OsO₄ in the presence of pyridine specifically modifies the structural distortions of the primary helix of supercoiled pRW777 near the (dA-dC)₃₂ sequence. Modification occurs at the same negative superhelix density value as required for formation of the Z-helix within the polymer block. Fine mapping of the distorted regions, which are probably the B-Z junctions, is presented. OsO₄ reactions provide a powerful and sensitive chemical approach to study DNA polymorphism in solution.

The complex nature of double-stranded DNA structures has attracted much attention in recent years. A large body of information has been obtained about the left-handed helix. Crystals of short deoxynucleotides composed of perfectly alternating guanine and cytosine residues were obtained, their structure was solved by x-ray methods, and the unusual Z-conformation was described (reviewed in Ref. 1). Other physiological methods like NMR, CD, and Raman spectroscopy (reviewed in Refs. 1-4) were also used to study the left-handed conformation of DNA in solution. The demonstration that the Z-type structure can co-exist in close proximity to the B-type helix in recombinant molecules and restriction fragments (2-6) represents the most striking example of the DNA polymorphism.

A number of factors are able to induce the Z-type helix within (dC-dG), or (dA-dC), sequences; the most powerful seems to be the free energy of negative supercoiling (2, 5, 6). Left-handed segments within the supercoiled molecules were detected by antibody-binding studies (7-10), supercoil relaxation studies (2, 5-13) and by using enzymic probes like S1 nuclease (5, 9, 10), BstI nuclease (12), and HhaI methylase (13, 14).

It has been shown that OsO₄ can be used as a site-specific chemical probe for distortions in the DNA helix caused by negative supercoiling (15-17). In the presence of the suitable ligand such as pyridine, osmium tetroxide adds to the C5-C6 double bond of the pyrimidine rings. Large modified regions on the linear molecules can be observed in the electron microscope as a denaturation bubble or can be specifically cleaved by the single strand specific nuclease S1 (15-17). We have recently demonstrated that OsO₄ specifically modifies the distorted regions caused by Z-DNA formation within the (dC-dG)ₙ and (dG-dC)ₙ blocks of the recombinant plasmid pRW751 (18).

In this paper, we present evidence that flipping the (dA-dC)₃₂ block from the B-Z conformation in the recombinant plasmid pRW777 (19) is associated with the appearance of a structural aberration of the primary helix near the (dA-dC)₃₂ block that can be specifically modified by OsO₄.

MATERIALS AND METHODS

Plasmid DNA—The recombinant plasmid pRW777 was prepared as previously described (19). It is a pBR322 derivative containing a 106-bp fragment from the mouse variable region gene cloned into the HindIII site (Fig. 1). Sequencing of the insert of the plasmid used in this study revealed that the (dA-dC) block is actually 6 bp longer than reported originally by Nishioka and Leder (20). The full sequence is shown in Fig. 5. The source of the discrepancy between the previously reported length of the (dA-dC) block and that found within the polymer block is not clear, but may be related to the recombinant events that occurred in the Escherichia coli host cells used to grow up and maintain the plasmid. For the sake of clarity and consistency with the previous work (9, 19, 20), we will designate the polymer sequence of pRW777 in the same way as shown previously, that is (dA-dC)₃₂. Topoisomeric samples of pRW777 with defined mean superhelical densities were obtained according to Singleton and Wells (21).

Modification Reaction with OsO₄—Unless stated otherwise, the reaction was performed with 10 μg of DNA in 2% pyridine, 25 mM Tris-HCl buffer, pH 8.0, 2.5 mM EDTA, 0.2 mM NaCl, and 1 mM OsO₄ to a final reaction volume of 100 μl. After 15 min at 25°C, the modification reaction was stopped by chloroform extraction, and DNA was precipitated with ethanol and dissolved in 10 μl of distilled water.

Enzyme Reactions—OsO₄-modified DNA was digested to completion with BstI (an isoschizomer of BamHI, Pharmacia L-Biochemicals, or SfiI, Department of Genetics, Warsaw University), extracted with phenol and ether, precipitated with ethanol, and dissolved in 10 μl of distilled water.

Acrylamide Gel Electrophoresis—Electrophoresis was performed on 3% acrylamide gels using 40 mM Tris-HCl buffer, pH 8.3, 20 mM sodium acetate, and 2 mM EDTA, at ambient temperature. Gels were stained with ethidium bromide and photographed under the UV light (2, 5, 22).

The abbreviation used is: bp, base pair.
was recovered, dissolved in 30 μl of 1 M piperidine, and incubated at 90 °C for 30 min. Piperidine was evaporated under vacuum and the DNA was dissolved in formamide and loaded on the sequencing gel next to the Maxam and Gilbert sequencing reactions (22) of the unreacted fragment. Mapping of the OsO₄-modified thymine residues on the 3' strand within the EcoRI-SalI fragment was done in a similar way, except for the polynucleotide kinase step which was replaced by a "filling in" reaction (24) using the Klenow fragment of E. coli DNA polymerase, TTP, and [α-³²P]dATP.

**RESULTS**

In order to visualize the site-specific modification of pRW777 with osmium tetroxide in the presence of pyridine as a ligand, we used an assay similar to that which was applied to observe the selective modification of the cruciform structures in CoIE1 plasmid (17, 18). It was shown previously that the OsO₄-modified regions in relaxed or linear molecules are sensitive to S1 nuclease (16, 17). Therefore, after gel electrophoresis, it is possible to detect specific bands in the digestion products which are limited by a restriction site on one end and the S1-sensitive site on the other end. The length of the band(s) enable localization to the osmium tetroxide modification spot(s) on the double-stranded and supercoiled molecule. Thus, the modified DNA was cleaved with an appropriate restriction enzyme to obtain linear molecules, digested with S1 nuclease, and the resulting products were separated on acrylamide gels.

OsO₄ Modification at One B-Z Junction Is Correlated with the Superoxo-dependent Z-DNA Formation within the (dA-dC)₁₂ Block of pRW777—Fig. 1 shows the restriction map of pRW777 (19) which contains the tract of (dA-dC)₁₂ originally found in the mouse immunoglobulin gene (20). The perfectly alternating (dA-dC)₁₂ block in this plasmid was shown previously to adopt a left-handed conformation after N-acetoxy-N-acetyl-2-aminofluorene modification (19) or under the torsional strain of negative supercoiling (9). A titratable negative superhelix density of about 0.06 was found to be sufficient to stabilize the left-handed helical state of (dA-dC)₁₂ as revealed by S1 nuclease, antibody binding, and supercoil relaxation studies (9).

Fig. 2A shows 2% agarose gel electrophoretic analyses of the topoisomeric samples of pRW777 prepared according to Singleton and Wells (21). It is possible to count individual topoisomers in the samples run in lanes 1–7. The observed mean −Δ values of these samples were in perfect agreement with those expected. The slower migration of the topoisomeric samples shown in lanes 8–10 may represent the expected relaxation due to the Z-helix formation within the (dA-dC)₁₂ block in good agreement with previous studies (9).

Fig. 2B shows nuclease digestion products of S1I followed by S1 nuclease treatment of the OsO₄-modified topoisomeric samples of pRW777 separated on a 5% acrylamide gel. Samples of the modified DNA after restriction with S1I were carefully analyzed on a 1% agarose gel to make sure that complete digestion was achieved (data not shown). The subsequent cleavage with S1 nuclease and the gel electrophoresis revealed the presence of a specific band (lanes 1–6) whose length was estimated to be about 620 bp. This band is not present in lanes 1–3 where the −Δ value of the substrate DNAs was relatively low (0.009, 0.037, and 0.064, respectively). Therefore, we conclude that negative supercoiling is able to induce a change in the structure of the double helix which is sensitive to OsO₄ modification, and that the modified region is sensitive to S1 nuclease after the DNA is linearized by S1I.

The supercoiled-dependent OsO₄-modified region maps 620 bp away from the S1I site or 355 bp from BstI site (Fig. 3). Thus, it must be located very close to the alternating (dA-dC)₁₂ tract (Fig. 1). In a control experiment, we demonstrated that no modification occurs under identical conditions on pBR322 DNA (data not shown). As stated above, the B-Z transition of the (dA-dC)₁₂ block occurs in pRW777 at −Δ around 0.06 (9). The site-specific OsO₄ modification in our experiment is not yet observed at −Δ = 0.054 (Fig. 2B, lane 3), but is clearly detected at −Δ = 0.072 and correlates well with the formation of a Z-helix within the (dA-dC)₁₂ sequence. We think that the change in the structure of pRW777 specifically recognized by OsO₄ is mediated by the B-Z transition within the (dA-dC)₁₂ block and is likely to be the junction region between the left-handed and right-handed helices.

Close inspection of Fig. 2B revealed the presence of other intense and longer bands than the 620-bp fragments indicating that other OsO₄-sensitive and supercoil-dependent structural distortions occur in the double helix of the plasmid. The very intense band whose length is about 2400 bp may correspond to the OsO₄-modified sites located at the major, minor, and subminor cruciforms (reviewed in Ref. 25) on the pBR322 vector. Unfortunately, resolution by the acrylamide gel in that size range does not permit a precise localization of the sites.

**Concentration Dependence of the OsO₄ Modification—Site-specific and Covalent Binding of OsO₄ to Double-stranded DNA**

is known to depend on the concentration of this chemical in the reaction mixture and the modification time (15–17). Under certain conditions, it is possible to observe the modified regions in the electron microscope as a denaturation bubble (16). The size of the bubble is correlated with the extent of modification. It is believed that the very first steps of the reaction are site-specific and depend on local distortions of the double helix structure. Initially modified regions become more sensitive to further modification, as compared with the rest of the molecule, even after the molecule is relaxed or linearized, and may result in strand separation which is detectable over relatively long distances. The precise mapping of the OsO₄-supersensitive structural aberrations by S1 nuclease cleavage may, therefore, depend on the extent of modification of the plasmid. We performed systematic studies in order to correlate the extent of modification at the B-Z junction region and its S1 nuclease sensitivity.

Fig. 3 shows topoisomeric samples of pRW777 (−Δ = 0.091) modified under standard conditions with increasing concentrations of OsO₄, linearized with BstI, cleaved with S1 nuclease, and electrophoresed on a 3% acrylamide gel. It can be seen that the intensity of the S1/BstI 335-bp band increases
as the OsO$_4$ concentration in the modification mixture is raised. No substantial change in the length of this band was detected. Therefore, we conclude that, under the reaction conditions used, the length of the modified sequence is very short and does not depend sharply on OsO$_4$ concentration. The increase in S1 nuclease sensitivity, however, is a function of the extent of OsO$_4$ modification.

**Mapping of OsO$_4$-modified Loci Depends on the Digestion Time with S1 Nuclease**—Fig. 4 shows that the electrophoretically separated products of pRW777 ($\bar{c} = 0.091$) reacted with OsO$_4$ under standard conditions, linearized with BstI, and treated with S1 nuclease for time periods up to 20 min. The length of the specific S1/BstI band, which is formed due to the OsO$_4$ modification near one end of the Z-block, is significantly reduced as the S1 nuclease digestion time increases. Prolongation of the S1 digestion time from 2 min to 20 min resulted in shortening of the fragment from 344 to 300 bp. Substantial broadening of this band was also observed at longer incubation times. Thus, precise mapping of the supercoil-induced OsO$_4$-sensitive structural aberration near the (dA-dC)$_{32}$ block in the Z-form greatly depends on the S1 nuclease digestion time.

**DNA Sequencing of the OsO$_4$-modified Thymine Residues at the B-Z Junctions**—The very unexpected feature of the results described thus far is that only one B-Z junction, proximal to the HindIII site, is recognized by S1 nuclease due to the OsO$_4$ modification, whereas the other junction is not. Clearly, two B-Z junctions must be present on either side of the (dA-dC)$_{32}$ block in the left-handed state. These results raise the following important questions. Is the second B-Z interface, proximal to the EcoRI site, insensitive to OsO$_4$ modification or is it modified, but remains insensitive to S1 nuclease digestion? To answer this question, we searched for a direct determination of the modified sites on both strands of the EcoRI-HindIII segment of pRW777.

It was reported that OsO$_4$-reacted pyrimidines, in the absence of pyridine, are selectively oxidized and degraded (26, 27) permitting the DNA chain cleavage. Data concerning the possible chemical degradation of the polynucleotide strand containing bis(pyridine)-osmate esters of thymine and cytosine are not available. In alkaline media the bis(pyridine)-osmate ester of thymine may be converted into thymine glycol (29, 33), i.e. the same product as that formed due to the treatment of DNA by OsO$_4$ alone. It might be possible to selectively degrade the DNA strand at the OsO$_4$-modified thymine nucleotides by treatment of the DNA with piperidine according to the protocol developed by Maxam and Gilbert (22), which is widely used for DNA sequencing.

A sample of pRW777 ($\bar{c} = 0.091$) was reacted with OsO$_4$, under standard conditions, cleaved with EcoRI, and the 5' end of the DNA was sequenced by the method of Maxam and Gilbert (22) using the following restriction enzyme digests: HindIII, EcoRI, HindIII + EcoRI, and PvuII. No substantial change in the length of this band was detected. Therefore, we conclude that under the reaction conditions used, the length of the modified sequence is very short and does not depend sharply on OsO$_4$ concentration. The increase in S1 nuclease sensitivity, however, is a function of the extent of OsO$_4$ modification.

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A sample of pRW777 ($\bar{c} = 0.091$) was reacted with OsO$_4$, under standard conditions, cleaved with EcoRI, and the 5'
strand was labeled using \([\gamma-^{32}P]ATP\) and polynucleotide kinase. The plasmid was then digested with Sall, electrophoresed on a 3% acrylamide gel, and the EcoRI-Sall fragment was extracted and reacted with 1 M piperidine at 90 °C for 30 min. The resulting products were separated on a sequencing gel next to the sequencing reactions of the same fragment prepared identically from the Os04-untreated plasmid (23). This experiment allowed us to map Os04-modified nucleotides around both B-Z junctions of the \((\text{dA-dC})_{32}\) block on the 5′ strand. In a similar approach, the Os04 modification sites were mapped on the 3′ strand since the modified plasmid after linearization with EcoRI was labeled with \([\alpha-^{32}P]dATP\) and the Klenow fragment of DNA polymerase in the presence of dTTP. The combined results of both experiments are presented in Fig. 5. It is clear that both junction regions located around the \((\text{dA-dC})_{32}\) segment were modified in the supercoiled plasmid and the modification was detected by piperidine treatment.

Similar mapping studies were performed with pRW777 at a lower supercoil density (−\(\sigma = 0.065\)). The observed results were identical with those presented in Fig. 5. In addition, the pattern of the chemical cleavage of the Os04-pyridine-modified 3′ strand was confirmed by labeling the BstNI (an isoschisomer of EcoRII) site located 99 bp away from HindIII site on the vector sequence with polynucleotide kinase and \([\gamma-^{32}P]ATP\). The EcoRI-BstNI fragment excised from the modified and supercoiled pRW777 (−\(\sigma = 0.065\)) was degraded with piperidine and run on the sequencing gel. The positions of the modified thymines as well as the relative intensities of the bands on the sequencing gel were in perfect agreement with those shown on Fig. 5. No specific degradation was detected when pRW777 at a supercoil density (−\(\sigma = 0.037\)) below the B-Z transition was used.

Only thymine residues were found to undergo modification with Os04, pyridine detectable by piperidine treatment. This may be explained if we assume that (a) only thymine residues are modified under the given experimental conditions or (b) both thymine and cytosine residues are modified, but the polynucleotide strand is cleaved only at thymine sites. Our preliminary results suggest the latter explanation as a more probable one. To check the possibility whether the DNA chain cleavage was obtained due to the formation of pyridine-osmate esters or due to the modification occurring with Os04 alone (although the DNA samples were always treated with Os04, pyridine reagent), we performed a control experiment. We modified topoisomeric samples of pRW777 (−\(\sigma = 0.072\)) as described under “Materials and Methods” except that pyridine was omitted. After modification, the DNA was cleaved with EcoRI, labeled with \([\alpha-^{32}P]dATP\) and dTTP using the Klenow fragment of DNA polymerase, and redigested with Sall. The EcoRI-Sall fragment, after extraction from the acrylamide gel, was incubated with piperidine at 90 °C for 30 min. This sample was run on a sequencing gel next to the Maxam and Gilbert sequencing reactions of the same, but Os04-unreacted, fragment (otherwise prepared in the same way). The same sequencing gel also contained the sample of the Os04-untreated EcoRI-Sall fragment which was incubated with piperidine. As expected, no specific cleavage due to the piperidine treatment was observed when pRW777 was treated with Os04 without pyridine. Traces of degradation were detected, however, at the G residues. This degradation background corresponded to the identical background result-
Os04 Reaction at B-Z DNA Junctions

The structure of the B-Z junction is not well understood at present. However, it is recognized by S1 nuclease (5, 9, 10, 34) and is energetically unfavorable (2, 6, 11, 34, 35). These and other studies (NMR, Raman spectroscopy, and supercoil relaxation determinations) indicate that the structural aberration is only a few base pairs in length (2, 11, 34–36).

In this paper, we demonstrate that formation of the left-handed structure within the (da-dc)32 block of pRW777 is correlated with formation of Os04-sensitive distortions within the primary helix of the supercoiled plasmid near or at the junction regions. The chemically modified junctions revealed an unusual sensitivity toward S1 nuclease. One Os04-modified B-Z region was found to be sensitive toward S1 nuclease, whereas the other one was not. Similar observations were made previously (18) using pRW751 which contains blocks of (dc-dc)3 and (dg-dc)13 (22). These two blocks in the left-handed state were flanked by four B-Z junctions, but only two of them were found to be sensitive to S1 nuclease digestion after the Os04-pyridine reaction. This behavior may be explained by the different sensitivity of the B-Z interfaces toward the chemical used.

It was possible to map the osmium-reacted bases by piperidine cleavage of the modified DNA chain (Fig. 5). We found that both B-Z junctions around the (da-dc)32 block of pRW777 were chemically modified. The region proximal to the HindIII site and sensitive to S1 nuclease after modification was Os04-reacted on both strands, whereas the other junction was extensively modified on the 3' strand only. This difference in the modification pattern represents the most probable explanation of the different sensitivity of the modified B-Z junctions described herein.

These observations, combined with the fact that thymine residues exclusively were found to be reactive toward Os04, make it possible to speculate that S1 sensitivity of the modified B-Z junctions may depend on their sequence, that is the number of thymines present within the junction area as well as their location on both or just on one strand.

Detection of the Os04-modified junction by S1 nuclease cleavage was correlated with the supercoil-induced Z-helix near the (da-dc)32 sequence. Modification detected by piperidine cleavage was also supercoil-related since we were able to demonstrate it at the \(-\bar{\omega} = 0.091\) and 0.065 but not at \(-\bar{\omega} = 0.037\). Thus, Os04 is the first sensitive and specific chemical probe that can be used to detect the structural distortions of the double helix accompanying the formation of the Z-segment within the B-type sequences. We propose that the very initial hits of osmium tetroxide are at the most distorted thymines of the B-Z interface. While this contribution was in the final stages of preparation, a paper appeared (37) which also indicates that Os04 may be used to detect B-Z junctions.

Os04 is known to be very reactive toward single-stranded but not double-stranded DNA (17, 38). It also specifically modifies cruciform structures stabilized by supercoiling (17) which are known to be sensitive to single-stranded nucleases (39–41 and reviewed in Refs. 25 and 42). Our data presented in this paper and those obtained with pRW751 (18) indicate that both Os04 and single strand-specific enzymes may recognize similar but not identical features, such as local helical distortions and/or alterations in hydrogen bonds.

Bromosacetaldehyde has been used recently as a probe to study the conformation of the B-Z junctions within supercoiled plasmids (43). In contrast to the results described herein, modification occurred only at very high \(-\bar{\omega}\) values, substantially higher than required to form the left-handed helices. Thus, it was concluded (43) that B-Z junctions contain few, if any, unpaired bases at physiological superhelical densities but that the B-Z interfaces could be "pried open" at much higher densities, consistent with prior conclusions (5, 9, 10, 34, 40). Our results described herein are consistent with prior studies since:

(a) The precise structure required for the conformation-dependent Os04 modification of the pyrimidine rings within the double helix is uncertain. In fact, the B-Z interface and cruciforms are the only well defined aberrations of the primary helix within supercoiled plasmids that are sensitive to this chemical.

(b) The B-Z interface and cruciforms are not the only modified loci found in pRW777 in our studies. A clear example is shown on Fig. 5 where thymine residues in position 21 on the 3' strand and in position 26 on the opposite strand were...
found to be Os42-sensitive. Inspection of the sequence in this area does not indicate any obvious reason for this behavior.

(c) The mechanisms by which Os42 and bromoacetaldehyde react at the structural aberrations within the double helix are not the same. Os42 plus pyridine reacts with the 5,6-double bond of C and T (nonpairing positions), whereas bromoacetaldehyde reacts with sites on A and C which are involved in hydrogen bonding.

The chemical approach described in this paper offers a very powerful and sensitive tool to study the supercoil-stabilized structural aberrations of the primary helix. In contrast to enzymic approaches, the reaction is selective for pyrimidine rings (26, 33, 44, 45) and does not result in cleavage of the DNA chain, which results in a rapid relaxation of the supercoiled DNA. Thus, several distorted regions can be modified by Os42 at the same time on the same molecule. It is also important that this chemical modification is not limited to the specific conditions which are required in enzymic reactions. Thus, our work offers a valuable alternative to other assays used so far in studying DNA polymorphism in solution.

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