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The supercoil-stabilised cruciform of ColE1 is hyper-reactive to osmium tetroxide

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Supercoiled pColIR215 contains a site of pronounced hyper-reactivity towards modification by osmium tetroxide, a reagent known to be single-strand-selective. The site of hyper-sensitivity has been mapped to the ColE1 inverted repeat, believed to extrude a cruciform in supercoiled DNA. Linear or relaxed plasmids are not modified by the reagent. We conclude that cruciform formation is responsible for the site-selective modification. Fine mapping of the modification site as a function of time has revealed that the initial reaction occurs at the centre of the inverted repeat, i.e., the unpaired loop of the cruciform, but that the modification region rapidly expands outwards from this point.

Key words: cruciform/ColE1 inverted repeat/osmium tetroxide/supercoiled DNA/chemical modification

Introduction

The three dimensional structure of DNA is a function of its nucleotide sequence (Dickerson and Drew, 1981), and such structural polymorphism may be enhanced by negative supercoiling (Vinograd and Lebowitz, 1966). Structural perturbation which results in a net negative twist locally will be relatively stabilised in a supercoiled DNA molecule (Hsieh and Wang, 1975; Benham, 1980; Vologodskii and Frank-Kamenetskii, 1981). Thus the formation of melted regions, left-handed Z DNA and cruciforms should be promoted in underwound circular DNA, and all have been observed (Vinograd *et al.*, 1968; Gellert *et al.*, 1979; Lilley, 1980; Klysik *et al.*, 1981; Panayotatos and Wells, 1981; Peck *et al.*, 1982).

Cruciform structures have been revealed by two experimental approaches. The first is direct topological measurement, in which the writhing change concomitant with the twist change on cruciform formation is revealed as a band shift in gel electrophoresis. This method is both direct and informative and has been used chiefly for artificial DNA sequences by Gellert *et al.* (1979) and Courey and Wang (1983). There is, however, a potential problem in that the gel systems used do not resolve small changes in writhing close to native superhelical densities, and therefore rather long inverted repeats are necessary. A second approach, without this limitation, is to use enzyme or chemical probes of the resulting cruciform structure. Thus, enzymes which cleave the unpaired loop (Lilley, 1980, 1981; Panayotatos and Wells, 1981), the stem base (Mizuuchi *et al.*, 1982; Lilley and Kemper, 1984) and chemicals which modify the region (Lilley, 1983a) have been used. In the case of the inverted repeat of ColE1, cruciform formation has been revealed by all these methods (Lilley, 1980, 1981, 1983a; Panayotatos and Wells, 1981; Lilley and Hallam, 1983; Lilley and Kemper, 1984), and confirmed by a skilfully modified band shift method (Lyamichev *et al.*, 1983).

Chemical probes are particularly valuable as they obviate concern arising from the possible perturbation caused by the enzyme binding itself. Unfortunately the reagent used previously (Lilley, 1983a), bromoacetaldehyde, was used at pH 4.5, thereby creating another source of structural modification. A new probe of choice would therefore be a substance of low mol. wt. which reacts selectively with single-stranded DNA at a pH close to neutrality. We report here just such a probe, osmium tetroxide.

Osmium tetroxide has been exploited as a probe of secondary structure in ribonucleic acids (Kim *et al.*, 1973; Rosa and Sigler, 1974), as a potential heavy atom label on single-stranded DNA (Beer *et al.*, 1966; Chang *et al.*, 1977) and as an electroactive marker for DNA (Paleček *et al.*, 1981; Lukášova *et al.* 1982, 1984). Osmium tetroxide reacts with thymine and cytosine bases in DNA, and the nature of the chemical reaction with thymine is shown in Figure 1. In contrast to bromoacetaldehyde, the hydrogen bonding donor and acceptors are not modified, and it is not immediately obvious why this reaction might be single-strand-selective. The crystal structure of this adduct has been solved (Neidle and Stuart, 1976), from which it is seen that the osmium atom lies well removed from the plane of the pyrimidine ring, and inspection of models of DNA show that it is not possible to accommodate this into regular B-DNA. In addition, the adduct formation saturates the C5-C6 bond, causing a loss of aromaticity and hence a likely distortion of planarity and alteration of the electronic properties of the base. A major reason for suspecting that osmium tetroxide might selectively modify a cruciform was the study of Glikin *et al.* (1984). These authors mapped sites of osmium tetroxide modification in a plasmid comprising *Drosophila* histone genes cloned in ColE1. They noted a major site of hyper-reactivity of unknown DNA sequence in the intergenic spacer between the H1 and H3 genes, and a minor site ~100 bp from the *Eco*RI site. Previous studies have shown that an inverted repeat centred at 100 bp from the unique *Eco*RI site of ColE1 has strong cruciform potential (Lilley, 1980, 1981; Panayotatos and Wells, 1981).

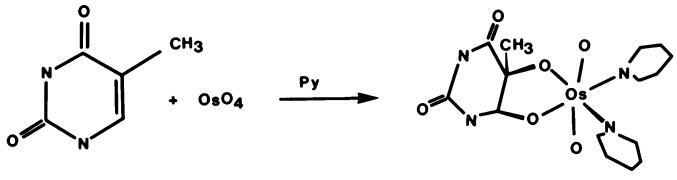
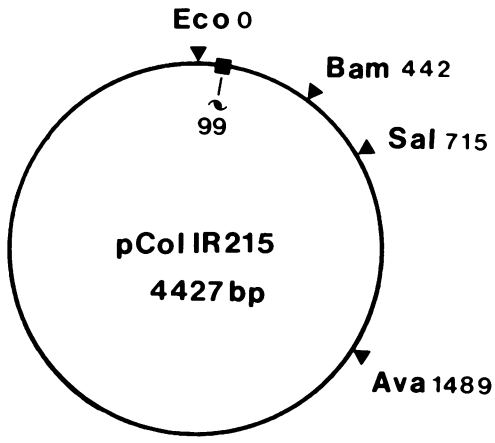


Fig. 1. Chemistry of adduct formation between thymidine and osmium tetroxide-pyridine. Osmium tetroxide adds to the C5-C6 double bond to give a *cis* ester in which the osmium is approximately octahedral. The stereochemistry indicated is taken from the crystallographic study of Neidle and Stuart (1976).



AAAGTCCTAGCAATCCAAATGGGATTGCTAGGACCAA
TTTCAGGATCGTTAGGTTTACCCTAACGATCCTGGTT

Fig. 2. Map of pColIR215 showing the relative positions of restriction targets used in these studies, and the ColE1 inverted repeat shown as a filled box. The nucleotide sequence of the ColE1 inverted repeat is shown below, with symmetrically related nucleotides in larger type.

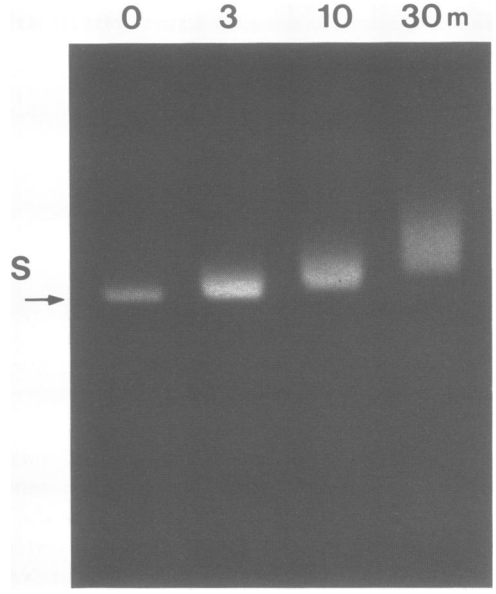


Fig. 3. Osmium tetroxide modification of supercoiled pColIR215. Agarose gel electrophoresis of supercoiled pColIR215 after reaction with 1 mM osmium tetroxide at 25°C for 0, 3, 10 or 30 min. Note the progressive reduction in mobility of the plasmid DNA as the reaction proceeds. The position of unmodified supercoiled pColIR215 is shown by the arrow labelled S.

We therefore decided to investigate the possible modification of this sequence in supercoiled pColIR215. This is a clone of the inverted repeat-containing region of ColE1 in pBR322 (Lilley, 1981), whose sequence is completely known. A map of this plasmid and the sequence of the inverted repeat are shown in Figure 2. We show here that this sequence is strongly hyper-reactive to osmium tetroxide, provided that the plasmid is negatively supercoiled.

Results

Site-selective modification of supercoiled pColIR215 by osmium tetroxide

Supercoiled plasmids may react with osmium tetroxide, resulting in partial relaxation of writhing, which is shown by a retardation on gel electrophoresis (Glikin *et al.*, 1984). Analogous behaviour by supercoiled pColIR215, after reaction with 1 mM osmium tetroxide for various times, is revealed by agarose gel electrophoresis (Figure 3). Higher reagent concentrations result in even greater reductions of mobility. We conclude that adduct formation by deoxyribonucleotide bases disrupts the normal double helical DNA structure, thereby effecting a net negative twist and hence a relaxation of tertiary writhing. The progressive increase in relaxation as a function of reaction time indicates a continuous spread in the extent of modification. This would be the result expected if, e.g., osmium tetroxide-base adduct formation opened a 'bubble' of un-base-paired DNA which might then increase in size by modification of contiguous nucleotides.

To test for possible site-selectivity of modification, we used a procedure analogous to that used for bromoacetaldehyde modification of supercoiled plasmids (Lilley, 1983a). The essence of this approach was the use of a restriction enzyme to provide sequence-specific cleavage, and S1 nuclease to remove nucleotides rendered permanently single-stranded by virtue of the base modification. A three stage protocol is used, with DNA purification by ethanol precipitation between each step. These are (i) osmium tetroxide modification. This reaction may be terminated by extraction in chlorinated solvent; (ii) restriction enzyme cleavage; and (iii) incubation with S1 nuclease at 37°C.

The DNA is then examined by gel electrophoresis. Specific fragments (i.e., discrete bands) will only result from the creation of permanently modified regions due to site-selective adduct formation in the supercoiled plasmid. We should stress the importance of the order of reactions (ii) and (iii). Specific DNA fragments of less than full length can only arise by S1 cleavage on the specifically linearised molecule. S1 nuclease does not cleave linear DNA with any significant sequence preference in the absence of chemical modification (Lilley, 1980).

Figure 4 shows such an experiment performed with pColIR215. Supercoiled plasmid was reacted with a range of osmium tetroxide concentrations up to 1.6 mM, the purified DNA samples each cleaved at the unique *Sal*I site, followed by S1 nuclease incubation. Osmium tetroxide at concentrations of 0.32 mM or higher gives site-selective modification of pColIR215 at a location ~600 bp from the *Sal*I site. The right-most track on this gel contains unmodified pColIR215 which has been incubated with S1 nuclease followed by *Sal*I cleavage. A 4090-bp band is produced, resulting from S1 nuclease cleavage at the centre of the ColE1 inverted repeat, and it may be seen that this fragment migrates at a very similar position to those arising from osmium tetroxide modification.

The ColE1 inverted repeat is the primary target for modification by osmium tetroxide

The results presented in Figure 4 are consistent with selective osmium tetroxide modification at the ColE1 inverted repeat, but do not prove it since the restriction mapping is ambiguous. To remove this ambiguity the use of two or more restriction enzymes is necessary (Figure 5). Supercoiled pColIR215 was reacted with 1.6 mM osmium tetroxide, then cleaved by either *Ava*I, *Bam*HI or *Sal*I before S1 nuclease in-

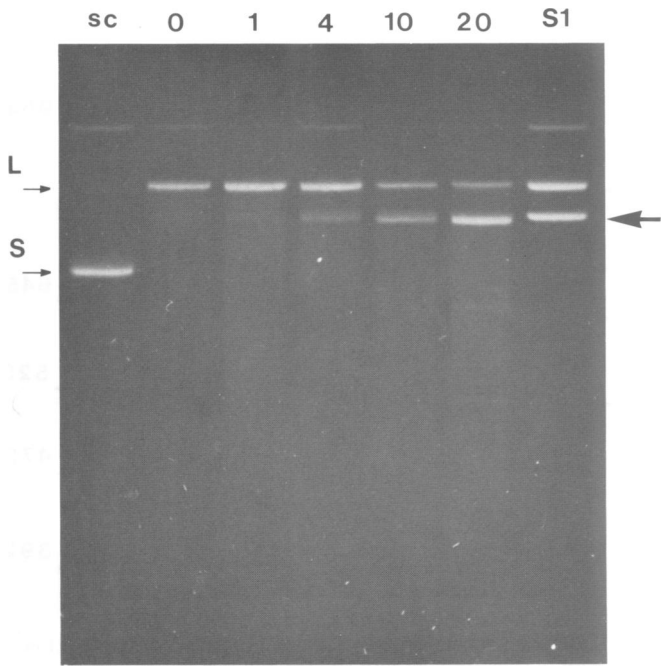


Fig. 4. Site-selective modification of supercoiled pColIR215 by osmium tetroxide. Agarose gel electrophoresis of pColIR215 reacted with various concentrations of osmium tetroxide at 25°C for 10 min, followed by *Sal*I-cleavage and S1 digestion. The figures above **tracks 2–6** indicate molarities of osmium tetroxide x 8 x 10^{-5}. **Track 7**, labelled S1, contains unmodified pColIR215 incubated with S1 nuclease followed by *Sal*I-cleavage. Unmodified supercoiled pColIR215 was electrophoresed in **track 1** (sc). The arrows on the left denote the migration positions of supercoiled (S) and linear (L) plasmid, whilst the arrow on the right indicates the major fragment produced as a result of site-selective reactions by osmium tetroxide or S1 nuclease.

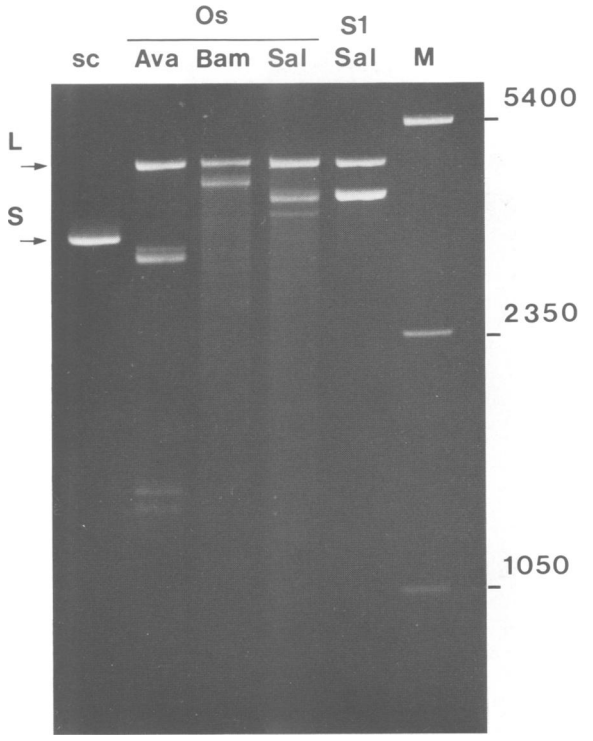


Fig. 5. Supercoiled pColIR215 is selectively modified by osmium tetroxide at the ColE1 inverted repeat. Supercoiled pColIR215 was reacted with 1.6 mM osmium tetroxide for 5 min at 25°C. The modified DNA was cleaved by either *Ava*I, *Bam*HI or *Sal*I followed by S1 nuclease digestion and electrophoresed in an agarose gel. For comparison, **track 5** contains S1 nuclease-digested pColIR215 cleaved by *Sal*I. **Track 1** (sc) contains supercoiled pColIR215 whilst track 6 (M) contains marker fragments of DNA derived by *Hind*III cleavage of phage PM2 DNA, the sizes of which (bp) are indicated on the right. The positions of supercoiled (S) and linear (L) pColIR215 are denoted by the arrows on the left.

cubation. In each case one major fragment is produced, consistent with a single site of modification in the supercoiled plasmid. Some of the shorter fragments are resolved into doublets however, seen particularly clearly for the shorter *Ava*I fragment (see below). Measurement of fragment lengths by means of the *Hind*III-cleaved phage PM2 marker fragments confirms that the site of modification is located 336 ± 26 bp from the *Bam*HI site, and fixes it on the side proximal to the *Eco*RI site. Within experimental error, this places the hyper-sensitive site coincident with that for S1 nuclease, i.e., 100 bp from the *Eco*RI site. This point is further illustrated by track 5, in which S1 nuclease-cleaved pColIR215, restriction-cleaved by *Sal*I, is run in parallel to the equivalent osmium tetroxide modification sample. Once again the close similarity in fragment migration is seen. The agreement is not exact, however, with the osmium tetroxide fragment appearing to be slightly shorter than that of S1 nuclease. This is explored further below.

The modification at this location occurs under a fairly wide range of conditions. We have observed site-selective reaction at temperatures between 12°C and 37°C, although no specific modification could be detected at 0°C. Furthermore, reaction at this site appears to proceed in acetate buffer at pH 5 and 6 and Tris buffer at pH 7 and 8.

Negative supercoiling is essential for site-selective modification

We have shown so far that supercoiled pColIR215 is modified by osmium tetroxide at, or near to, the ColE1 inverted repeat. In the light of previous investigations of structural perturbation at this locus (Lilley, 1980, 1981; Panayotatos and Wells, 1981; Lilley, 1983a; Lilley and Hallam, 1983; Lilley and Kemper, 1984), it is clearly important to establish whether or not this modification is supercoil-dependent. Identical modification reactions by 1.6 mM osmium tetroxide were performed on three pColIR215 samples: (i) negatively supercoiled plasmid at native superhelix density i.e., as prepared from *Escherichia coli*; (ii) linear plasmid prepared by complete digestion by *Ava*I; and (iii) relaxed plasmid prepared by complete incubation with chicken topoisomerase I.

Following the modification reaction the purified DNA samples were then cleaved by *Ava*I [samples (i) and (iii)] followed by S1 nuclease (all samples) (Figure 6). The supercoiled sample gives the bands corresponding to site-selective modification, identical to the results in Figure 5. These fragments are totally absent for the linear and relaxed samples however. Clearly, negative supercoiling is an essential prerequisite for osmium tetroxide modification at this site.

Formation of a well-defined modification 'bubble'

The previous figures show that the shorter fragments are frequently resolved into doublets. This point is clearly illustrated in Figure 7, which shows the shorter, i.e., ~615 bp, *Sal*I fragments arising from the S1 or osmium tetroxide reactions on supercoiled pColIR215, resolved on polyacrylamide gel electrophoresis. Whilst S1 nuclease, known to cleave highly specifically at the centre of the ColE1 inverted repeat, still on-

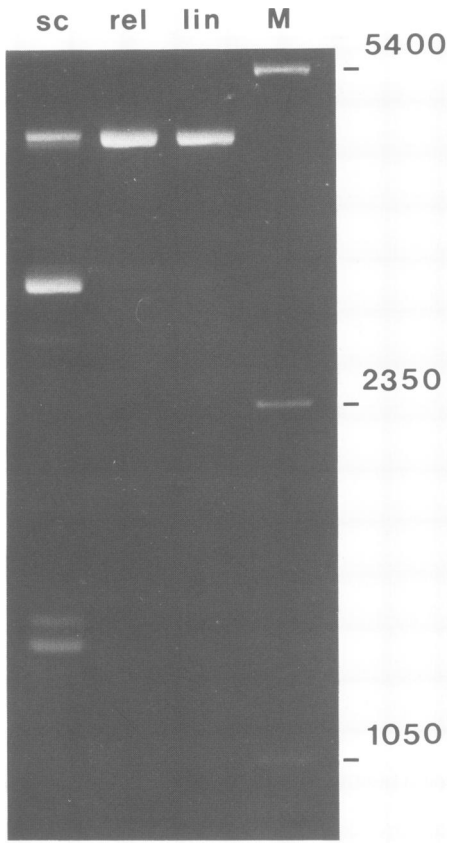


Fig. 6. Supercoiling is essential for site-selective modification by osmium tetroxide. Supercoiled (sc), topoisomerase I relaxed (rel) and *Ava*I-linearised (lin) pColIR215 were identically reacted with 1.6 mM osmium tetroxide for 10 min at 25°C. Supercoiled and relaxed samples were then cleaved by *Ava*I and finally all three samples were digested by S1 nuclease. They were each electrophoresed in an agarose gel together with marker DNA fragments (M) derived by *Hind*III-cleavage of phage PM2 DNA, the sizes of which (bp) are indicated on the right.

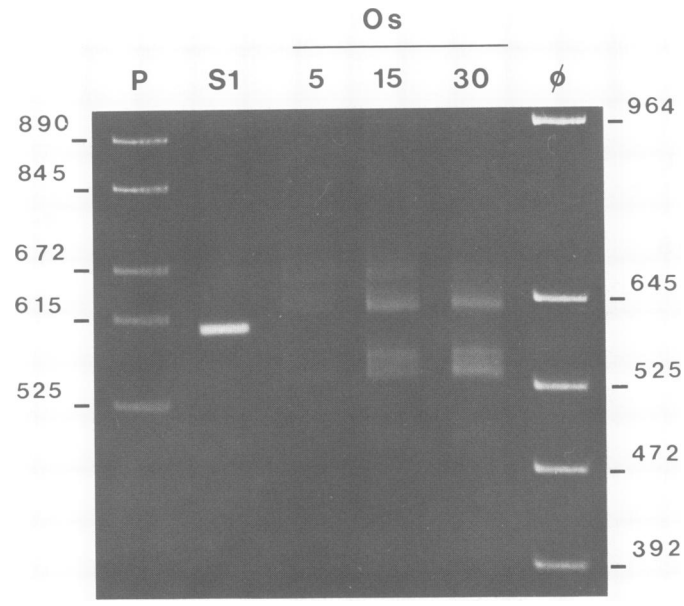


Fig. 7. Distribution of osmium tetroxide modification about the ColE1 inverted repeat of supercoiled pColIR215. Plasmid was reacted with 1 mM osmium tetroxide at 25°C for 5, 15 or 30 min, cleaved with *Sal*I followed by S1 nuclease digestion. These samples were then electrophoresed on a 5% polyacrylamide gel to resolve the shorter, i.e., 615-bp fragments. For comparison, the track labelled S1 contains the result of *Sal*I-cleavage of S1 nuclease digested pColIR215. The outer tracks contain marker DNA fragments and were derived by *Hae*III-cleavage of phage PM2 DNA (P) or by *Rsa*I-cleavage of phage ϕX174 RF DNA (ϕ), the sizes of which (bp) are indicated on the left and right respectively.

ly gives a single fragment, several fragments are resolved from the osmium tetroxide-modified samples, forming two distinct groups 30–50 bp on either side of the S1 cleavage site.

This has been investigated using radioactive labelling to examine the shorter DNA fragments resulting from *Bam*HI cleavage, high osmium tetroxide concentration (3.2 mM) and shorter times. The ~340-bp fragments were separated by polyacrylamide gel electrophoresis, an autoradiograph of which is shown in Figure 8. At 3 min, the major site of modification is coincident with the S1 nuclease hypersensitive site, i.e., the centre of the inverted repeat, with flanking positions also modified. At 20 min reaction, however, the central position is not seen at all and the only fragment remaining is that cleaved ~60 bp from the S1 nuclease site.

The general kinetic behaviour observed in these and similar experiments suggest that the primary modification event occurs at the centre of the inverted repeat, which then causes a rapid propagation of a modification 'bubble' outwards. Two points emerge from this analysis. Firstly, to account for the longer fragments remaining after the S1 nuclease cleavage, it is necessary to propose either that osmium-modified DNA is relatively protected against nucleolysis, or that the junction between modified and unmodified DNA is especially sensitive. Secondly, the 'bubble' does not appear to expand smoothly but to move to specific pause points, particularly that centred around 70 bp from the inverted repeat on the *Bam*HI site. This may be related to variations in base composition in this abnormally A + T-rich region of DNA.

Discussion

The ColE1 inverted repeat of pColIR215 is hyper-sensitive to modification by osmium tetroxide when, and only when, the DNA is negatively supercoiled. This sequence has previously been shown to be peculiarly sensitive to nucleases including S1 nuclease (Lilley, 1980, 1981; Panayotatos and Wells, 1981), T7 gene 3 endonuclease (Panayotatos and Wells, 1981), micrococcal nuclease (Dingwall *et al.*, 1981; Lilley, 1983b), BAL 31 (D.M.J.Lilley and L.H.Hallam, in preparation) and the Holliday junction resolving endonuclease VII of phage T4 (Lilley and Kemper, 1984), to be resistant to *Ava*I cleavage at the site partially contained within the inverted repeat (Lilley and Hallam, 1983), and to be hyper-sensitive to modification by bromoacetaldehyde (Lilley, 1983a). Small molecule probes are particularly significant as they obviate a specific objection to enzyme probes, namely that the DNA binding domains of enzymes may induce structural alteration prior to reaction. Small molecules possess no such substrate binding regions and it is not likely that structural transition would result from non-covalent interaction. Thus, demonstration of a specific site of elevated reactivity implies a pre-existing structural perturbation of some kind. The ColE1 inverted repeat, is believed to extrude a cruciform structure for the following reasons: the loop formed from the central nucleotides is sensitive to single-strand-selective reagents including osmium tetroxide; the four-way junction at the base of the stems should be structurally equivalent to a Holliday junction (Holliday, 1964), and is recognised as such by T4

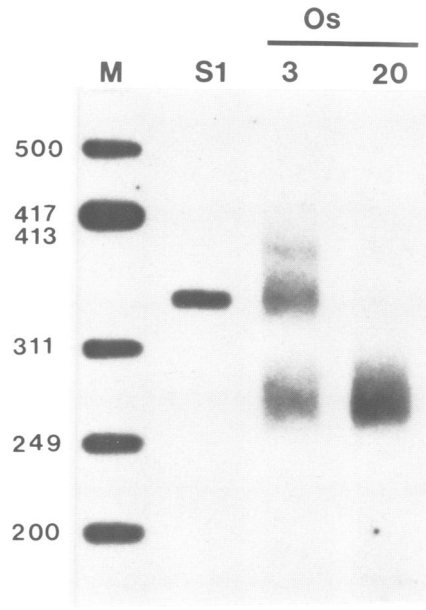


Fig. 8. Distribution of osmium tetroxide modification about the ColE1 inverted repeat of supercoiled pColIR215 as a function of time. Plasmid was reacted with 3.2 mM osmium tetroxide at 25°C for 3 and 20 min, cleaved with *Bam*HI followed by S1 nuclease digestion. Following radioactive labelling of 5′-termini by [^{32}P], the DNA was electrophoresed on a 5% polyacrylamide gel to resolve the ~340 bp fragments. The track labelled S1 contains ^{32}P-labelled DNA derived by S1 nuclease digestion of supercoiled pColIR215 followed by *Bam*HI digestion. Marker DNA fragments (M) were derived by *Hinf*I cleavage of phage φX174 RF DNA, the sizes of which (bp) are indicated on the left.

endonuclease VII; in addition to probing experiments, topological studies of this sequence have revealed properties expected of a cruciform. The S1 nuclease sensitivity is a sharp function of plasmid linking differences (Singleton and Wells, 1982; Panyutin *et al.*, 1982; Lilley and Hallam, 1983) and this transition may be correlated with writhing changes seen by means of gel electrophoresis (Lyamichev *et al.*, 1983; D.M.J. Lilley and L.R.Hallam, in preparation).

Osmium tetroxide is a significant addition to cruciform probes. Firstly, it is a small molecule probe which may be used at neutral pH, in contrast to bromoacetaldehyde which requires low pH for reaction. Both S1 nuclease and bromoacetaldehyde are used at pH 4.6, which is sufficiently close to the p*K* of some nucleotide base groups to create some anxiety as to the possible facilitation of structural perturbation. In addition, although both bromoacetaldehyde and osmium tetroxide are single-strand-selective reagents, their chemical mechanisms are quite distinct from one another. Secondly, the reagent concentration, in the millimolar region, is considerably lower than that for bromoacetaldehyde. Thirdly, we have evidence that osmium tetroxide may exhibit considerable selectivity in the inverted repeats with which it can react. The synthetic inverted repeat of pIRbke8 (Lilley and Markham, 1983) and the major inverted repeat of pBR322 (Lilley, 1980, 1981) are both cleaved by S1 nuclease and T4 endonuclease VII, and yet neither are measurably modified by osmium tetroxide. However, we have preliminary evidence that the minor pBR322 inverted repeat (Lilley, 1980, 1981) may be hyper-sensitive to this reagent. We suspect that the crucial difference between modifiable and unmodifiable cruciform structures may be loop size, but this requires further study. However, it is apparent that careful investigation of these differences should allow greater insight into the detailed geometry of cruciform structures.

Materials and methods

Preparation of plasmid

pColIR215 (Lilley, 1981) was isolated from *E. coli* K12 HB101 after growth at A_{600} = 0.6 in the presence of 150 μg/ml chloramphenicol. Cells were lysed by lysozyme, EDTA and Triton X-100 and supercoiled plasmid banded in CsCl and ethidium bromide isopycnic gradients. Plasmid DNA was recovered by side puncture, butan-1-ol extraction, dialysis and two ethanol precipitations.

Osmium tetroxide

Osmium tetroxide (Johnson Matthey) was dissolved in glass distilled water as an 8 mM stock solution. Unless otherwise indicated, reactions were performed in 10 mM Tris pH 7.8, 1 mM EDTA, 1% (v/v) pyridine at 25°C. Typical reactions were performed in a total volume of 50 μl containing 5 μg plasmid DNA. After cleavage by restriction enzymes, osmium tetroxide-modified DNA was incubated with 5 U S1 nuclease (BRL) in 50 mM sodium acetate pH 4.6, 50 mM NaCl, 1 mM $ZnCl_2$ at 37°C for 60 min.

Enzyme reactions

S1 nuclease cleavage of supercoiled pColIR215 was performed in 50 mM sodium acetate pH 4.6, 50 mM NaCl, 1 mM $ZnCl_2$ at 15°C for 60 min using 3 U of S1 nuclease (BRL) in a 20 μl reaction volume. Restriction enzymes were purchased from BRL or New England Biolabs and used as directed. 5′-termini of DNA molecules were radioactively labelled with [^{32}P] using T4 polynucleotide kinase (BRL) and the exchange labelling procedure (Berkner and Folk, 1977). Supercoiled plasmid was relaxed using chicken topoisomerase I, a gift of T. Kimura, in 10 mM Tris pH 8.0, 200 mM NaCl, 10 mM EDTA at 25°C.

Electrophoresis

Agarose and polyacrylamide gel electrophoresis was performed using 90 mM Tris pH 8.3, 90 mM borate, 10 mM EDTA buffer at ambient temperature. For autoradiography, polyacrylamide gels were dried on 3 MM paper (Whatman) and exposed to Kodak X-omat S film at −70°C using an Ilford fast tungstate intensifier screen.

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