Anthramycin inhibition of restriction endonuclease cleavage and its use as a reversible blocking agent in DNA constructions

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ABSTRACT

Anthramycin can form a stable complex with DNA which does not dissociate upon repeated ethanol precipitations. The complex forms in less than one hour at pH 5.5. Bound anthramycin seems to be located in the minor groove of the DNA helix in the anthramycin-DNA complex, since methylation of adenosine residues at N-3 by dimethylsulfate is reduced. The anthramycin-DNA complex is resistant to digestion by an excess of a number of restriction enzymes. Anthramycin can be removed from DNA by incubation at acid pH. The released DNA can then be cleaved by restriction enzymes. Anthramycin-DNA complexes can be acted upon by T_4 polynucleotide ligase to form longer DNA molecules. The ability of anthramycin to form a stable but reversible complex which is not cleaved by restriction enzymes but can engage in joining reactions may allow a wider variety of DNA fragments to be more readily constructed in vitro.

INTRODUCTION

Several antibiotics which bind DNA have been used to partially protect cleavage sites from restriction endonuclease action.^{1,2} Netropsin, actinomycin D, and distamycin bind non-covalently in the minor groove and prevent cleavage by a number of restriction enzymes. The inhibition relies on the equilibrium between the DNA-antibiotic complex and antibiotic in solution so the amount of inhibition depends on the concentration of antibiotic. Certain DNA binding drugs have been used to increase the T_m of the DNA duplex during ligation reactions, enabling the joining of small DNA fragments at enzymatically favored high temperatures.³

For certain DNA constructions it would be useful to carry out a ligation reaction followed with cleavage by an enzyme in only one of the fragments undergoing the ligation. If restriction sites are present within both fragments, cleavage would occur in both segments, but if one fragment can be protected from cleavage before the ligation, subsequent cleavages will take place only in the unprotected fragment. A procedure similar in concept has been used in the cloning of randomly generated fragments using Eco RI linkers by methylating the large DNA fragments, but not the linker molecules, with Eco

RI methylase.⁴ While this technique is suitable for cloning applications, it is not a general method, since many specific methylases are not readily available and the protection is not reversible. A more general type of protection would block cleavage of a DNA segment from a variety of nucleases, but be removable so the segment of DNA could be cleaved at a later stage of manipulation if desired.

With these ideas in mind, the study of the interactions of DNA complexed with anthramycin was undertaken. Anthramycin (An) has been previously studied and seemed to have several features required for such a protecting agent (for a recent review see Hurley, ref. 5). It rapidly forms a stable complex with DNA through formation of a metal linkage with the N-2 amino group of guanine.⁶ It can protect native DNA from degradation by nuclease^{7,8} and it can be removed from DNA under low pH conditions which should not extensively degrade the DNA molecule.⁹

In this report experiments are presented which show how anthramycin can form a stable complex with DNA and that the location of the complex can be determined by its inhibition of methylation by dimethyl sulfate. The DNA-An complex is resistant to a variety of restriction endonucleases, but still can ligate to other DNA fragments using T₄ polynucleotide ligase. Combinations of ligation and restriction cleavage were carried out as examples of constructions made easier with the aid of anthramycin protection.

MATERIALS AND METHODS

11-MeO-anthramycin was obtained from Hoffmann-La Roche, Nutley, N.J. Restriction enzymes were obtained from Bethesda Research Labs, Rockville, Md., or New England Biolabs, Beverly, Mass. Calf intestinal phosphatase and T₄ polynucleotide kinase were from Boehringer-Mannheim, Indianapolis, Ind. T₄ polynucleotide ligase was purified from an overproducing strain¹⁰ by the procedure of Panet et al.¹¹ BspR I¹² was isolated using the Cibacron-blue column technique.¹³

pBR322¹⁴ DNA was isolated from plasmid bearing RRI cells grown and amplified with chloramphenicol¹⁵ by lysis with Brij 58-deoxycholate,¹⁶ precipitation with polyethyleneglycol 6000,¹⁷ RNase A treatment, phenol extraction, ethanol precipitation and passage over an Agarose A 0.5 M column (BioRad Laboratories, Richmond, Calif.).

Polyacrylamide gels were composed and run as described by Maniatis et al.¹⁶ 0.8% horizontal agarose gels¹⁹ were run in 0.05 M Tris-Borate-EDTA at 100 V. for 30 min to 2 hr. DNA bands were detected by ethidium bromide

staining and photography under ultraviolet illumination.²⁰ ³²P-labeled DNA bands were detected by autoradiography on Kodak XR-5 or Sakura A film with exposure time reduced by the use of DuPont Lightning-Plus intensifying screens.²¹

DNA fragments were isolated from polyacrylamide gels, using high salt and SDS, treated with phosphatase and labeled using γ -³²P-ATP and polynucleotide kinase as described.²² Conditions for DNA sequencing reactions using dimethyl sulfate and hydrazine and the acid, alkali, and piperidine workup have been described.²²

Restriction enzyme digestions were carried out in 6 mM Tris-Cl, pH 7.4, 6mM DTT, 6 mM MgCl₂, 100 μ g/ml bovine serum albumin at a DNA concentration of 20-40 μ g/ml. The NaCl concentration recommended for each enzyme was used. The amount of enzyme used in the digestions was routinely a 3-5 fold excess of units per microgram DNA and incubations were for 2 hr. Ligation reactions were carried out in 60 mM Tris-Cl, pH 7.6, 10 mM MgCl₂, 10 mM DTT, 0.4 mM ATP for 2-5 hr at 12° with T₄ polynucleotide ligase in a volume of 25 μ l which contained approximately 10 pmoles of DNA fragments. More extensive ligation was observed if higher concentration and longer times of incubation were used.

An-protected plasmid DNA was prepared by incubating 50 μ g of DNA with 0.8-1 mM 11-MeO-anthramycin in 0.25 ml of 0.03 M sodium acetate pH 5.5 for 20-40 minutes at 24°. The DNA was precipitated with ethanol, reprecipitated, and washed with ethanol before it was resuspended in 10 mM Tris-Cl, 0.1 mM EDTA, pH 7.9 (TE). Aliquots were removed for restriction endonuclease experiments. Radioactively labeled DNA fragments were treated with anthramycin in the same manner using a molar ratio of anthramycin:nucleotide of approximately 10.

An was removed using conditions as previously described.⁹ An overnight dialysis of the An-DNA complex against 200 volumes SSC (0.015 M sodium citrate, 0.15 M NaCl) at pH 3 or 4 removed most of the anthramycin. The DNA was recovered by ethanol precipitation or by dialysis into TE buffer. An was removed in some experiments by a 15 min incubation in the low pH SSC buffer followed by two extractions with n-butanol.⁹

Transformation of *E. coli* RRI cells was carried out essentially by the procedure of Kushner.²³ Colonies were selected on nutrient broth plates containing 20 μ g/ml tetracycline or ampicillin.

RESULTS

Reaction of Anthramycin-treated DNA with DNA Sequencing Reagents.

A 85 bp fragment Hha I-BspR I from pBR322²⁴ labeled at the BspR I end was subjected to the DNA sequencing reactions of Maxam and Gilbert²² before and after treatment with An. From Figure 1, lanes A and H, one can see no difference in the reaction with hydrazine, an agent which attacks DNA in the major groove. From a comparison of lanes B and I, it is observed that several adenine residues are much less reactive (indicated by arrows) in the An-treated DNA than in the control. These adenines are near guanine residues, where An reacts with the N-2 position of guanine in the minor groove.⁶ Since the antibiotic fills a space in the minor groove corresponding to several base pairs, inhibition of methylation at N-3 of nearby adenines is easily interpreted.

In order to see if any alkali or acid labile linkages were introduced by An, the DNA was treated at pH 2.8 under conditions which can be used to remove An^{9,25} and no significant cleavage was observed (lanes D,E). In addition, treatment with 1 M piperidine produced no increased cleavage on DNA treated with An (lane F). Also linkages susceptible to alkali were not generated by the pH 2.8 treatment (lane E) and no increase in acid labile linkages were observed following piperidine treatment of An-DNA (lane G).

Inhibition of Restriction Enzyme Cleavage by Anthramycin.

pBR322 DNA was treated with An, recovered by ethanol precipitation and aliquots were incubated with individual restriction enzymes. The product was electrophoresed on an agarose or acrylamide gel adjacent to the corresponding digest of untreated DNA (Fig. 2). From part A, the inability of Hind III, Bam HI and Cla I to produce the normal linear band from the An-treated DNA is apparent. Upon enzymatic digestion the An-treated plasmid DNA does produce some of a band which migrates below that of undigested plasmid DNA but not at the location of the normal linear form. This band may be anthramycin coated nicked plasmid. It does not appear if the plasmid is cleaved and then treated with An. An-complexed DNA fragments run at approximately the same location but in somewhat more diffuse bands than uncomplexed DNA. In part B, of Fig. 2 digestion of An-treated DNA with Mbo II, Hpa II, Hinf I and BspR I fails to produce the normal band pattern, and only a faint smear of partial cleavage is observed in addition to undigested material near the well. In part C, Taq I and Hpa II also fail to produce proper banding patterns in the treated DNA. To see if larger amounts of an enzyme might produce complete cleavage, larger amounts of BspR I were tried (lanes 6-8). The larger amounts of BspR I used

Figure 1. The 85 bp fragment was subjected to the following reactions and treatments as in the Maxam-Gilbert sequencing technique.²² (A) C + T Hydrazine reaction, 1M piperidine cleavage reaction; (B) Dimethyl sulfate reaction, acid, then NaOH workup (A>G); (C) Dimethyl sulfate reaction, 1M piperidine cleavage reaction (G); (D) Sodium citrate, pH 2.8, 25°, 1 hr, then piperidine cleavage reaction; the 85 bp fragment was incubated with anthramycin, ethanol precipitated and aliquots were subjected to the following treatments; (E) Sodium citrate, pH 2.8, 25°, 1 hr, then piperidine cleavage; (F) Piperidine cleavage reaction only; (G) Piperidine cleavage reaction, then incubated at pH 2.8, 25°, 1 hr; (H) C + T Hydrazine reaction, piperidine cleavage reaction; (I) Dimethyl sulfate reaction, acid then NaOH cleavage (A>G); (J) Dimethyl sulfate reaction, then piperidine cleavage (G).

Figure 2. Digestion of DNA treated with anthramycin. A. 0.8% Agarose gel (1) pBR322 treated with anthramycin, (2) Hind III digest of pBR322 control, (3) Hind III digest of anthramycin-treated pBR322, (4) Bam HI digest of pBR322 control, (5) Bam HI digest of anthramycin-treated pBR322, (6) Cla I digest of pBR322 control, (7) Cla I digest of anthramycin-treated pBR322. B. 5% TBE polyacrylamide gel (1) Mbo II digest of anthramycin-treated pBR322, (2) Mbo II digest of pBR322 control, (3) Hpa II digest of anthramycin-treated pBR322, (4) Hpa II digest of pBR322 control (5) Hinf I digest of anthramycin-treated pBR322, (6) Hinf I digest of pBR322 control (7) Bsp I digest of anthramycin-treated pBR322, (8) Bsp I digest of pBR322 control. C. 5% TBE Polyacrylamide gel (1) Taq I digest of pBR322 control, 3 units/ μ g, 2 hr; (2) Taq I digest of anthramycin-treated pBR322, 3 units/ μ g, 2 hr; (3) Hpa II digest of pBR322, 2 units/ μ g, (4) Hpa II digest of anthramycin-treated pBR322, 2 units/ μ g, 2 hr; (5) Bsp I digest of pBR322, 5 units/ μ g, 2 hr; (6) Bsp I digest of anthramycin-treated pBR322, 5 units/ μ g, 2 hr; (7) Bsp I digest of anthramycin-treated pBR322, 100 units/ μ g, 2 hr; (8) Bsp I digest of anthramycin-treated pBR322, 500 units/ μ g, 2 hr.

(up to 500 units) did not produce a significant improvement in the banding pattern. Other enzymes tested and found to give similar results were Alu I, Mbo I, Hha I, Bgl II, Hinc II, Rsa I and Pvu II. EcoR I was not as completely inhibited under these conditions. It was shown that the inhibition was not due to direct action on the enzyme, since untreated DNA could be added to a labeled An-blocked DNA sample and the blocked DNA was not digested as detected by autoradiography while the untreated DNA was digested to completion as revealed by ethidium bromide staining.

Ability of DNA Complexed with Anthramycin to be Ligated

A 147 bp Hpa II fragment of pBR322 was 5'-end labeled and treated with anthramycin. Ligation of the treated fragment to another Hpa II fragment (527 bp) by T_4 polynucleotide ligase was compared to the reaction using untreated 147 bp fragment. Figure 3, lane 1 shows almost complete change in mobility of

Figure 3. The labeled 147 bp Hpa II fragment was incubated with T_4 polynucleotide ligase and 527 bp Hpa II fragment. The products were run on a 7% polyacrylamide gel and detected by autoradiography. X indicates the location of the xylene cyanol marker dye. (1) An-treated 147 bp fragment (~ 3 pmole) was incubated with the 527 bp fragment (~ 20 pmoles) in the presence of T_4 polynucleotide ligase in a volume of 25 μ l. (2) The An-treated 147 bp fragment digested with Cfo I. (3) The untreated 147 bp fragment plus T_4 polynucleotide ligase. (4) The untreated 147 bp fragment plus Cfo I, complete digestion products of 38 bp and 9 bp were expected. (5) The untreated 147 bp fragment (~ 3 pmoles) incubated with the 527 bp fragment (~ 7 pmoles) in the presence of T_4 polynucleotide ligase in a volume of 25 μ l.

the label to a position of higher molecular weight fragments. The extent of ligation is similar to that observed with the untreated fragment (lane 5). The 147 bp fragment was resistant to endonuclease attack, as shown by the inability of Cfo I to digest the An-treated fragment (lane 2) compared to the untreated fragment (lane 4). In parallel analyses, it was observed that pBR322 DNA added to the Cfo I reaction mixture was digested to completion as judged by its band pattern upon ethidium bromide staining, while the radioactive fragment remained intact.

Experiments in which the concentration of An was increased during preparation of the complex yielded DNA which was not so readily ligated. A range of 4-15 anthramycin molecules per nucleotide in the complexing reaction yielded DNA which was still ligated well, while cleavage with restriction

enzymes was substantially blocked. Thus it may be that an anthramycin molecule very near the end of the DNA molecule will inhibit the DNA ligase reaction.

Anthramycin-treated DNA can be Deblocked at Low pH

Anthramycin-treated pBR322 was dialyzed at pH 4.0 and pH 3.0 in SSC buffer. After recovery of the DNA, its sensitivity to digestion by restriction enzymes was tested. Figure 4 shows that after dialysis at pH 4.0 or pH 3.0, the DNA is cleaved by Rsa I to give the same bands as observed for untreated DNA. The anthramycin-treated DNA was not sensitive to Rsa I digestion after dialysis at pH 7.0, however.

A similar experiment with 5'-labeled DNA (Fig. 5) shows the effect of a 15 min. incubation at pHs 3.0-2.6 followed by extraction of the anthramycin with n-butanol. The ability of the fragment to be cleaved by Cfo I is re-

Figure 4. pBR322 treated with anthramycin was dialyzed vs sodium citrate buffer at various pHs, the DNA was recovered and digested with Rsa I. (1) pBR322 stock; (2) pBR322 stock cut with Rsa I; (3) pBR322 stock dialyzed at pH 7.0, mostly nicked form observed; (4) pBR322 dialyzed at pH 7.0 and cut with Rsa I; (5) pBR322 dialyzed at pH 4.0 and cut with Rsa I; (6) pBR322 dialyzed at pH 3.0 and cut with Rsa I; (7) pBR322 treated with anthramycin, dialyzed at pH 7.0, and cut with Rsa I; (8) pBR322 treated with anthramycin, dialyzed at pH 4.0 and cut with Rsa I; (9) pBR322 treated with anthramycin, dialyzed at pH 3.0 and cut with Rsa I; (10) pBR322 dialyzed at pH 7; (11) pBR322 dialyzed at pH 7, digested with Rsa I; (12) Anthramycin-treated pBR322 dialyzed at pH 7; (13) Anthramycin-treated pBR322 dialyzed at pH 7, digested with Rsa I.

Figure 5. A 5'-end labeled 147 bp DNA fragment containing Hha I (Cfo I) sites 38 bp and 9 bp from the ends was treated with anthramycin. The DNA-anthracycline complex was incubated with sodium citrate buffer for 14 min, extracted with *n*-butanol and precipitated with ethanol. After digestion of aliquots with Hha I the DNA was run on a 7% polyacrylamide gel. X indicates the location of the xylene cyanol marker dye. (A) 147 bp fragment; (B) 147 bp fragment + Hha I; (C) anthramycin-treated 147 bp fragment; (D) anthramycin-treated 147 bp fragment + Hha I; (E) pH 3 deblocked anthramycin-treated fragment; (F) pH 3 deblocked anthramycin-treated fragment + Hha I; (G) pH 2.8 deblocked anthramycin-treated fragment; (H) pH 2.8 deblocked anthramycin-treated fragment + Hha I; (I) pH 2.6 deblocked anthramycin-treated fragment; (J) pH 2.6 deblocked anthramycin-treated fragment + Hha I. The end of the 147 bp fragment which produced the smaller Hha I fragment is less effectively labeled.

gained following the low pH treatment, lanes F, H and J.

A more sensitive measure of biological DNA integrity during blocking and deblocking steps might be the ability of the DNA to transform E. coli cells.

In an initial experiment, pBR322 DNA was saturated with anthramycin and its transformation frequency compared with untreated DNA before and after the low pH anthramycin removal step. Anthramycin modified DNA transformed less than 0.2% as well as the control DNA; however after dialysis at pH 4 both control and anthramycin-treated DNA transformed with similar efficiency (approximately 50-60% as well as the original untreated pBR322). In a second experiment, DNA treated with anthramycin was digested with BspR I, 5 units/ μ g, prior to dialysis at low pH. The data below shows the ability of the anthramycin to protect the transforming ability of the DNA from digestion by BspR I and that the antibiotic can be removed to yield DNA capable of transforming with a reasonable frequency.

	<u>Ap^R Transformants per μg</u>
untreated pBR322	1.4×10^6
pBR322 dialyzed at pH 4	0.7×10^6
<u>Bsp</u> R I digested pBR322	$<4 \times 10^3$
Anthramycin-treated pBR322 DNA digested with <u>Bsp</u> R I then dialyzed at pH 4	0.2×10^6

Samplings of Ap^R transformants were 100% Tc^R in all experiments.

Figure 6. Scheme for construction of DNA fragments using anthramycin. To construct a fragment containing X and the section 3-2 of Y. Fragment X could be treated with anthramycin and cloned to Y. The site for enzyme 2 in X would be protected, but the site for 2 within Y is sensitive to cleavage. After cleavage the fragment (1-2-3-2) could be deblocked by a low pH treatment.

Construction of DNA Fragments using Anthramycin

The ability to reversibly block restriction enzyme cleavage of DNA within a fragment can allow new types of fragments to be prepared. Figure 6 illustrates how a product fragment with ends produced by cleavage with enzyme 1 and 2 can be constructed from two fragments X and Y, containing sites for restriction enzyme 2. One of the fragments (X) can be blocked with An prior to ligation then restriction cleavage with enzyme 2 occurs only in the fragment (Y) which was not protected with An. This series of reactions results in a product DNA fragment formed with an end specified by enzyme 2, but still containing sites for this enzyme within it.

Several experiments were carried out to demonstrate the feasibility of the scheme. In Figure 7, the ability of an An-treated 5'-³²P-labeled Hpa II 147 bp fragment to be ligated to an untreated non-radioactive 527 bp Hpa II fragment and be subsequently cleaved only at sites within the unprotected 527 bp segment is illustrated. (see diagram below for restriction maps, numbers refer to the center of the restriction site ²⁴)

Aliquots of the ligation reactions presented in Figure 3 were subjected to digestion and the product fragments were compared on a single gel. Lanes 1 and 2 (with the untreated fragment) vs lanes 7 and 8 (with An-treated DNA) show that the An-treated 147 bp fragment is resistant to Cfo I digestion. The extent of ligation of An-147 and untreated 147 with the 527 bp fragment was approximately the same. These ligation products are illustrated in Fig. 3, lanes 1 and 5 and an aliquot of the ligation of untreated 147 with 527 is shown in lane 5 (Fig. 7) for reference. In order to easily identify the products of Cfo I cleavage which arise from ligation of 147 to itself, the product of ligation of the untreated 147 bp fragment without any 527 present

Figure 7. A 147 bp 5'-end labeled Hpa II fragment containing Cfo I (Hha I) sites 9 and 38 bp from its ends was joined to an unlabeled 527 bp Hpa II fragment which had Cfo I sites 24 and 29 bp from its ends. After various treatments the material was electrophoresed on a 7% polyacrylamide gel and products detected by autoradiography. X indicates the position of the xylene cyanol marker dye, approximately 170 bp, B indicates the location of the bromphenol blue dye, approximately 35 bp. (1) the untreated 147 bp fragment; (2) the untreated 147 bp fragment digested with Cfo I, major bands of 9,38 bp are present as well as a partial digestion product of 138 bp; (3) ligation of untreated 147 bp fragment to itself; (4) the self ligation product digested with Cfo I, major bands include 47, and 76 bp products; (5) product of ligation of untreated 147 bp fragment with 527 bp fragment; (6) products from digestion of the material in lane 5 with Cfo I, in addition to the bands present in lanes 2 and 4, major bands occur at 62-67 bp and faint bands appear due to the less intensely labeled Cfo I 9 end at ~33 bp (24 + 9) and 38 bp (29 + 9); (7) the 147 bp fragment treated with An; (8) An-147 digested with Cfo I, only very faint

bands corresponding to those found in lane 2 are observed; (9) Cfo I digestion of the product of ligation of An-147 with the 527 bp fragment, major band of approximately 170 bp (147 + 26); (10) an aliquot of the material in lane 9 was deblocked briefly using the pH 3-n-butanol extraction procedure and subjected to Cfo I digestion, in addition to bands at the positions found in lane 9 the 62-67 bp bands appear as in lane 6.

is shown lane 3, and lane 4 shows a Cfo I digest of this material. The appearance of labeled fragments of 38 + 9 (47 bp) and 38 + 38 (76 bp) which arise from either different end joining or same end joining about the 2681 Hpa II end indicate that both types of intermolecular ligation occurred. A similar Cfo I digest of the product of ligation of the untreated 147 to the 527 is shown in lane 6. In addition to the products of digestion of unligated 147 bp fragment (lane 2) and the products of digestion of the self-ligated form (lane 4), major bands appear in the size range corresponding to 62-67 bp, the size expected for a fragment composed of the 38 bp end of Hpa II 147 plus the 24 or 29 bp Hha II-Cfo I end of the 527 bp fragment. In lane 9, however, which is a Cfo I digest of the ligated product of An-147 to the 527 bp fragment, these 62-67 bp fragments do not appear and instead material somewhat larger than the original An-147 bp fragment is the major product. After partial deblocking at low pH, digestion with Cfo I yields the 62-67 bp fragments found in the Cfo I digestion of the joined untreated fragments (lane 6). Thus, the procedures outlined in Fig. 6 were demonstrated with the An-blocked 147 bp fragment corresponding to fragment X and the 527 bp fragment corresponding to fragment Y. Cfo I cleavage of the ligated material yielded a fragment longer than 147 bp which contained a Cfo I site. Sensitivity of the Cfo I site within the 147 bp fragment could be regained after low pH treatment.

In another test of the construction scheme (Fig. 6) a 5'-³²P-labeled BspR I-Taq I fragment was treated with An and joined to the Hpa II 527 bp fragment used above. These can join via a Hpa II/Taq I junction since Hpa II and Taq I both generate 5'-CG protruding ends. The ligated product was then cleaved within the untreated segment and separated by polyacrylamide gel electrophoresis. After elution, the fragment contained a segment arising from the untreated 527 bp fragment, which could be digested with restriction enzymes Mbo II and Cfo I and a section, composed of the An-BspR I-Taq I fragment, which was not cleaved by these enzymes although it contains recognition sites for both of them. This experiment demonstrated that An-DNA could be run on a gel, eluted and still retain its ability to protect the DNA from restriction enzyme cleavage. An aliquot of the product of ligation of the An-BspR I-Taq I fragment was subjected to dialysis vs SSC pH 3.0

overnight. After this deblocking step, which gave more complete An removal than was usually found with the n-butanol procedure, the restriction cleavage products were the same as those found if an untreated Bsp I - Taq I fragment were taken through the procedure. This experiment demonstrated that An could be removed from DNA after ligation to yield a product which could be cleaved by restriction endonucleases in the expected manner.

DISCUSSION

Anthracycline-treated DNA has been shown to be resistant to a number of restriction endonucleases. Previous investigations of minor groove binding molecules have shown an inhibition of several restriction endonucleases.^{1,2} The antibiotic anthracycline binds the DNA covalently through the N-2 amino group of guanine.⁶ This adduct is quite stable and is not dependent on several residues of specific sequence for its formation. Since virtually all restriction endonuclease recognition sequences contain one or more G-C base pairs, the technique should be suitable for use at almost any restriction site. Suitable protection could usually be obtained by using a 10 fold molar excess of An to nucleotide in 0.03 M NaAc pH 5.5 for approximately 20 min.

The ability to regain sensitivity to restriction enzymes and to transform E. coli after removal of anthracycline indicates the procedures for blocking and deblocking do not drastically affect the integrity of the DNA molecule. Protected plasmid DNA attacked by Bsp I still transformed competent E. coli cells fairly efficiently after removal of the antibiotic. Overnight dialysis vs pH 3 SSC gave the most dependable deblocking. The ability to remove anthracycline from DNA allows this antibiotic to be used as a general reversible blocking agent for a large number of restriction enzymes.

The action of T_4 polynucleotide ligase on the complexed DNA demonstrates that protected DNA can be used in DNA constructions while the antibiotic remains in the same location on the DNA during the usual manipulations associated with these procedures. The rate or extent of ligation with An-DNA seemed to be approximately that of normal DNA although excess An treatment did inhibit ligation. The conditions may need to be adjusted for various DNA fragment lengths, base composition or types of ends. These features of the An-DNA complex should allow anthracycline to be useful in many DNA constructions which might otherwise pose problems.

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