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Diethyl pyrocarbonate: a chemical probe for DNA cruciforms

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ABSTRACT

Two palindromic DNA sequences were analyzed with respect to their chemical reactivities with diethyl pyrocarbonate. In negatively supercoiled plasmid templates enhanced N7 carbethoxylation was found with individual purines located in presumptive single-stranded loops of DNA cruciform structures. No enhanced reactivity at these positions was observed in linear, relaxed or low superhelical density plasmids. Hyperreactivity was found over a narrow region only, indicating that stable cruciforms contain loops of minimal size. No enhanced chemical reactivity was found with the four-way junction at the base of cruciforms. Diethyl pyrocarbonate has proved a sensitive structural probe for the analysis, with single nucleotide resolution, of DNA cruciform structures.

INTRODUCTION

Conformational transitions of the DNA double-helix that lead to a local reduction in helical twist are energetically favored in negatively supercoiled, circular DNA molecules (1). Such altered DNA conformations include melted or single-stranded DNA segments, slippage structures, left-handed Z-DNA, and DNA cruciforms. The nucleotide composition of a particular DNA segment exhibiting conformational flexibility greatly determines which of these alterations in structure will be stabilized. Palindromic DNA sequences (or inverted repeats) can undergo a supercoil induced conversion of interstrand base-pairing to intrastrand base-pairing, which results in the formation of DNA cruciform structures (2-4). The three structural components that build up a DNA cruciform include the four-way junction, the stem segment and the single-stranded loop (see Figures 1,4). A variety of approaches to identify cruciforms have been developed including physical (2,5-9), enzymatic (3,4,7,10-14) and chemical (15-17)

protocols. Single-strand specific probes have allowed identification of cruciform loop regions (3,4,15-17) and the four-way junction is recognized by T4 endonuclease VII (13).

In order to identify DNA conformational changes at the nucleotide level, chemical modification followed by strand scission at the modified sites has been applied successfully. The experiments of Herr (18) have demonstrated that DNA segments capable of forming left-handed Z-DNA exhibited an increased reactivity to diethyl pyrocarbonate in a supercoil dependent fashion. This selective hyperreactivity was used to develop a chemical footprinting method to characterize binding of proteins to Z-DNA (19). Johnston and Rich (20) showed that in addition to diethyl pyrocarbonate the chemicals dimethylsulphate and osmium tetroxide detect DNA conformational changes in the vicinity of Z-DNA segments.

Diethyl pyrocarbonate carbethoxylates purines at the N7 position. In double-stranded DNA this chemical has shown high reactivity with the left-handed Z-form and reduced, but significant reactivity with right-handed B-form (18-20). In RNA, however, purines were found to be reactive with diethyl pyrocarbonate only if they assumed a single-stranded but not a double-stranded conformation (21). These observations led us to test whether diethyl pyrocarbonate could be used as a probe for single-stranded, nonpaired regions within a DNA segment. The experiments presented here illustrate that diethyl pyrocarbonate can be used as a probe to detect unpaired bases in the loops of DNA cruciforms. No reactivity could be detected in the region of the four-way junction at the base of the cruciform stem. Our experiments demonstrate diethyl pyrocarbonate to be a specific probe for the identification and characterization of DNA cruciform extrusion.

MATERIALS AND METHODS

Plasmids

Plasmid pColIR515 (12) (kindly provided by D. Lilley, Dundee University) is a deletion derivative of pBR322 which carries between its EcoRI and BamHI sites a 442 bp ColE1 fragment containing the 13 bp long inverted repeat sequence shown in Figure 1.

Plasmid pPS11 was constructed by ligating a 70 bp long HindIII fragment composed of the perfect palindromic sequence shown in Figure 4 into the polylinker of the pUC18 cloning vector (22). The palindrome represents the original EcoRI-HindIII fragment of pBR322 in head-to-head orientation and was obtained by HindIII-excision from the plasmid pHD127-16 (23) (kindly provided by H.-J. Fritz, München). Plasmid pPS11 was found to be genetically somewhat unstable in that recombination derivatives tended to occur in DNA preparations of this plasmid (as seen with the identical palindrome in (7)). The cloning steps were performed according to standard procedures (24).

Plasmid DNAs were prepared according to standard procedures (24) employing Triton X-100 lysis and two CsCl/ethidium bromide equilibrium gradient centrifugations. Amplification of plasmid copy numbers by treatment of bacteria with chloramphenicol was avoided.

Generation of topoisomeric plasmid preparations.

pColIR515 plasmid preparations of different superhelical densities were generated by treatment with calf thymus DNA topoisomerase I (a gift of M. Darby and H.-P. Vosberg, MPI Heidelberg) in the presence of different amounts of ethidium bromide as described (25). The superhelical densities obtained were determined on agarose gels containing chloroquine (26).

Chemical DNA modification with diethyl pyrocarbonate.

For chemical modification of plasmid DNAs with diethyl pyrocarbonate, the protocol of Herr (18) was closely followed. Plamids were linearized with EcoRI (pColIR515) or BamHI (pPS11) either before or after chemical DNA modification, depending on the desired topology of the DNA substrate. To 1.5 ug DNA in 200 ul reaction mixture (10 mM Tris-HCl, pH=8, 60 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA) 3 ul diethyl pyrocarbonate (Kodak) were added, vortexed and incubated for 20 min. at 20°C. Chemical modification was terminated by DNA precipitation from ethanol. Chemically modified and linearized plasmids were ^{32}P-endlabeled as indicated in the figure legends and cleaved with a second restriction enzyme (BamHI/pColIR515, HaeIII/pPS11) to generate uniquely endlabeled fragments. These fragments with length of 442 bp (from pColIR515) and 102 bp (from pPS11) were separated electropho-

retically and isolated by electroelution (the purification of the 102 bp fragment of pPS11 also eliminated contaminating fragments from pPS11-generated plasmid recombi-nation derivatives). Subsequent backbone cleavage with piperidine and denaturing gel electrophoresis was as described (18). Chemical DNA sequencing reactions were performed as published (27). General protocols were used for restriction cleavage and ^{32}P-endlabeling (24).

RESULTS

Two types of palindrome, differing in nucleotide sequence, length and symmetry characteristics, were analyzed in this study. The shorter palindrome of plasmid pColIR515 (12) (Figure 1) contains a central AAATG sequence which is not part of the symmetry element. After cruciform extrusion this 5 bp sequence is not expected to form intrastrand base pairing. The longer palindrome of plasmid pPS11 (Figure 4) is of perfect symmetry, not containing a central interruption of the inverted repeat sequences.

Reactivity of diethyl pyrocarbonate with the palindromic region of plasmid pColIR515.

To study the reactivity of diethyl pyrocarbonate with the different structural components of a DNA cruciform the plasmid pColIR515, containing a 13 bp inverted repeat sequence which readily extrudes the cruciform upon negative supercoiling (12, 14), was investigated.

Plasmid pColIR515, in either negatively supercoiled or linear form, was treated with diethyl pyrocarbonate, cleaved with piperidine at the modified positions, and the degree of chemical modification of individual purines was analyzed across the region of inverted repetition on both DNA strands. Figure 2 shows that a general and low reactivity with diethyl pyrocarbonate was found on both DNA strands with most purines of both linear and supercoiled DNA (Figure 2, lanes 5, 6, 9 and 10). However, enhanced reactivity was found at selected purines on supercoiled but not linear templates. These sites of hyperreactivity include positions A15u and A16u on the upper strand (Figure 2, lane 5) as well as position A17l on the lower strand (Figure 2, lane 9). Reactivity above background levels, although significantly lower than those found with above listed residues, was also seen with

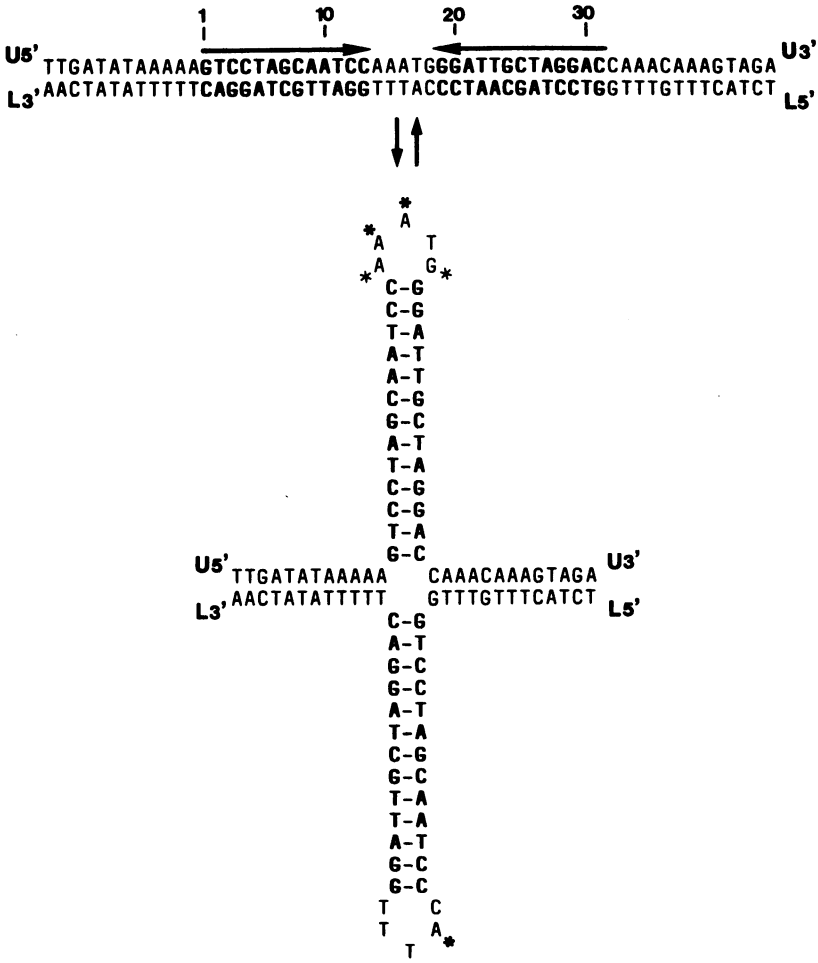
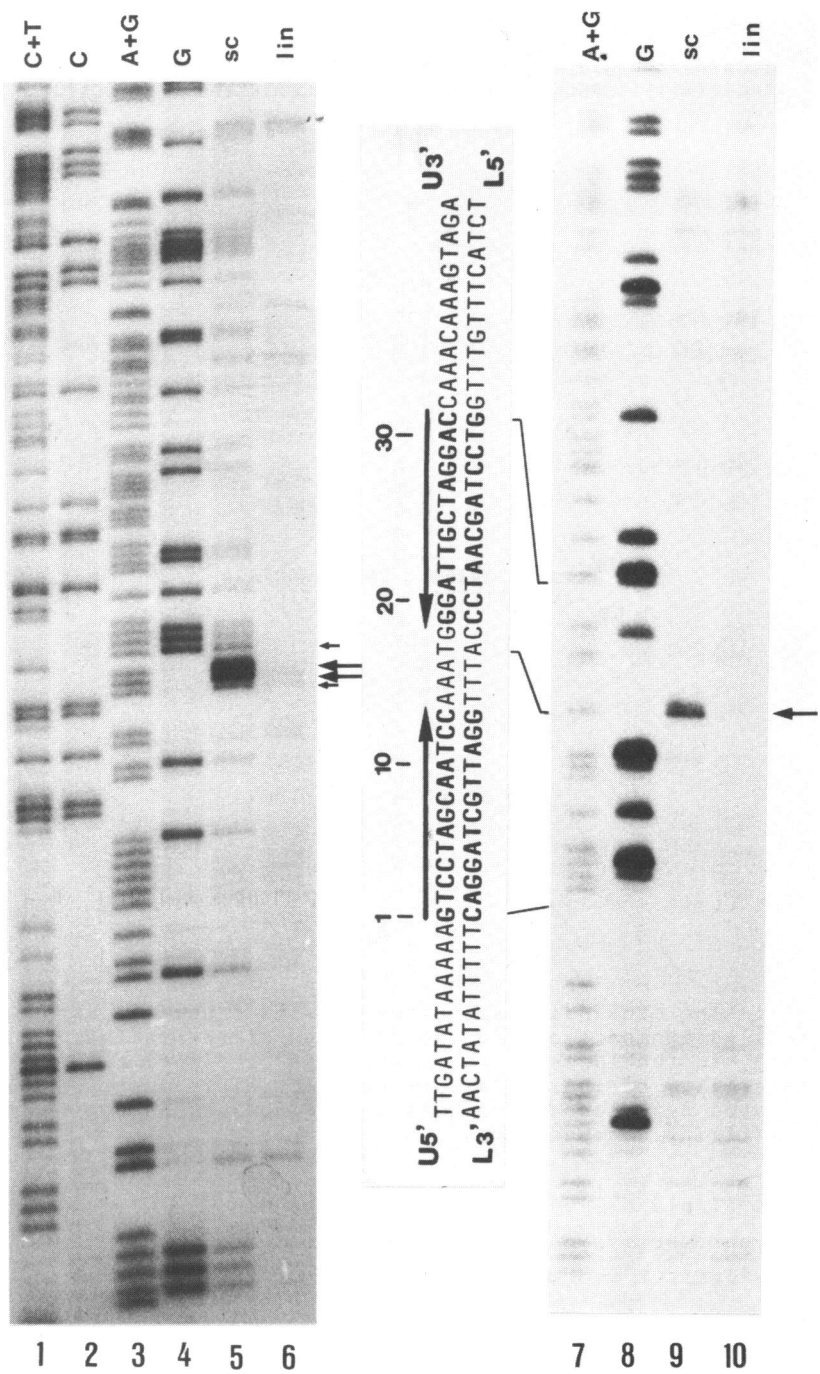


Figure 1: The pColIR515 palindrome and its cruciform structure. Numerical assignments of individual bases of the palindrome are indicated. In the loops of the postulated cruciform the hyperreactivity of individual purines with diethyl pyrocarbonate is indicated by thick and thin asterisks for strong and moderate hyperreactivity, respectively.

positions A14u and G18u of the upper strand.

The hyperreactive purine residues identified in the experiment shown in Figure 2 represent all purines of the central 5 bp element which is not part of the symmetrical 13 bp sequences (Figure 1, with positions of hyperreactive purines emphasized by asterisks). No hyperreactivity was observed within or flanking


C+T
C
A+G
G
sc
lin
U5' TTGATATAAAAAGTCCTAGCAATCCAAATGGGATTGCTAGGACCAAACAAAGTAGA U3'
L3' AACTATATTTTTCAGGATCGTTAGGTTTACCCTAACGATCCTGGTTTGTTTCATCT L5'
1
10
20
30
A+G
G
sc
lin
1 2 3 4 5 6 7 8 9 10

the inverted repeat sequences.

Supercoil dependence of the diethyl pyrocarbonate reactivity on plasmid pColIR515.

The comparison in Figure 2 of reactivity patterns of negatively supercoiled versus linear DNA templates indicated that specific hyperreactivity developed as a consequence of negative torsional stress existing within the plasmid. To further investigate the role of DNA supercoiling in the generation of the hyperreactive structure, pColIR515 plasmid preparations of four different negative superhelical densities were generated and subjected to modification by diethyl pyrocarbonate. The obtained modification pattern is displayed in Figure 3. Although a generally higher background level in reactivity was generated with most purines of all four types of DNA in this particular experiment, specific hyperreactivity was again found with residues A15u and A16u (and to a lesser extend with A14u and G17u) once a sufficiently high level of negativ supercoiling was reached (Figure 3, lanes 3, 4). At the superhelical density of $-\sigma$ =0.055 (Figure 3, lane 3) strong hyperreactivity was found, whereas the DNA sample with the density of $-\sigma$ =0.021 (lane 2) did not yet exhibit enhanced chemical modification at these positions. These values of superhelical densities necessary to generate diethyl pyrocarbonate hyperreactivity closely approximated those previously determined for extrusion of the cruciform structure in the segment analyzed (14).

Figure 2: Chemical reactivity of the pColIR515 palindrome with diethyl pyrocarbonate. Plasmid pColIR515 in linear (lanes 6, 10) and negatively supercoiled forms (lanes 5, 9) were treated with diethyl pyrocarbonate and analyzed according to the protocol of Herr (18) as detailed in Materials and Methods. The supercoiled plasmid was of the superhelical density as obtained directly after bacterial lysis and plasmid preparation. Reactivity of upper strand sequence (lanes 1-6) was visualized by 5'-end-labeling of the EcoRI-end with polynucleotide kinase, and lower strand reactivity (lanes 7-10) was visualized by 3'-endlabeling of the same site with the Klenow fragment of DNA polymerase I. DNA sequencing reactions are shown in lanes 1-4 and lanes 7-8 as indicated on top of the gel autoradiographs. Arrows point toward the positions of purines with enhanced reactivity to diethyl pyrocarbonate.

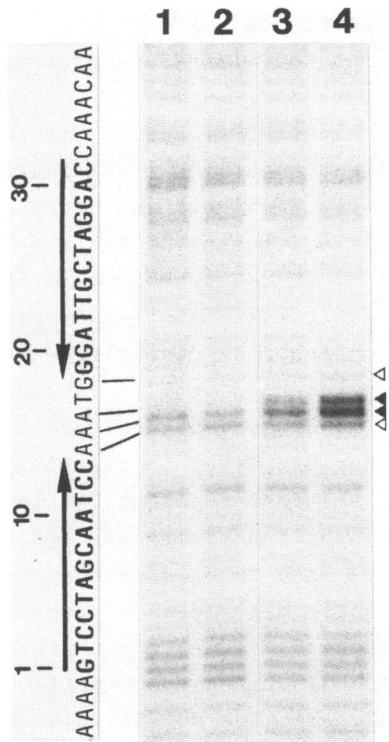


Figure 3: Chemical diethyl pyrocarbonate reactivity of pColIR515 at different superhelical densities. Reactivity of the upper strand sequence in the palindromic region is shown. The plasmid preparations of lanes 1 to 4 had superhelical densities ($-\sigma$) of 0, 0.021, 0.055, and 0.070, respectively. Purines of strong and moderate hyperreactivity are indicated by filled and open triangles, respectively.

Reactivity of diethyl pyrocarbonate with the palindromic region of plasmid pPS11.

Analysis of the pattern of diethyl pyrocarbonate modification on a second palindromic sequence was carried out on plasmid pPS11 which carried a 35 bp long inversely repeated, palindromic element (Figure 4) in the polylinker of the vector pUC18 (22). Figure 5 shows that treatment of negatively supercoiled pPS11 with diethyl pyrocarbonate results in specific hyperreactivity exclusively with residues A36l and A37l at the centre of the palindrome symmetry (lane 2). On the upper DNA strand enhanced reactivity was found with residues A24u and A35u (data not

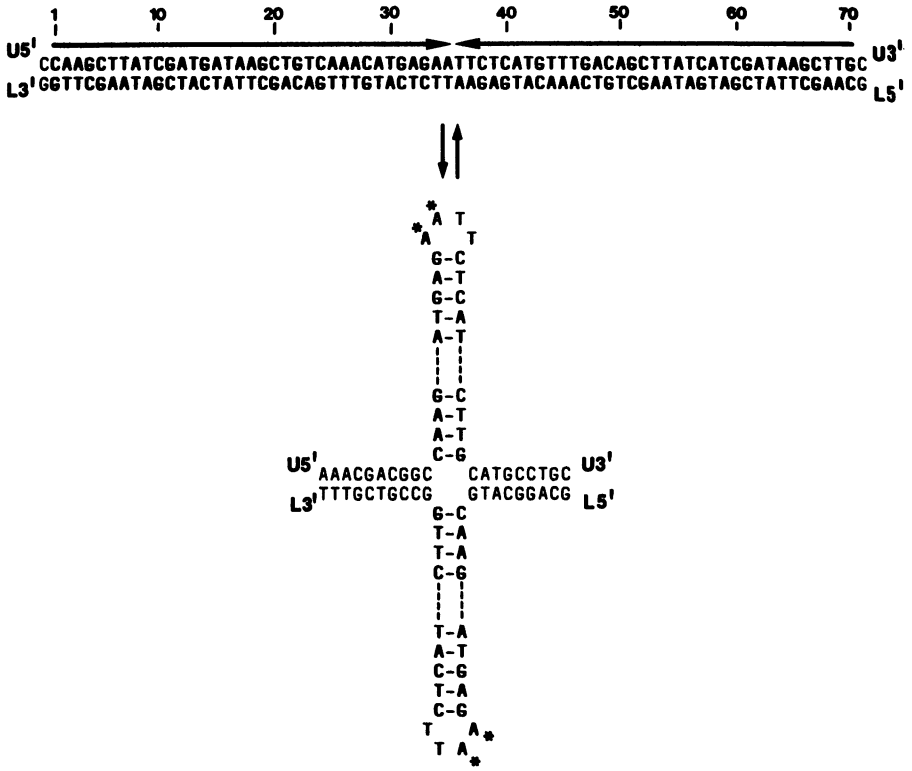
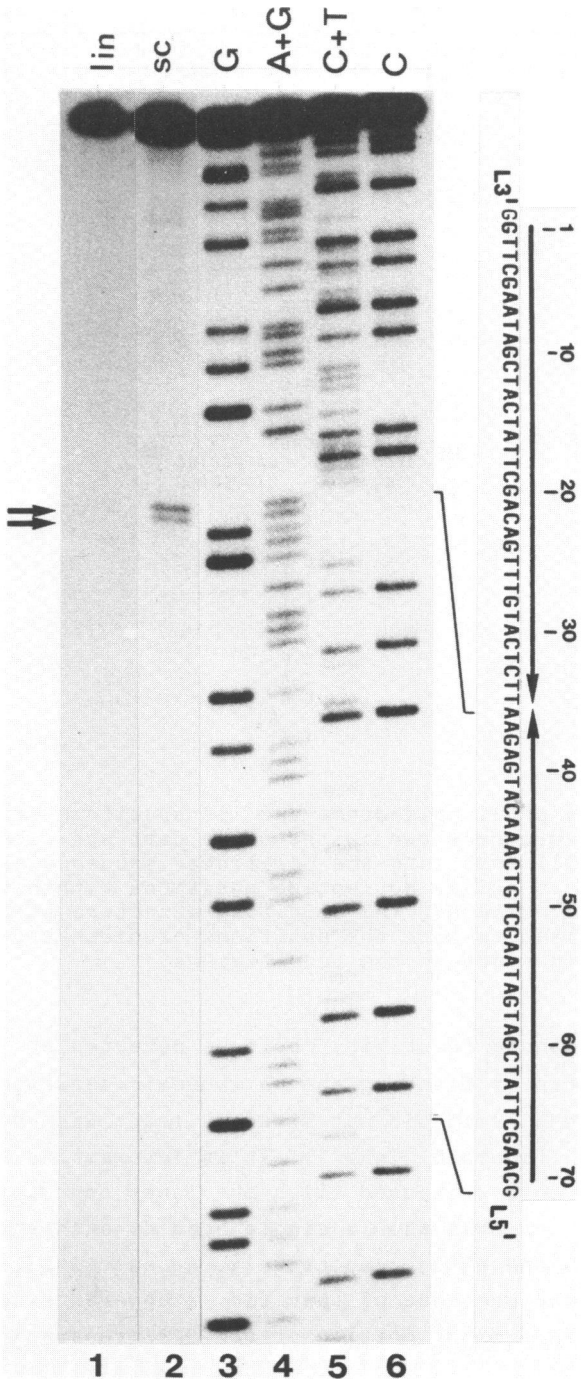


Figure 4: The pPS11 palindrome and its cruciform structure. The palindromic sequence shown in the upper part was cloned with its terminal HindIII ends into the polylinker sequence of pUC18 to generate plasmid pPS11. Nucleotide positions within the palindrome are numbered. The cruciform structure at the bottom carries asterisks to mark the positions of diethyl pyrocarbonate hyperreactive purines in the loop regions.

shown). No enhanced reactivity could be detected at other positions within or flanking the palindromic sequence of pPS11. The linearized plasmid did not strongly react with diethyl pyrocarbonate (Figure 5, lane 1). It is interesting to note, however, that on overexposed films the lanes containing endlabeled linear DNAs show a single band on each strand at positions A35u and A361, respectively. We speculate, that these bands reveal the presence of specific, staggered single-stranded cuts present in a minor portion of our DNA preparations.


lin
sc
G
A+G
C+T
C
1
2
3
4
5
6
L3' GGTTCGAATAGCTACTATTCGACAGTTTGTACTCTTAAGAGTACAAACTGTCGAATAGTAGCTATTCGAACG L5'
1
10
20
30
40
50
60
70

DISCUSSION

We have demonstrated that diethyl pyrocarbonate reacts to an enhanced extent with purines that are positioned at the center of inverted repeat sequences in both palindromes studied here. The hyperreactivity is found only with negatively supercoiled templates. The superhelical densities required to elicit this effect approximated those values determined earlier to induce extrusion of cruciform structures at the palindromic sequences (14). It is therefore plausible that the observed hyperreactivity to diethyl pyrocarbonate occurs at the purine residues of the single-stranded loops of cruciform structures. The single-stranded character of these loops has been previously determined by enzymatic and chemical probes (3,4,15-17).

In the case of RNA, diethyl pyrocarbonate only modified unpaired purines, the double-stranded segments remaining refractory to modification (21). With double-stranded DNA it has already been demonstrated that purines showed much higher reactivity with diethyl pyrocarbonate in the Z-conformation than in the B-conformation (18,20). The present study now indicates that unpaired purines are more sensitive to diethyl pyrocarbonate modification than base-paired purines of B-DNA.

The modification data obtained in this study reveal information concerning the geometry and size of cruciform loops. In the case of plasmid pColIR515 only the central five purine residues, which are not part of the symmetry element, exhibit enhanced diethyl pyrocarbonate reactivity. Due to the arrangement of the palindrome these five bp can not form intrastrand base-pairing in the cruciform. No base of the inverted sequence shows increased reactivity. This suggests that the cruciform loop of pColIR515 is generated by the central five bp only. It should be noted, however, that lack of enhanced diethyl pyrocarbonate reactivity does not necessarily argue for the presence of base-pairing between complementary bases. In the case of the perfectly

Figure 5: Pattern of purine reactivity in the palindrome region of linear (lane 1) and supercoiled (lane 2) plasmid pPS11. Analysis of the lower strand is shown after 5´-endlabeling of the BamHI site with polynucleotide kinase. Symbols were used as in the legend to Figure 2.

symmetrical palindrome of pPS11 only the central four A residues are hyperreactive. The loop reactivities of both cruciforms studied here suggest that energetic costs of cruciform formation are kept at a minimum by maintaining the smallest possible loop region. It is of additional interest that we did not observe any enhanced diethyl pyrocarbonate reactivity associated with purines near the four-way junction at the base of the cruciform stem. This may suggest that major stacking rearrangements in this structural element are kept at a minimum. These conclusions are in complete agreement with the results obtained from bisulfite mutagenesis experiments (17).

Diethyl pyrocarbonate is a sensitive chemical probe to study, with single nucleotide resolution, the supercoil induced transition of a palindrome sequence to its cruciform structure. The use of this technique will greatly aid further analyses of mechanistic, thermodynamic, and functional aspects regarding cruciform extrusion *in vitro* and *in vivo*.

The selective hyperreactivity of cruciforms with diethyl pyrocarbonate was also recently observed in the group of Dr. D.M.J. Lilley, Dundee University (pers. comm.).

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