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Site-specific cleavage of left-handed DNA in pBR322 by Λ-tris(diphenylphenanthroline)cobalt(III)

(Z-DNA/chiral specificity/photoactivated DNA cleavage)

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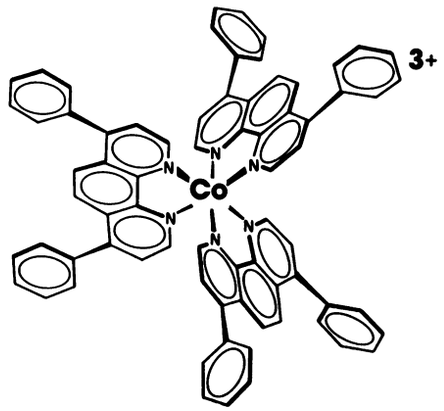
ABSTRACT **The chiral complex tris(4,7-diphenyl-1,10-phenanthroline)cobalt(III), Λ-Co(DiP)$_3^{3+}$, binds to and, with photoactivation, cleaves left-handed DNA helices, thereby providing a unique molecular probe for local DNA conformation. We have mapped the specific left-handed sites where Λ-Co(DiP)$_3^{3+}$ cleaves in the plasmids pBR322 and pLP32, which is the derivative of pBR322 containing a Z-form d(C-G)$_{16}$ insert. For pLP32, a primary cleavage is at the insert; for native pBR322, cleavage occurs at four discrete sites: 1.45, 2.3, 3.3, and 4.2 kilobase pairs. These sites correspond to segments of alternating purine-pyrimidines. Moreover, these positions map to the ends of the three distinct coding regions in pBR322: the tetracycline-resistance gene, the origin of replication, and either end of the ampicillin-resistance (β-lactamase) gene. The locations of these left-handed segments suggest to us that Z-DNA might serve as a conformational punctuation mark to demarcate the ends of genes.**

Numerous spectroscopic and x-ray crystallographic studies have shown that DNA may adopt a range of conformations, from the right-handed A- and B-forms to the striking left-handed Z-DNA helix (1–7). Regions of conformational heterogeneity along the strand, such as cruciform structures, single-stranded loops, and left-handed segments, have been detected by using DNA enzymes (8–10), and it has been suggested that local DNA conformation might play some role in regulating gene expression. Chiral metal complexes that can intercalate into the helix are particularly advantageous in probing local DNA conformation (11–14). Tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II) complexes [Ru(DiP)$_3^{2+}$] provide a spectroscopic probe for helix handedness; the Λ isomer, which does not bind B-DNA owing to steric constraints, binds avidly to Z-DNA (13). Upon photoactivation, the analogous cobalt isomers, Co(DiP)$_3^{3+}$, furthermore cleave DNA stereospecifically, providing a sensitive assay for local regions in the Z form (14). In this report we describe the specific cleavage by Λ-Co(DiP)$_3^{3+}$ of discrete sites in the plasmid pLP32 (15), which contains a well-defined Z-DNA segment, and in pBR322 (16, 17). The chiral complex serves as a conformation-specific tool to map these left-handed DNA helical segments. The structure of Λ-Co(DiP)$_3^{3+}$, the photoactivated DNA cleaving agent, is shown here.



MATERIALS AND METHODS

Chemicals. 4,7-Diphenylphenanthroline was purchased from Aldrich. [Co(DiP)$_3$]Cl$_3$ was synthesized, and tartrate diastereomers were separated as described (14). The plasmid pBR322 and all enzymes used were obtained from Bethesda Research Laboratories. PLP32 was a gift from A. Nordheim.

Instrumentation. Irradiations were with a 1000-W Hg/Xe lamp (Oriel Corporation of America, Stamford, CT) narrowed to 315 ± 6 nm with a monochrometer. Densitometric scans were done with a Cary 219 spectrophotometer with a gel-scanning attachment. Band sizes and intensities were calculated on an IBM personal computer.

Mapping Procedure. [Co(DiP)$_3$](tartrate)$_3$ (10 μM) was added to plasmid DNA (100 μM nucleotides) in 50 mM Tris acetate buffer containing 18 mM NaCl (pH 7.0). The 20-μl sample was then irradiated at 315 nm for 90 sec and ethanol-precipitated. The ethanol wash removes unreacted Co(DiP)$_3^{3+}$ and the metal and ligand products of the reaction. After resuspension in Tris acetate buffer containing 50 mM NaCl and 10 mM MgCl$_2$ (pH 7.0), restriction enzyme was added (*Eco*RI, *Bam*HI, *Ava* I, or *Nde* I) in at least a 3-fold excess to ensure complete linearization. This was incubated at 37°C for 45 min. The pH of the reaction mixture was then lowered to 5.0, and 10 mM Zn(NO$_3$)$_2$ was added along with 4 units of nuclease S1, and the samples were incubated for 5 min at 37°C. This step causes cleavage of the DNA by nuclease S1 opposite the site nicked by Co(DiP)$_3^{3+}$. Then electrophoresis on 1% agarose gels (50 mM Tris acetate/18 mM NaCl, pH 7.0) resolved the double-stranded fragments produced. Gels were stained with 5 μg of ethidium bromide per ml for 0.5 hr and then destained in buffer for 2 hr. Gels were photographed with a Polaroid 600 camera equipped with a red filter and 615 positive/negative film, irradiated from below. In these experiments pBR322 sequences are numbered beginning at the *Eco*RI site as described by Sutcliffe (16, 17). The superhelical density of pBR322 was determined to be −0.053.

Densitometric scans were performed on photographs, and band sizes were quantitated by using markers of known molecular weight. Note that since the bands were stained

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Abbreviations: DiP, 4,7-diphenyl-1,10-phenanthroline; bp, base pairs.

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with ethidium bromide, the band intensities are weighted by their length.

RESULTS AND DISCUSSION

Irradiation at 315 nm of Λ-Co(DiP)$_3^{3+}$ (10 μM) solutions containing supercoiled pLP32 or pBR322 yields nicked, circular form II DNAs. Photoreduction of Co(DiP)$_3^{3+}$ enantiomers bound to DNA leads to oxidative single-strand scission at the DNA binding site (14). In order to establish that cleavage and therefore binding occurs at discrete sites, the scheme outlined in Fig. 1 was used. After irradiation of Co(DiP)$_3^{3+}$–DNA samples and the production of nicked circles, the DNAs were linearized by using a restriction enzyme that is known to cleave the plasmid at only one site along the strand. Subsequent treatment with nuclease S1, which is specific for single-stranded regions, cleaved the DNA at sites only opposite to the cobalt-induced nick producing a pair of linear fragments. From the sizes of these fragments, determined based upon their gel electrophoretic mobilities relative to markers, the distance of the cleavage site from the restriction site origin may be obtained.

In order to distinguish whether the site is clockwise or counterclockwise to the origin, at least two restriction enzymes that cut at sufficiently distinct locations were examined. It is important to notice that this procedure yields distinct fragments only if binding and subsequent cleavage occurs at discrete sites. This leads to the bands evident in Fig. 2 *Top* for the fragmentation of pBR322 by Λ-Co(DiP)$_3^{3+}$ and *Ava* I. Nonspecific cleavage produces fragments of all sizes and, hence, a smear on the gel; thus, the presence of some contaminating form II DNA just alters the background intensity. Control experiments of sample irradiated without cobalt or with cobalt binding but without irradiation yielded no distinct bands. Nonspecific DNA damage as a result of irradiation was negligible. However, controls showed that full linearization of the plasmid was essential to avoid mapping nuclease S1-hypersensitive sites (8–10). Some restriction enzymes did not yield complete linear digests because of either thymine dimer formation at the restriction site or inhibition due to Co(DiP)$_3^{3+}$ reaction, and these were not used. Finally, samples were irradiated only for short times so that no more than one nick per plasmid would occur. The fact that the sizes of pairs of fragments must add to 4363 base pairs (bp) provided a useful experimental redundancy. By this general procedure the coarse map of Λ-Co(DiP)$_3^{3+}$ cleavage sites in any plasmid may be obtained.

We examined first the plasmid pLP32, which contains a Z-DNA segment at a well-defined location (15, 18). The plasmid had been constructed by inserting a d(C-G)$_{16}$ fragment into the filled-in *Bam*HI site (position 375) of pBR322 (16). The densitometric scan of the *Ava* I digest after reaction with Λ-Co(DiP)$_3^{3+}$ is shown in Fig. 2. In addition to the linear

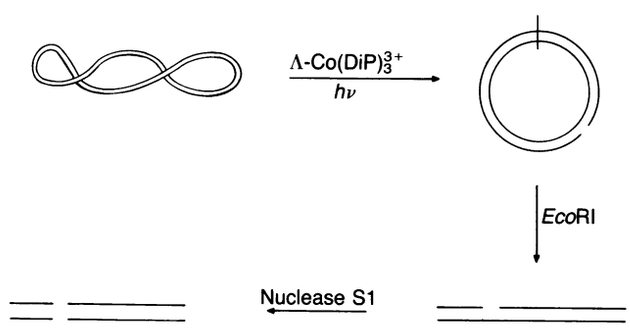

FIG. 1. Experimental scheme used to map the single-stranded cleavage sites of Co(DiP)$_3^{3+}$. For details see *Materials and Methods*.

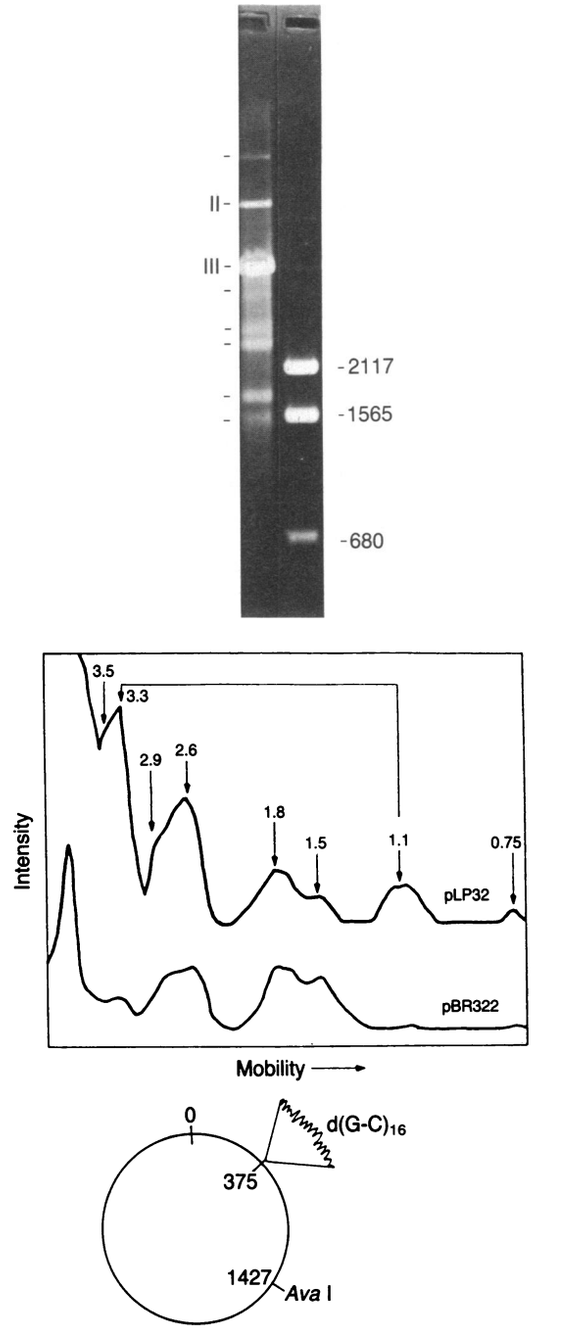

FIG. 2. Agarose (1%) gel (*Top*) and densitometric scans (*Middle*) of Λ-Co(DiP)$_3^{3+}$ fragmentation using the scheme depicted in Fig. 1. (*Top*) Fragmentation of pBR322 by Λ-Co(DiP)$_3^{3+}$ and nuclease S1 (*Ava* I was used for linearization) (left lane) and *Rsa* I (for molecular weight markers) (right lane). Cleavage with Λ-Co(DiP)$_3^{3+}$ yields discrete bands. (*Middle*) The pairs of double-stranded fragments produced in pBR322 are 3.5 and 0.75, 2.9 and 1.5, 2.6 and 1.8 kbp. Note that these pairs of bands sum to 4.4 kbp, the length of the plasmid. In pLP32, a pair of bands not present in pBR322 are seen at 3.3 and 1.1 kbp. (*Bottom*) These bands indicate cleavage at the d(C-G)$_{16}$ insert at position 375 of pBR322 (1.1 or 3.3 kb from the *Ava* I site at 1427).

form, several bands and shoulders are evident; their sizes in kilobase pairs (kbp) are indicated in the figure. The appearance of the pair of fragments at 3.3 and 1.1 kbp from the *Ava* I site (position 1427) shows that a major cleavage point is indeed at the Z site. Parallel digestion with *Nde* I established this position uniquely. More interesting, perhaps, is the comparison to the *Ava* I digest of pBR322, the same plasmid

but lacking the insert. The pattern here is identical except that it lacks the 3.3- and 1.1-kbp fragments. This same digest is shown in Fig. 2 *Top*. In pLP32, then, the cobalt complex must recognize and cleave a site not present in pBR322, the Z-form $d(C\text{-}G)_{16}$ insert. The result demonstrates that Λ-$Co(DiP)_3^{3+}$ can cleave specifically at a left-handed site.

Other conformations are not similarly accessible to the chiral cobalt complex. Λ-Tris(diphenylphenanthroline) complexes of ruthenium(II) and cobalt(III) do not react as assayed spectrophotometrically (12, 13) (for ruthenium) and by cleavage assays (14) (for cobalt) with B-form helices. The Δ isomer in contrast can bind both B and Z forms, and photolysis experiments using Δ-$Co(DiP)_3^{3+}$ showed nonspecific cleavage of the linear DNA but some specific band formation. We also found that tris(phenanthroline)ruthenium(II) complexes do not bind to double-stranded RNA (unpublished results); hence, it is not likely that Λ-$Co(DiP)_3^{3+}$ could bind preferentially to an A-form helical conformation. Finally, we examined racemic $Co(DiP)_3^{3+}$ cleavage of single-stranded phage ϕX174 DNA. Here, after photolysis, we observed $\approx$12% cleavage, less than the 15% double-stranded content in the ϕX174 sample, calculated based upon hypochromicity. It is unlikely then that $Co(DiP)_3^{3+}$ enantiomers could recognize open looped regions of a plasmid. Instead, the only DNA conformation for which appreciable binding and cleavage by Λ-$Co(DiP)_3^{3+}$ has been found is Z-DNA.

Photolysis and digestion of both pLP32 and pBR322 actually yielded several distinct fragments (Fig. 2). Therefore, additional cleavage sites for Λ-$Co(DiP)_3^{3+}$, similar structurally to the left-handed $d(C\text{-}G)_{16}$ insert, may be present in these plasmids. Based upon numerous trials with *Eco*RI, *Bam*HI, *Ava* I, or *Nde* I for linearization, there appears to be four discrete cleavage sites in pBR322: given in order of reactivity, $1.45 \pm 0.05 \approx 3.3 \pm 0.1 > 4.24 \pm 0.02 > 2.25 \pm 0.07$ kbp. The standard deviations are based upon averaging at least seven experiments.† The observed inhibition of some restriction enzymes, mentioned earlier, is consistent with the locations of these cobalt binding sites. The plasmid pLP32 showed cleavage at these same positions in addition to cleavage at the insert. Fig. 3 *Upper* shows a typical *Eco*RI digest. Fragment pairs are evident and, because *Eco*RI linearizes at the origin, the lengths of one fragment of the pair shows the position of the site in kbp. The relative intensities, weighted by the fragment molecular weight, reflects either the relative site affinity for Λ-$Co(DiP)_3^{3+}$ or relative cleavage efficiency at a site. We found variations in relative site intensities as a function of irradiation time and also as a function of salt concentration in the incubation mixture. No significant cleavage was observed at sodium concentrations >150 mM. This effect resembles the dependence of specific anti-Z-DNA antibody binding to pBR322 on salt (19–21). We also found a dependence of cleavage on supercoiling. Plasmid samples linearized prior to irradiation with Λ-$Co(DiP)_3^{3+}$ showed a loss of 81% of the band intensity found with the supercoiled substrate, and relaxed, closed, circular DNAs showed even greater reductions in cleavage. Thus, recognition may be related to the DNA secondary structure. Table 1 summarizes the specific sites in pBR322 that we found with cleavage by Λ-$Co(DiP)_3^{3+}$.

What are the features unique to these recognition sites? Λ-$Co(DiP)_3^{3+}$ is not a sequence-specific reagent, and there is

†The estimated width of the bands in Figs. 2 and 3 *Upper* is 100 bp. The band is broadened for several reasons: first, nonspecifically nicked DNA initially present in the sample also may be cleaved by nuclease S1, resulting in some background intensity that reduces band resolution; second, for the specifically cleaved site, nuclease S1 activity may extend beyond the cobalt-induced lesion; and finally, ethidium bromide staining in general does not yield sharp bands.

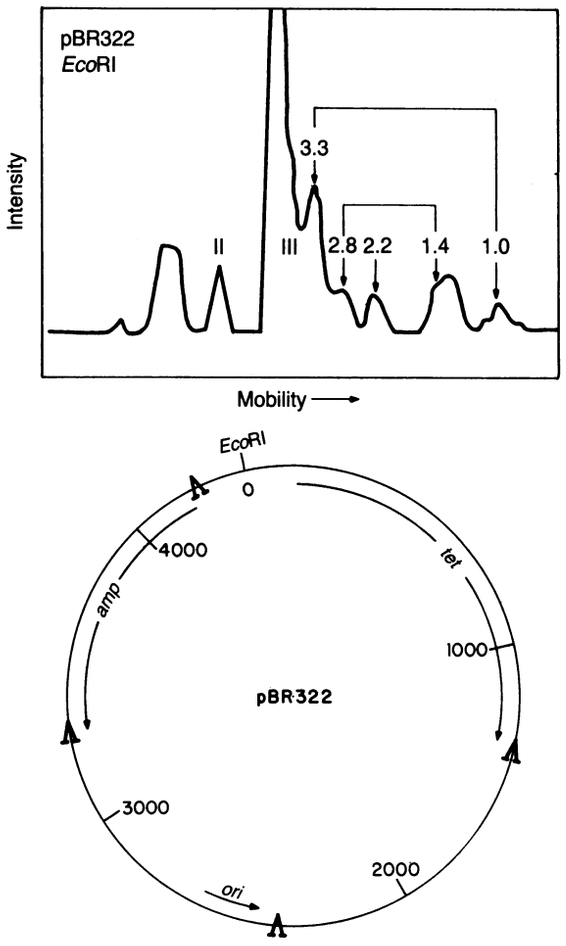

FIG. 3. Coarse map of left-handed sites in pBR322. (*Upper*) Densitometric scans of Λ-$Co(DiP)_3^{3+}$ fragmentation after linearization with *Eco*RI. Bands are produced at 3.3 and 1.1, 2.75 and 1.45, and 2.2 kbp. Since *Eco*RI cleaves at position zero, these fragments correspond to recognition sites at 3.3, 1.45, and 2.2 kbp. (*Lower*) Biological map of pBR322 showing positions of cleavage by Λ-$Co(DiP)_3^{3+}$ (Λ). *amp*, ampicillin-resistance gene; *tet*, tetracycline-resistance gene; *ori*, origin of replication.

no sequence homology evident at these positions in pBR322. Instead, the Λ enantiomers of the tris(phenanthroline) complexes appear to bind selectively to left-handed DNA sites, owing to the asymmetry of the DNA helical groove. Thus, perhaps it is a common left-handed conformation at these locations that is being recognized by the cobalt complex. Alternating purine-pyrimidine sequences have been shown (3–7, 22, 23) to adopt the Z-DNA conformation most readily because alternative residues in Z-DNA have bases in the *syn*

Table 1. Λ-$Co(DiP)_3^{3+}$ cleavage sites

Fragment size, kbp	Corresponding alternating purine-pyrimidine positions and sequences
1.45 ± 0.05	1447–1460
	C-A-C-G-G-G-T-G-C-G-C-A-T-G
2.25 ± 0.07	2315–2328
	C-G-C-A-C-A-G-A-T-G-C-G-T-A
3.32 ± 0.11	3265–3277
	G-T-A-T-A-T-A-T-G-A-G-T-A
4.24 ± 0.02	4254–64
	T-C-C-G-C-G-C-A-C-A-T

Underlined residue identifies the base out of register.

conformation. Inspection of the pBR322 sequence revealed that the Λ-Co(DiP)$_3^{3+}$ recognition sites included the longest runs of alternating purines and pyrimidines, allowing for one base out of alternation. Table 1 shows also the alternating sequences that appear within one standard deviation of each measured recognition site. At positions 1447, 2315, 3265, and 4254 begin, respectively, 14-, 14-, 13-, and 11-bp regions with alternating purine and pyrimidine residues having one mistake. These regions correspond essentially to one helical turn in a Z-DNA conformation and are the longest of such conformation homology within the plasmid.

What sequences are not recognized by Λ-Co(DiP)$_3^{3+}$? There are several other sequences, beginning at 1171, 1533, and 1709, that constitute 11-bp segments with one mistake that are not significantly cleaved by Λ-Co(DiP)$_3^{3+}$, and the longest sequence of alternation in the plasmid with no mistakes—10 bp beginning at position 2785—is also not cleaved. At this stage, we do not know whether the flanking sequences at these sites affect Z-DNA formation or Λ-Co(DiP)$_3^{3+}$ recognition. The sequences within the recognition sites detected do have a range of G+C contents. It is interesting that the 1447 site in pBR322 has been shown to adopt the Z conformation in low-salt buffers at bacterial superhelical densities by a completely different route—crosslinking studies (19–21) with anti-Z-DNA antibodies. Thus, specific Λ-Co(DiP)$_3^{3+}$ cleavage sites appear to correlate with Z-sites observed by other methods—the d(G-C)$_{16}$ insert in pLP32, seen by supercoiling experiments, and the 1447 site in pBR322, detected by anti-Z-DNA antibodies. It is possible then that all four sites of alternating purine-pyrimidine residues that are specifically recognized and cleaved by Λ-Co(DiP)$_3^{3+}$ adopt the Z conformation at low-salt concentrations in native supercoiled pBR322.

It is interesting, finally, to ask whether these segments share some common biological function in this plasmid. PBR322, assembled from three naturally occurring plasmids, contains three genetically distinct coding regions—the tetracycline-resistance gene, the β-lactamase gene conferring ampicillin resistance, and the origin of replication. Fig. 3 *Lower* shows the map of these genes in pBR322. It is curious to notice the correspondence in position between the ends of these discrete coding elements and the Λ-Co(DiP)$_3^{3+}$ recognition sites. A single polypeptide in pBR322 appears to be necessary for tetracycline resistance (24). The 3′ end of the region encoding this peptide is thought to be near the *Ava* I site at 1425 bp; sequences upstream from the tetracycline-resistance promotor (which begin at 45 bp) were lost in construction from pSC101. The β-lactamase gene is defined upstream by the start site at 4201 with the −35 consensus region for the promoter ending at position 4236, 18 bp away from the Λ-Co(DiP)$_3^{3+}$ cleavage site (25). The 3′ end of the region encoding β-lactamase is found at position 3295, which is 22 bp upstream of the alternating purine-pyrimidine cleavage site. Finally, the essential region constituting the origin of replication in pBR322 extends from the RNA/DNA junction at 2536 to position 2360, 32 bp from the weak site detected with Λ-Co(DiP)$_3^{3+}$ (16, 17, 26). Thus, there appears to be a remarkable correspondence between sites recognized by the cobalt complex and the ends of genetic coding elements.

It is tempting to suggest that the Z-DNA conformation might provide a general structural signal or punctuation mark that demarcates the ends of these genes. Consistent with this idea, the Z-conformation has been shown to provide a poor template for transcriptional activity with *Escherichia coli* RNA polymerase (27), and further, the d(C-G)$_{16}$ sequence in supercoiled plasmids causes a severe block to transcription *in vitro* (28). This notion is consistent also with the location of alternating purine-pyrimidine tracts in simian virus 40 DNA regulatory sequences that bind anti-Z-DNA antibodies (29), of the d(G-T)$_n$ tracts at the ends of yeast chromosomes (30) and of the alternating purine-pyrimidine long terminal repeats in mouse mammary tumor virus (31). Recent experiments with mung bean nuclease in the malaria parasite *Plasmodium* have demonstrated that a particular conformation, rather than a sequence, appears to be shared by gene termination sites (32). Moreover, anti-Z-DNA antibody binding sites in φX174 replicative form I DNA, mapped recently by darkfield immuno-electron-microscopy, show a striking correlation with regions of transcriptional attenuation at the ends of genes (33). Finally in simian virus 40, a region, rather than a sequence, downstream of the poly(A) tail consensus sequence has been shown to be required for the formation of the viral late mRNA 3′ termini in frog oocytes (34), and this region contains a 9-bp alternating purine–pyrimidine stretch having one mistake. These correlations of location with conformation are intriguing and support the notion that DNA secondary structure, as well as sequence, may be important to the regulation of gene expression.

In summary, based upon cleavage experiments with chiral cobalt complexes, several conformationally discrete sites occur in pBR322 under native physiological conditions, and these sites are likely to be in the left-handed Z conformation. The positions of these sites mark the ends of genetically distinct coding elements in the plasmid. Λ-Co(DiP)$_3^{3+}$ provides a photoactivated site-specific cleavage agent that is useful to map left-handed sites and may be helpful in establishing a relationship between the locations of Z-DNA segments and its biological function.

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