic effect on DNA replication, transcription and recombination (Lilley 1984; Kmiec and Holloman 1986). Due

Abbreviations: bipy — 2,2′-bipyridine, CAA — chloroacetaldehyde, bp — base pairs to superhelicity some structural forms of DNA are stabilized (e. g. cruciforms and left-handed Z-helices), which do not occur in non-superhelical DNA under physiological conditions (Rich et al. 1984). These local perturbations of double-helical DNA which occur in regions with specific nucleotide sequences probably play an important role in the regulation of gene expression, and have therefore been studied intensively in recent years. Until recently the research on local conformational changes in superhelical DNA was mainly undertaken using single-strand selective nucleases (especially nuclease S1). Their use is based on the fact that these conformational changes usually include a portion of bases more accessible to interaction with the environment than those contained in B form. The interpretation of results obtained using these nucleases is made more difficult by the fact that the mechanism of their action on DNA is not known in detail and that the interaction of protein with DNA can induce DNA structural changes.

In studying the polymorphism of double helical DNA in the past, the methods of electrochemical analysis (Paleček 1976; Paleček 1983) have been found to be advantageous, though they do not allow the location of the structural change in the DNA molecule to be determined. Several years ago we turned our attention to the development of chemical probes of DNA structure which would form electroactive markers in the polynucleotide chain detectable by modern electrochemical analysis and which would extend significantly the methodical repertoire of DNA structure research. We found OsO_4 with pyridine (Paleček et al. 1981; Lukášová et al. 1982; Paleček and Hung 1983) to be an electroactive marker and a probe of DNA structure. Using it we detected structural changes in linear (Lukášová et al. 1982; Paleček and Hung 1983; Lukášová et al. 1984; McClellan et al. 1986) and superhelical (Lukášová et al. 1984; Glikin et al. 1984; Lilley and Paleček 1984; Nejedlý et al. 1985) DNA molecules. We have recently shown that this structural probe is capable of recognizing structural distortions at the junction between B and Z segments in supercoiled plasmid DNA. To map the osmium binding site we used subsequent cleavage with restriction and single-strand selective nucleases and gel electrophoresis of the DNA fragments (Nejedlý et al. 1985). The precision of this mapping is limited by the fact that it is not known how exactly nuclease S1 (used as a single-strand selective nuclease) recognizes and cleaves the chemically modified region. Moreover, it is rather doubtful whether this enzyme is able to recognize a single solitary osmium modified nucleotide. We therefore tried to apply other methods to ensure higher precision and/or sensitivity of mapping (Paleček 1986; Galazka et al. 1986; McClellan et al. 1986). One of these approaches is based on the inhibition of restriction cleavage due to chemical modification of the recognition site (Nejedlý et al. 1985). In this work we have applied this method to study the B-Z junctions in the superhelical plasmid pRW751. In addition to OsO_4 with pyridine we have used OsO_4 with 2,2′-bipyridine and chloroacetaldehyde to study changes in DNA cleavage with BamHI restriction endonuclease, which recognizes the sequence GGATCC placed in two positions at the boundaries of the $(dC\text{-}dG)_{13}$ and $(dC\text{-}dG)_{16}$ segments in pRW751 (Fig. 1). Our results show that this method is a powerful tool in DNA structure studies and suggest that the DNA B-Z junctions in pRW751 contain bases with disturbed hydrogen bonding or non-Watson-Crick base pairs at physiological superhelical densities.

Materials and Methods

Plasmid DNAs. Recombinant plasmid pRW751 was prepared as described (Klysik et al. 1981; Klysik et al. 1982). Topoisomeric samples with defined mean superhelical densities ($\bar{\sigma}$) were generated according to Singleton and Wells (1982).

Chemical modifications. Typical reaction took place in a total volume of 50 µl, containing 1 µg of plasmid DNA. The reaction was terminated by ethanol precipitation; the pelleted DNA was extracted with ether and dissolved in distilled water.

Osmium tetroxide (Fisher Scientific Co.). Unless stated otherwise, reactions were performed in 25 mmol/l Tris-HCl buffer (pH 7.8), 2.5 mmol/l EDTA, 0.2 mol/l NaCl, indicated concentration of OsO_4 (up to 2 mmol/l) plus either 0.2 mol/l (2 %/v/v/) pyridine or 4 mmol/l 2,2′-bipyridine at 26 °C for 1 h.

Chloroacetaldehyde (Fluka). Reactions proceeded in the same medium as above with 0.1 mol/l chloroacetaldehyde at 37 °C for 1 h. The above-mentioned purification was preceded by chloroform extraction.

Enzyme reactions. Restriction endonucleases were purchased from BRL (AvaI) or the Institute of Sera and Vaccines, Prague (BamHI, EcoRI, HindIII, PstI). Modified DNAs were linearized and subsequently cleaved with restriction endonucleases in 0.1 mol/l NaCl, 10 mmol/l Tris-HCl buffer (pH 7.5), 10 mmol/l $MgCl_2$ and 1 mmol/l mercaptoethanol.

Electrophoresis. Agarose (Serva) gel (1 %) electrophoresis was performed using 40 mmol/l Tris-acetate buffer (pH 8.0), 2 mmol/l EDTA at ambient temperature (20 h, 2.5 V/cm). Gels were stained in ethidium bromide and photographed in midrange UV light (302 nm).

Results

In pRW751 the 157 bp insert (Fig. 1) was cloned into the BamHI site of pBR322 in such a way that the BamHI recognition sequence (G-G-A-T-C-C) was regenerated at both ends of the insert (Klysik et al. 1982). Thus the BamHI recognition sites lie on the border between the $(dC\text{-}dG)_n$ segments and the pBR322 sequences (the first guanine of the recognition sequence being the last guanine of the $(dC\text{-}dG)_{13}$ segment and the first cytosine of the $(dC\text{-}dG)_{16}$ being the last cytosine of the recognition sequence (Fig. 1)).

Binding of osmium to DNA results in substantial permanent chemical changes as well as local changes in the spatial organization of DNA (Lukášová et al. 1984; Glikin et al. 1984). It may thus be expected that binding of osmium to any thymine or cytosine in the recognition sequence will result in inhibition of the BamHI cleavage.

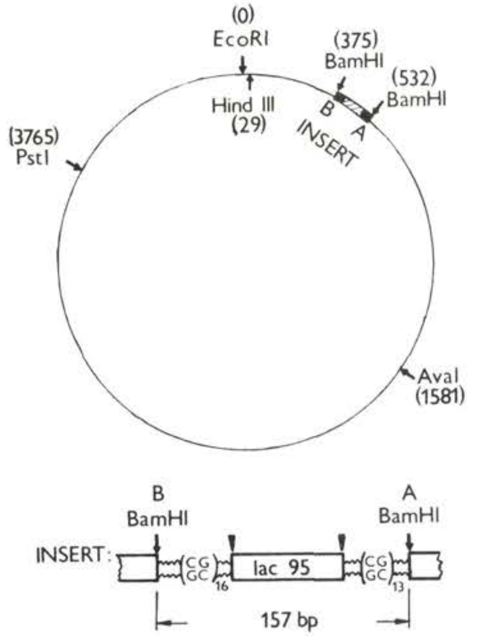

Fig. 1. Map of the plasmid pRW751 (a recombinant 4519 bp plasmid derived from pBR322 which contains a 157-bp insert in the BamHI site; Klysik et al. 1982) showing the relative positions of cleavage sites of restriction endonucleases used in this study. A and B denote the BamHI sites which are referred to in the text; ▼ and ↓ denote the "inner" and "outer" junctions, respectively.

We first tested the effect of low OsO_4, pyridine concentrations on BamHI cleavage of unmodified pRW751 DNA. The presence of 0.025 mmol/l OsO_4, 3 mmol/l (0.025 %) pyridine (i. e. eighty times diluted compared to the most concentrated reaction mixture used in our further experiments) in an environment where enzyme reaction was taking place did not affect DNA cleavage with BamHI (not shown). 0.05 mmol/l OsO_4 with 6 mmol/l pyridine slightly inhibited restriction cleavage. In order to exclude the possibility of an effect on restriction cleavage by the traces of reaction mixture following ethanol precipitation we introduced a further purification step after DNA precipitation, consisting in the extraction of pellets with ether (see Materials and Methods). Using this method the addition of reaction mixture (2 mmol/l OsO_4, 0.25 mol/l/ /2 %/pyridine) into a test tube containing a mixture of DNA with ethanol did not induce any inhibition of restriction cleavage.

Specific inhibition of BamHI cleavage due to treatment with OsO_4 and pyridine

Sample $-\bar{\sigma} = 0.07$ of pRW751 was modified with 2 mmol/l OsO_4 and 0.25 mol/l pyridine 60 min at 26 °C and digested with AvaI, EcoRI, HindIII, PstI and BamHI, respectively, i.e. restriction endonucleases each with the single cleavage site in pBR322. With the exception of BamHI all other restriction endonucleases cleaved the osmium modified DNA in the same way as the unomodified DNA, giving rise to linear full-length molecules (Fig. 2). BamHI inhibition was studied in dependence on the cleavage time in modified and unmodified DNA at $-\bar{\sigma} = 0.07$. Using 11 units of BamHI per µg DNA the unmodified DNA was fully linearized after only 30 min of incubation at 37 °C (not shown), while much of the supercoiled DNA in the modified sample remained uncleaved even after 120 min incubation. In agreement with previous results (Singleton et al. 1983) we observed in unmodified topoisomers with more negative superhelical density clear inhibition of BamHI cleavage. In view of this inhibition a three-step procedure was used to eliminate the influence of supercoiling on BamHI cleavage (Nejedlý et al. 1985): a) chemical modification and purification of DNA, b) linearization of DNA with the restriction endonuclease, which cleaves DNA at one site located far away (more than 300 bp) from the BamHI recognition sequence (e.g. EcoRI; Fig. 1), c) incubation with BamHI restriction endonuclease.

Fig. 2. The effect of osmium tetroxide with pyridine modification of pRW751 DNA on restriction endonuclease cleavage. Supercoiled DNA ($-\bar{\sigma} = 0.06$) was reacted with 2 mmol/l OsO_4 and 0.25 mol/l pyridine (60 min at 26 °C) and digested with BamHI, AvaI, EcoRI, HindIII and PstI, respectively (lanes 2—6). For comparison, lane 1 contains unmodified DNA cleaved with BamHI.

Under the modification conditions used in the previous experiment (Fig. 2), we applied this procedure to study the dependence of the BamHI cleavage (7 units per µg DNA) on the digestion time in the sample $-\bar{\sigma} = 0.02$ (in which left-handed DNA does not form) and $-\bar{\sigma} = 0.07$, containing $(dC\text{-}dG)_n$ blocks in the left-handed form. In the sample $-\bar{\sigma} = 0.02$ the chemical modification did not affect BamHI restriction cleavage: after 10 minutes' cleavage with BamHI only a small portion of the linearized pRW751 DNA remained uncleaved, and after 30 minutes' incubation the cleavage was completed, as in the case of unmodified DNA (not shown). Chemical modification of the sample $-\bar{\sigma} = 0.07$ resulted in a marked inhibition of BamHI cleavage even after 120 min incubation at 37 °C.

Other osmium tetroxide ligands

OsO_4 as a structural probe of superhelical plasmids has been used so far only in combination with pyridine in a concentration range of 0.13—0.63 mol/l pyridine. It cannot, however, be excluded that pyridine at this relatively high concentration may somehow influence the DNA structure. It is known that OsO_4 forms complexes with DNA not only in the presence of pyridine but also in the presence of other ligands (Marzilli 1977). OsO_4 alone (in the absence of any suitable ligand) is substantially less reactive to DNA than OsO_4 with pyridine, yielding mainly the *cis*-5,6-dihydroxy derivative. In an earlier work with linear DNA (Lukášová et al. 1982; Lukášová et al. 1984) we used in addition to pyridine 2,2′-bipyridine (bipy), CN^- or CSN^- as OsO_4 ligands. In this paper we attempted to find out whether application of these ligands in experiments with plasmids containing left-handed DNA may be advantageous.

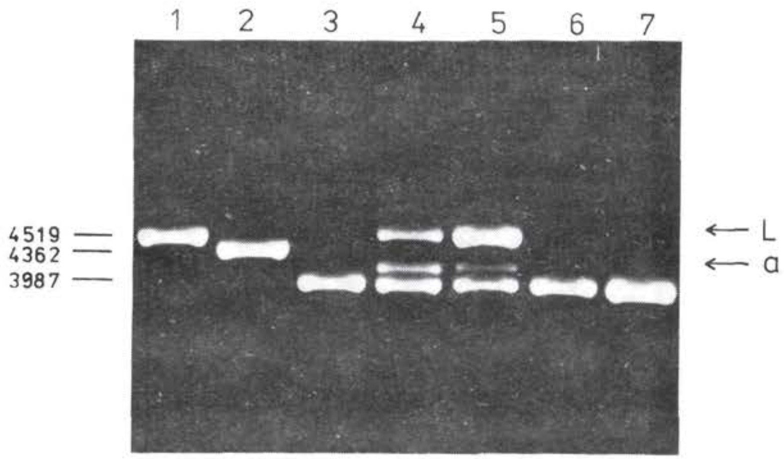

Fig. 3. Inhibition of BamHI cleavage of pRW751 DNA modified with osmium tetroxide in the presence of various ligands. Supercoiled DNA ($-\bar{\sigma} = 0.07$) was reacted with 2 mmol/l OsO_4 (lane 7), plus 0.25 mol/l pyridine (lane 4), 4 mmol/l bipy (lane 5) or 0.2 mol/l thiocyanate (lane 6), respectively, under condition as in Fig. 2, linearized with EcoRI and cleaved with BamHI. For comparison, lanes 1—3 contain unmodified DNA cleaved with EcoRI, BamHI, or EcoRI plus BamHI, respectively. The arrows denote positions of linear DNA (L) and fragment *a*.

The sample $-\bar{\sigma} = 0.07$ was treated with 2 mmol/l OsO_4, 0.25 mol/l pyridine or 4 mmol/l bipy and 0.2 mol/l NH_4CSN respectively, or in the absence of any ligand, for 60 min at 26 °C, and BamHI cleavage inhibition was tested. While the action of OsO_4 with pyridine brought about BamHI cleavage inhibition (Fig. 3, lane 4), OsO_4, CSN^- and OsO_4 on its own did not affect DNA cleavage under the given conditions (Fig. 3, lanes 6, 7). On the other hand treatment of pRW751 with OsO_4 and bipy resulted in a stronger inhibition of BamHI cleavage at both cleavage sites (Fig. 3, lane 5) in the DNA molecule (Fig. 1) than in the case of the reaction with OsO_4 and pyridine (Fig. 3, lane 4). In these two samples in addition to band L a band *a* appeared corresponding to about 4100 bp which

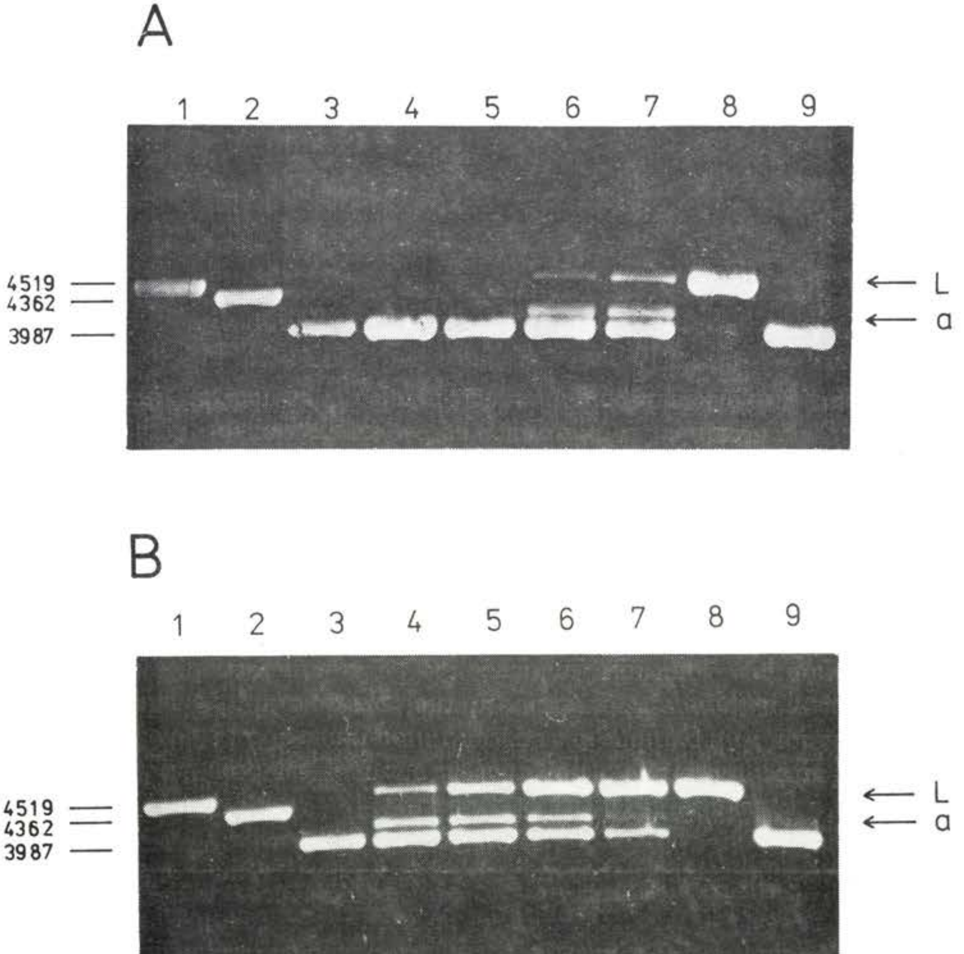

Fig. 4. Inhibition of BamHI cleavage of pRW751 DNA reacted with various concentrations of osmium tetroxide in the presence of pyridine (*A*) or 2,2′-bipyridine (*B*). Supercoiled DNA ($-\bar{\sigma} = 0.07$) was reacted with 0.2 (lane 4), 0.5 (lane 5), 1.0 (lane 6) and 2.0 (lane 7) mmol/l OsO_4 plus either 0.25 mol/l pyridine (*A*) or 4 mmol/l bipy (*B*) under conditions as in Fig. 2, linearized with EcoRI and digested by BamHI. Lane 8 represents DNA modified under the same conditions as in lane 7, cleaved by EcoRI only, lane 9 represents DNA to which 2 mmol/l OsO_4 (plus pyridine resp. bipy) was added in the course of precipitation. For comparison, lanes 1—3 are unmodified DNA cleaved with EcoRI, BamHI, or EcoRI plus BamHI, respectively. The arrows denote positions of linear DNA (L) and fragment *a*.

is consistent with the inhibition of cleavage at $(dC\text{-}dG)_{13}$ segment only (site A, Fig. 1). The greater effectiveness of OsO_4 with bipy was more apparent in an experiment where the dependence of BamHI cleavage inhibition on OsO_4 concentration was investigated at a constant pyridine (0.25 mol/l) or bipy (4 mmol/l) concentration (Fig. 4). When 0.25 mol/l pyridine was used with 0.2 or 0.5 mmol/l OsO_4 there was no band L apparent (Fig. 4*A*, lanes 4, 5). This band only appeared at 1 and 2 mmol/l concentrations of OsO_4 (Fig. 4*A*, lanes 6, 7). On the other hand even with 0.2 mmol/l OsO_4, bipy BamHI cleavage inhibition was induced (Fig. 4*B*, lane 4) comparable with the inhibition brought about by 1—2 mmol/l OsO_4 with pyridine.

Dependence of inhibition of BamHI cleavage on superhelical density

Topoisomeric samples with $-\bar{\sigma}$ in the range from 0.01 to 0.08 were reacted with 2 mmol/l OsO_4 and 4 mmol/l bipy, 60 min at 26 °C, linearized with EcoRI and incubated with BamHI 120 min at 37 °C. Sample $-\bar{\sigma} = 0.01$ and 0.02 showed no influence of this treatment on BamHI cleavage (Fig. 5, lane 4). At $-\bar{\sigma} = 0.06$ and 0.08 a band L of full-lenght linear pRW751 DNA was observed (Fig. 5, lanes 5, 6) corresponding to inhibition of cleavage at both BamHI sites (Fig. 1).

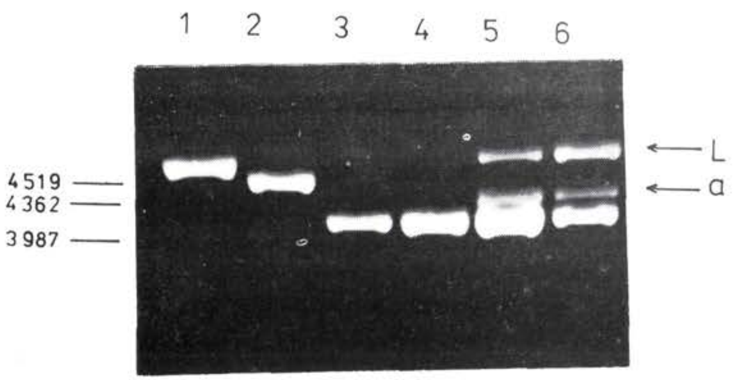

Fig. 5. Inhibition of BamHI cleavage of pRW751 DNA modified with osmium tetroxide and 2,2′-bipyridine at different superhelical densities. Superhelical DNAs with mean superhelical densities of −0.01 (lane 4), 0.06 (lane 5) and 0.08 (lane 6) were reacted with 2 mmol/l OsO_4 and 4 mmol/l bipy under conditions as in Fig. 2, linearized with EcoRI and cleaved with BamHI. For comparison, lanes 1—3 are unmodified DNA cleaved with EcoRI, BamHI or EcoRI plus BamHI, respectively. The arrows denote positions of linear DNA (L) and fragment *a*.

At $-\bar{\sigma} = 0.06$ band L was weaker than in sample with $-\bar{\sigma} = 0.08$. The weaker inhibition of BamHI cleavage at $-\bar{\sigma} = 0.06$ is in good agreement with our previous work (Nejedlý et al. 1985) where nuclease S1 cleavage was used for the detection of osmium binding sites in pRW751, as well as with those obtained with nuclease S1 cleavage of unmodified pRW751 DNA (Singleton et al. 1982) (according to which formation of left-handed DNA in pRW751 begins at about $-\bar{\sigma} = -0.04$ and is completed at $-\bar{\sigma} = 0.07$).

Similarly as in the case of OsO_4 with pyridine (Fig. 2), treatment of pRW751 with 2 mmol/l OsO_4 and bipy did not induce inhibition of cleavage, when other restriction endonuclease were used, i.e. AvaI, EcoRI, HindIII and PstI (not shown).

Chloroacetaldehyde

The osmium binding site is the 5,6 double bond of the pyrimidine ring, i.e. the site which is not involved in the Watson-Crick hydrogen bonding system. Structural distortions responsible for the hypersensitivity of the B-Z junction towards osmium tetroxide (Nejedlý et al. 1985; Galazka et al. 1986; Johnston and Rich 1985, Fig. 1) may thus not include hydrogen bond breakage and formation of single-stranded regions. In order to discover whether on the boundary between the right- and left-handed structures there are some unpaired bases we used chloroacetaldehyde (CAA). CAA reacts with N-1 and 6-aminogroup of adenine and with ring N-3 and 4-aminogroup of cytosine (Kusmierek and Singer 1982a, 1982b), i.e. with atoms involved in the formation of hydrogen bonds. Similarly as with OsO_4 we tested the influence of this agent on the BamHI cleavage of unmodified, linearized pRW751 DNA. No inhibition was observed up to 0.025 mol/l concentration; the presence of CAA at 0.06 mol/l concentration resulted in a strong inhibition of BamHI cleavage. In spite of the fact that CAA influenced the activity of BamHI to a lesser extent than OsO_4 with pyridine and/or bipy, respectively, we used in further experiments the same purification steps as with OsO_4.

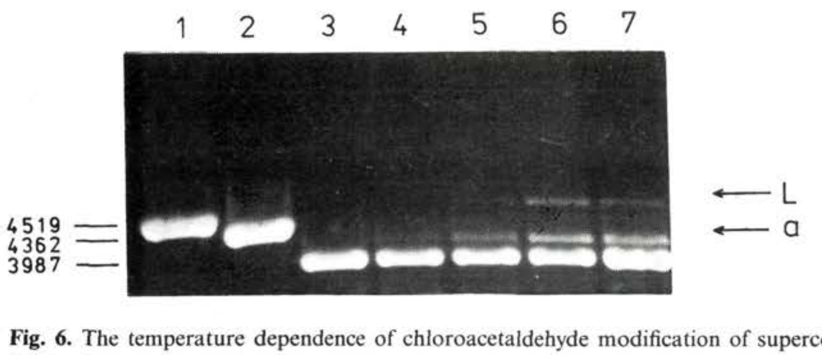

Fig. 6. The temperature dependence of chloroacetaldehyde modification of supercoiled pRW751 DNA. Supercoiled DNA ($-\bar{\sigma} = 0.07$) was reacted with 0.1 mol/l CAA for 1 h (lane 7) or 2 h (lanes 4—6) at 8 (lane 4), 26 (lane 5) or 37 (lanes 6, 7) °C. Modified DNA was linearized with EcoRI and cleaved by BamHI. For comparison, lanes 1—3 are unmodified DNA cleaved with EcoRI, BamHI, or EcoRI plus BamHI, respectively.

With CAA (0.1 mol/l, 60 min at 37 °C) the inhibition of BamHI cleavage was observed in samples with $-\bar{\sigma} = 0.07$ and 0.09 but not in 0.01 (not shown). The inhibition was manifested by band *a* and by band L suggesting inhibition at both BamHI cleavage sites (Fig. 1). Under the same conditions of modification no inhibition of EcoRI cleavage occurred (not shown). With sample $-\bar{\sigma} = 0.07$ the inhibition of BamHI cleavage was observed also after the modification with CAA at 26 and 8 °C (Fig. 6).

Discussion

Chemical probes have been used in the study of supercoiled DNAs for more than ten years. It has been shown that some probes, such as formaldehyde, methyl mercuric hydroxide and carbodiimide react with supercoiled DNA more rapidly than with the relaxed one (Paleček 1976). Until recently, however, it has not been possible to determine exactly the initial site of the reaction of the chemical probe in the supercoiled DNA. The situation changed only a few years ago when it was demonstrated that the reaction sites of bromoacetaldehyde (Lilley 1983; Kohwi-Shigematsu et al. 1983) and osmium tetroxide with pyridine (Lukášová et al. 1984; Glikin et al. 1984; Paleček et al. 1983; Vojtíšková et al. 1983) are recognized and cleaved with nuclease S1; at the same time it was shown that the osmium binding site can be visualized in electron microscope (Glikin et al. 1984). More recently structural analysis of supercoiled DNA has employed further chemical probes such as diethyl pyrocarbonate (Johnston and Rich 1985; Herr 1985), hydroxylamine (Johnston and Rich 1985), glyoxal (Lilley et al. 1985; Gough 1986), $NaHSO_3$ (Gough et al. 1986), etc. The accuracy of mapping of reaction sites has been greatly improved by the application of nucleotide sequencing techniques using hot piperidine instead of nuclease S1 to cleave the polynucleotide chain at the site of the chemical modification (Paleček 1986; Galazka et al. 1986; Johnston and Rich 1985; Herr 1985).

The method based on monitoring of restriction cleavage inhibition used in this work represents a further means of detection of site-specific chemical modification of structural distortions in supercoiled DNAs. A disadvantage of the method is that it requires the recognition sequence to be contained in the region where one expects structural distortion; compared with the method based on the nucleotide sequencing it is less accurate. On the other hand it has certain advantages thanks to which it would seem to offer good prospects. Among these advantages are the fast and simple procedure, the small amount of DNA sample required, and the high sensitivity of analysis.

Inhibition of restriction cleavage at recognition sequences placed in positions near to the B-Z junctions of chemically unmodified plasmids containing

$(dC\text{-}dG)_n$ inserts was studied earlier (Singleton et al. 1983; Azorin et al. 1984). Such an inhibition may be due, however, not only to the structural distortions at the junction but also to nucleotides in the recognition sequence included in the Z form. In contrast to that OsO_4 with pyridine has shown no preference for the Z DNA (Paleček, upublished data) and its site-specific binding to the B-Z junction can be interpreted only in terms of structural distortion. A combination of suitable chemical probes may in addition yield a more detailed picture of the structural distortion than the studies of restriction cleavage of unmodified DNA.

It should be pointed out that the mechanism of DNA cleavage by BamHI is not well understood. We do not know what is the exact size of the DNA segments which interact with the enzyme in addition to the six nucleotides of the recognition site and whether the chemical modification in these segments can result in the inhibition of BamHI cleavage. It can be expected, however, that these segments cannot be very large and that their conformation is of lesser importance for the restriction cleavage than the conformation of the recognition sequence. On the other hand a comparison of the inhibition of the restriction cleavage due to the chemical modification with the modification pattern at the nucleotide resolution may yield in a near future important information about the specific interaction of the restriction endonuclease with DNA.

Of the structural probes which have so far been used for the structural analysis of supercoiled DNAs, OsO_4 is applicable at the lowest molar concentrations. The pyridine concentration (0.13—0.63 mol/l) in reaction mixture is, however, about two orders of magnitude higher than OsO_4 concentration. The results of this work (Fig. 3, 4) show that pyridine can be replaced by bipy, which can be used in approximately the same concentration as OsO_4; moreover the substitution of bipy for pyridine makes it possible to work with considerably lower OsO_4 concentrations, as indicated by our results obtained in a study of BamHI inhibition in pRW751 (Fig. 4*A*, *B*). The better stability of DNA-OsO_4-bipy complex compared with the DNA-OsO_4-pyridine one has already been shown (Chang et al. 1977) in work with linear DNA and synthetic polynucleotides. The OsO_4-bipy complex is thus the first chemical probe of local changes in the conformation in supercoiled DNA which can be applied at submillimolar concentrations (Boublíková, unpublished data). The low concentrations of the reagents could be particularly advantageous in attempts to analyze DNA structure in vivo.

On the basis of a study of inhibition of BamHI cleavage in pRW751 it has been shown in this work that, in accordance with our previous results (Nejedlý et al. 1985), both "outer" junctions (Fig. 1) contain structural distortions which are site-specifically modified with osmium tetroxide if the $(dC\text{-}dG)_n$ segments are in left-handed form. These chemically modified regions are not, however, preferentially cleaved by S1 nuclease (Nejedlý et al. 1986) as in the case of "inner" junctions in the same plasmid. As follows from our experiments with pRW777 (Galazka et al. 1986) and pKK1 (Galazka et al. 1987), in which the site-specific osmium modification was studied using the sequencing technique, a condition of recognizing the osmium-modified site by the nuclease S1 is the modification of the group of nucleotides in both strands in close proximity. Where, for example, few thymines of the 5′-strand were modified, while modification in the opposite 3′-strand did not occur, the nuclease S1 did not cleave the modified site. Data on osmium modification of pRW751 plasmid on the level of nucleotide resolution are not yet available, but it is probable that the inhibition of BamHI cleavage observed is conditioned by chemical modification of a smaller number of nucleotides, not sufficient for recognition by nuclease S1, but sufficient for inhibition of restriction cleavage.

We have shown that in addition to osmium tetroxide (Fig. 2—5) modification of pRW751 with CAA also results in the inhibition of the BamHI cleavage (Fig. 6) provided the superhelical density is sufficiently negative to stabilize the $(dC\text{-}dG)_n$ segments in the left-handed form. CAA has been applied in this work for the first time to study local site-specific structural changes in a supercoiled plasmid but its analog bromoacetaldehyde has been used for the same purpose (Lilley 1983; Lilley 1984; Kohwi-Shigematsu et al. 1983) including the study of the B-Z junctions of plasmids pRW756 and pRW777 (Kang and Wells 1985). In the latter study nuclease S1 was used to recognize and cleave the DNA chains the sites of the chemical modification and no signs of site-specific modification of the B-Z junction were detected. As mentioned above nuclease S1 may not always recognize the osmium modification in the polynucleotide chain. In the light of our recent results (Galazka et al. 1986; Galazka et al. 1987) the data obtained with bromoacetaldehyde (Kang and Wells 1985) cannot thus be unambiguously interpreted in terms of absence of the bromoacetaldehyde reaction with the bases contained in B-Z junctions, as it can be hardly excluded that limitations in recognition of osmium-modified bases by nuclease S1 apply to some extent also for bases modified by bromoacetaldehyde.

We have demonstrated that CAA selectively modifies the B-Z junction in pRW751 (Fig. 6). Similar results were obtained with glyoxal (Paleček, unpublished data), i.e. another agent which requires unpaired bases for the reaction in the double helix with Watson-Crick base pairs. Do our results mean that some non-paired bases or non-Watson-Crick base pairs exist in the B-Z junction region? Taking in consideration that the results of glyoxal and bromoacetaldehyde modification of the cruciform structures (Lilley et al. 1985; Gough 1986) are in a good agreement with those obtained by other methods, together with the fact that the observed inhibition of the BamHI cleavage occurred also after modification with CAA at temperatures lower (8 and 26 °C, Fig. 6) than that used in cruciform studies (37 °C) we assume that our results reflect primary changes in the DNA structure. We thus believe that on the basis of our results we may conclude that the B-Z junction regions in pRW751 contain bases with disturbed hydrogen bonding or non-Watson-Crick base pairs at physiological superhelical densities.

To get a better insight into the structure of the B-Z junction, further work including the employment of other means of detection of the site-specific chemical modification of the B-Z junction, will be necessary. At this stage we do not find it appropriate to make any general conclusions concerning the presence or absence of non-paired bases in the B-Z junction. Considering the polymorphy of the DNA double helix we conclude that the B-Z junction might be nonuniform, its exact structure being dependent on the nucleotide sequence (including the flanking sequences), on superhelical density and possibly, also on environmental conditions.

Acknowledgements. The authors wish to express their gratitude to Professor R. D. Wells for his kind permission to use the recombinant plasmid pRW751 constructed in his laboratory.

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Final version accepted February 17, 1987