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# Reaction conditions affect the specificity of bromoacetaldehyde as a probe for DNA cruciforms and B–Z junctions

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ABSTRACT

The reaction of bromoacetaldehyde (BAA) was investigated further with recombinant plasmids containing tracts of $(CG)_{16}$, in pRW756, or $(CA)_{32}$, in pRW777, which adopt left-handed Z-structures under the influence of negative supercoiling. The cruciform structures adopted by the inverted repeat sequences near the replication origins of the pBR322 vectors served as internal controls for the number of unpaired bases. The extent of reaction with the B-Z junctions and the cruciforms was dependent on the reaction and analysis conditions, the method of preparation of BAA, ionic conditions, and the amount of negative supercoiling. In contrast to the previous results of Kang and Wells, B-Z junctions in addition to cruciforms do react with BAA. However, more forcing conditions are required to detect this reaction since B-Z junctions appear to be less reactive than the single stranded loops of cruciforms. The site of reaction with DNA was readily mapped with high precision at the nucleotide level. Also, a simple method is described for determining the concentration of BAA as well as its intrinsic reactivity in a given ionic medium.

INTRODUCTION

The reactions of halogenated acetaldehydes with adenine and cytosine nucleosides have been widely studied, and these compounds have been used as probes for structural perturbations (possibly regions of single-strandedness) in duplex DNA (1-5). The products of BAA or chloro-acetaldehyde (CAA) reaction are 1-$N^6$-etheno-A and 3-$N^4$-etheno C, and thus the reaction of BAA with a supercoiled DNA prohibits base pairing at the sites of covalent adduct formation, since the hydrogen bonding positions are blocked. The locations of these wedged open regions may be subsequently mapped, after linearization of the DNA, by S1 nuclease cleavage.

BAA offers several distinct advantages over the enzymatic probes (S1, P1, BAL 31 nucleases (6-19)) in spite of the need for a second step which is required for detection of reaction. First, enzymes require stringent reaction conditions for their activity, whereas BAA can be used over a range of conditions of pH, temperature, salt concentrations, etc. Second, BAA is

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extremely sensitive to small regions of distorted DNA structure and, using it, we have identified several structural features that S1 nuclease alone does not recognize (McLean and Wells, unpublished results). Previous investigations revealed that S1 nuclease itself specifically recognizes structural aberrations in an otherwise double stranded DNA (6-16).

We previously reported (5) that BAA does not react with the junctions between right-handed B- and left-handed Z-DNA, whereas it did react with the single stranded loops of cruciforms present in the same recombinant plasmid molecule. Herein, we report that BAA reacts with B-Z junctions in supercoiled plasmids by using a different method of preparation of the probe and by varying the conditions used to detect its reaction with DNA.

The concentration of the pure aldehyde was determined using the fluorescence emission of the tricyclic product of reaction of BAA and adenosine. Also, we have quantitatively determined the extent of its reaction with adenosine under varying conditions of ionic strength, thus enabling an investigation of DNA structure as a function of salt concentration and pH (McLean, Lee and Wells, unpublished results).

The sites of reaction of BAA with B-Z junctions were mapped to the nucleotide level in a precise manner, and we conclude that BAA is an extremely useful, sensitive and specific probe for unusual DNA secondary structures over a wide range of conditions.

## MATERIALS AND METHODS

### Bromoacetaldehyde Preparation and Characterization.

Equal volumes of bromoacetaldehyde diethyl acetal (Aldrich) and 0.1 N sulfuric acid were heated gently under reflux for 1 hr. and then allowed to cool. The pale yellow solution was then carefully distilled at atmospheric pressure over 3-4 hrs. and the fractions boiling between 75-80°C were collected. To this colorless solution was added dropwise with stirring, 1 N sodium hydroxide, until the pH of the turbid mixture was 7.0. Bromoacetaldehyde was extracted from the mixture by shaking with 3 volumes of *tert*-butyl methyl ether (Aldrich). The organic phases were collected, pooled, dried by addition of solid magnesium sulfate, filtered and evaporated under an air stream to give a mixture of BAA and ether in an approximately 1:2 ratio. This solution was divided into aliquots and the residual ether was evaporated under an air stream before each use.

Using a stock solution of chloroacetaldehyde (generous gift of Dr. Leo Hall, this department) whose concentration had been determined previously

(20), a calibration curve was prepared for the reaction of a haloacetaldehyde with adenosine (21,22). 50 μl aliquots of various dilutions of chloroacetaldehyde or bromoacetaldehyde were reacted with 5 mM adenosine in 50 mM potassium phosphate (pH 6.1) (100 μl volume) for 10 min. in a boiling water bath. The reaction mixtures were diluted with 350 μl of 100 mM Tris•HCl (pH 7.6), and fluorescence was read at an excitation wavelength of 312 nm and emittance of 420 nm. In four separate determinations, the BAA concentration in the stock solution was found to be 2.62 ± 0.18 M. Proton NMR (Bruker 400 MHz), infra-red spectroscopy (Perkin-Elmer Model 283B) and chemical analysis with Tollens' reagent, showed the absence of the diethyl acetal starting material and the presence of this halogenated aldehyde. Proton NMR analysis of BAA dimethyl acetal shows a doublet for the methylene protons centered at approximately 3.27 p.p.m., a singlet at 3.32 p.p.m. for the two methyl groups and a triplet for the single proton on carbon one at 4.43 p.p.m. Similar analysis of a sample of BAA prepared as above from the diethyl acetal shows resonaces due to protons on ethanol and _t_-butyl methyl ether, in addition to a singlet at 3.38 p.p.m., which we attribute to the methylene protons. The triplet at 4.43 p.p.m. (from the starting material) is not present. These resonances were the only significant peaks, although a broad signal between 2.3-2.05 p.p.m. is observed. No resonance for the aldehydic proton is observed, although infra-red spectroscopy showed a strong signal at 1640 $cm^{-1}$ characteristic for the C=O stretch. In addition, a shoulder on a peak at 2900 $cm^{-1}$ is attributed to the stretching mode of the aldehydic C-H bond.

Addition of a small amount of the BAA solution to Tollens' Reagent, revealed the presence of an aldehyde. In contrast, neither ethanol nor _t_-butyl ether (the only organic contaminants in the product) gave a positive result. BAA dimethyl acetal gave a weak positive result very slowly, which we attribute to a slow hydrolysis of this compound to BAA in the highly basic solution.

We conclude that the major products of the hydrolysis of BAA diethyl (or dimethyl) acetal are BAA and the corresponding alcohol.

Plasmids and Enzymes.

pRW756 (13) and pRW777 (6) were characterized previously and are shown schematically in Fig. 1.

Topoisomeric samples of the DNAs were generated using ethidium bromide and calf thymus topoisomerase as described previously (6,23). The 123 bp ladder and the 1 kbp ladder markers were purchased from Bethesda Research

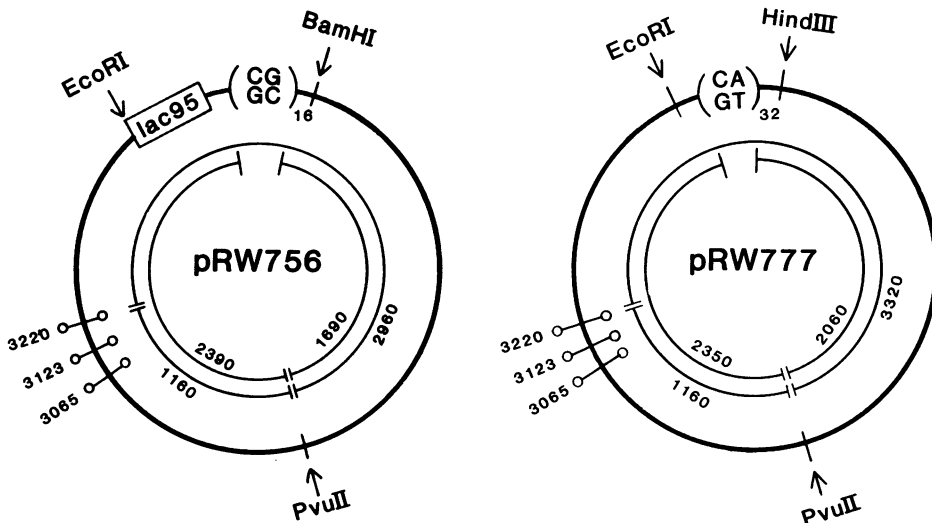


Figure 1. Physical maps of pRW756 and pRW777.
The pBR322 cruciform sites (3065, 3123, 3220) are shown. The sizes of the fragments from the PvuII sites to the B-Z junctions and to the cruciforms are shown for each plasmid.

Laboratories (BRL). All restriction endonucleases were from Boehringer Mannheim Biochemicals (BMB), S1 nuclease from Pharmacia/P-L Biochemicals, and P1 nuclease from BRL.

BAA Reactions.

For reaction of BAA with supercoiled plasmids, 1.5 μg of DNA in 100 μl of buffer (10 mM Tris•HCl, 0.1 mM EDTA, pH 7.6, with various additional salts) were incubated at 37°C for 2 h in the presence of 2% BAA. The excess BAA was removed by two extractions into diethyl ether; the DNA was precipitated with ethanol, redissolved in 15 μl of buffer containing 50 mM Tris•HCl (pH 7.4), 6 mM $MgCl_2$, 50 mM KCl, and 50 mM NaCl, and digested with PvuII for 2 h. This reaction mixture was brought to a final volume of 30 μl, (final concentrations of 40 mM sodium acetate, 50 mM NaCl, 1 mM $ZnSO_4$, pH 4.6), and digested with 14 U of S1 nuclease at 37° for 30 min. The S1 nuclease reaction was terminated by adding EDTA to 50 mM. The DNA fragments were then separated by 2% agarose gel electrophoresis as described (5,12-14).

For fine mapping experiments, the DNA pelleted after BAA reaction was redissolved in 30 μl of a suitable buffer and subjected to double digestion by restriction endonucleases which leave the fragment of interest with a

5'-overhang on one end and a 3'-overhang on the other. The 5'-overhang was filled-in with 5'-[$\alpha^{32}P$] dATP (Amersham) in the presence of cold dNTPs with DNA polymerase, Klenow fragment (BMB), and the labelled fragments were separated on 5% PAGE, eluted from the gel and precipitated. The DNA was redissolved in 30 µl of 40 mM sodium acetate, 50 mM NaCl, 1 mM $ZnSO_4$, pH 4.6, and pre-incubated at 37° for 15 min. Then 21 U of S1 nuclease were added, and the samples were incubated at 37°C for 0.3-1 min., when the reaction was stopped by addition of EDTA to 50 mM. The DNA was precipitated and redissolved in 80% formamide, 1% bromophenol blue, 1% xylene cyanol in TBE, and loaded onto a denaturing (7 M urea) polyacrylamide gel next to the appropriate sequencing ladder. Bands were visualized by autoradiography at -70°C using an intensifying screen.

## RESULTS

### Determination of BAA Concentration.

A major problem in the past with the use of BAA was that the concentration of the reagent in the reaction mixture was unknown. This was due to the fact that the free aldehyde is not commercially available and must be prepared by hydrolysis of the corresponding dialkyl acetal, with distillation of the aldehyde. This leads to a mixture of products from which the pure aldehyde is not readily obtained. Using a combination of the previously reported (4,24,25) methods for BAA preparation, we have obtained a high yield of product and have determined the concentration of free aldehyde. The products of reaction of CAA and BAA with adenosine are identical, namely 1-$N^6$-etheno A, and this compound fluoresces with an excitation wavelength of 312 nm and an emission wavelength of 420 nm. Using a stock solution of CAA whose concentration had previously been determined to be 4.0 M by a combination of titration procedures (20), we prepared a calibration curve of fluorescence at 420 nm of the product versus concentration of CAA in a solution containing an excess of adenosine. This enabled the determination of the concentration of BAA present in the distillation mixture by measuring the fluorescence produced due to its reaction with adenosine under forcing conditions such that rate differences between the two compounds could be ignored. Using this method we have determined the concentration of BAA as 2.62 ± 0.18 M.

### Effect of S1 Nuclease Cleavage Conditions on Detection of BAA Modified Regions.

The covalent modification of adenine and cytosine bases by BAA in a circular DNA does not lead to strand scission, hence a specific nuclease

(usually S1 nuclease) must subsequently be used to map the site(s) of reaction. By necessity, this step must be performed after linearization of the DNA with a suitable restriction enzyme, otherwise it would be impossible to distinguish fragments due to S1 nuclease reaction alone (6-17) from those due to S1 cleavage of BAA modified regions. The reaction of BAA occurs at the positions of hydrogen bonding with A and C. Thus it is envisaged that after linearization of the DNA, a region that was "single stranded" in the supercoiled molecule (and so reacted with BAA) will remain unpaired. This becomes a potential substrate for S1 nuclease which will first introduce a nick in the DNA and then cut across from the nick (10) to produce a fragment whose length is indicative of the site of initial reaction.

It is not known how much single-strandedness is necessary for BAA reaction to occur, nor is it known how much BAA modification is needed before S1 nuclease will recognize a putatively bulged-out region as a substrate. Hence, a quantitative determination of the relative amounts of single-strandedness (either in absolute terms or as a function of a dynamic equilibrium) present in different structural forms has not been feasible.

We previously reported (5) that BAA does not react with the junctions between right-handed B-DNA and left-handed Z-DNA in plasmids where reactions were detected with cruciform structures (which were present simultaneously). Thus, we concluded that B-Z junctions contain fewer than three unpaired nucleotides.

However, S1 nuclease alone recognizes and cleaves B-Z junctions (6,12, 13,15), and recent studies indicated (McLean and Wells, unpublished results) that the BAA/S1 combination is more sensitive to regions of structural perturbation than is S1 nuclease _per se_, provided that the concentration of S1 nuclease used to detect BAA modified bases is appreciably higher than that used in experiments involving nuclease treatment of supercoiled plasmids.

Therefore, we re-investigated the reaction of BAA with plasmids containing Z helices. For this study we used the same plasmids (pRW756 and pRW777) as those studied previously (5) and varied each of the reaction conditions in a controlled and quantifiable manner.

Fig. 2 shows the effect of varying both the concentration of S1 nuclease and the time of nuclease digestion, on the detection of BAA reaction with specific sites in supercoiled pRW756. In this experiment, the superhelical density of the plasmid was as isolated from the cell (determined by chloroquine gel electrophoresis to be $0.085 \pm 0.005$). At

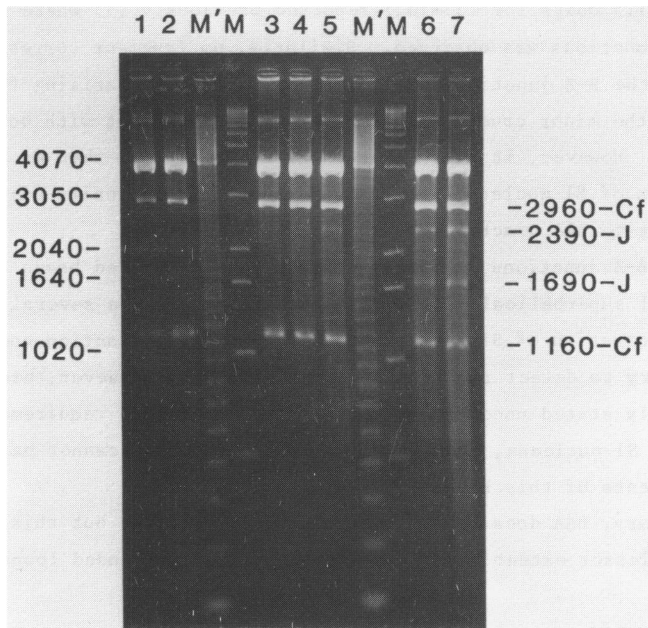


Figure 2. Effect of S1 nuclease conditions on the detection of BAA modified sites. pRW756 was incubated with BAA and cut with PvuII, as described in Materials and Methods, and divided into aliquots for treatment with S1 nuclease. Lanes 1-4, 1.4, 3, 5, and 7 units of S1 nuclease, respectively, for 15 minutes; lanes 5-7, 7, 14, and 28 units, respectively, for 30 minutes. M' is a 123 bp ladder (BRL) and M is a 1 kbp ladder (BRL). Size markers on the left refer to the 1 kbp ladder expressed in base pairs. Numbers on the right refer to the approximate sizes (in bp) of fragments arising from BAA reaction at the B-Z junctions (J) or at the cruciforms (Cf).

this superhelical density, both the 32 bp Z helix (and therefore the B-Z junctions) and at least one of the three possible cruciforms will be present (5). The BAA treatment of DNA was performed in one reaction which was then divided into aliquots after PvuII digestion, so that the only difference between the samples was the S1 nuclease treatment.

If BAA reacted at the B-Z junctions, PvuII digestion followed by treatment with S1 nuclease should give rise to two new fragments (approximately 1690 and 2390 bp), in addition to full length linearized DNA. Reaction with the single stranded loop of the minor cruciform of pBR322 (map position 3220) from which this plasmid is derived will give two fragments of length 1160 and 2960 bp.

Lane 1 in Fig. 2 represents the identical S1 nuclease reaction

conditions (1.5 units for 15 min.) reported previously (5) where no reaction of the B-Z junctions was observed. Similarly, no fragment corresponding to reaction at the B-Z junctions was found, but a fragment arising from BAA reaction at the minor cruciform was observed in agreement with our past results (5). However, it is evident (Fig. 2, lanes 2-7) that as the concentration of S1 nuclease increases, so does the intensity of two bands corresponding to BAA reaction at or near the Z helix.

Hence, B-Z junctions do indeed contain few non-paired bases at physiological superhelical densities, since it requires a several fold higher concentration of S1 nuclease to detect the BAA reaction compared to that necessary to detect reaction at the cruciforms. However, because of the previously stated uncertainty in the single stranded requirements of both BAA and S1 nuclease, such quantitative conclusions cannot be derived from experiments of this kind.

In summary, BAA does react at or near B-Z junctions but this reaction occurs to a lesser extent than that with the single stranded loops of cruciforms.

Effect of BAA Concentration and Method of Preparation.

Using these more forcing S1 nuclease conditions, we repeated our previous studies (5) using the same BAA sample that had been used for those experiments. Surprisingly, no reaction was observed with the B-Z junctions. This implies some difference in the nature of the two samples of BAA. An experiment which demonstrates this difference is shown in Fig. 3. Lanes 1-4 show the results of reaction of pRW756 at native superhelical density with varying concentrations of the BAA sample prepared previously (5) by a harsh (50% $H_2SO_4$) hydrolysis method. Fragments arising from reaction with the minor cruciforms are present at all BAA concentrations used in this study, whereas only the highest concentration of BAA (8%) gave rise to bands specific for reaction at the B-Z junctions.

Lanes 5-8 show the same experiment performed with a sample of BAA prepared by a much milder method (see Materials and Methods); lanes 6 and 7 show that reaction at B-Z junctions occurs at a much lower BAA concentration than with the previous BAA sample (lanes 1-4). In lane 8, no specific fragments due to reaction at the loop of the minor cruciform are visible, whereas bands are seen for this reaction at the lower concentration.

The following explanation is proposed. The average superhelical density of the plasmid in this study (as determined by gel electrophoresis in the presence of chloroquine) is $-0.060 \pm 0.005$. The B-Z transition for

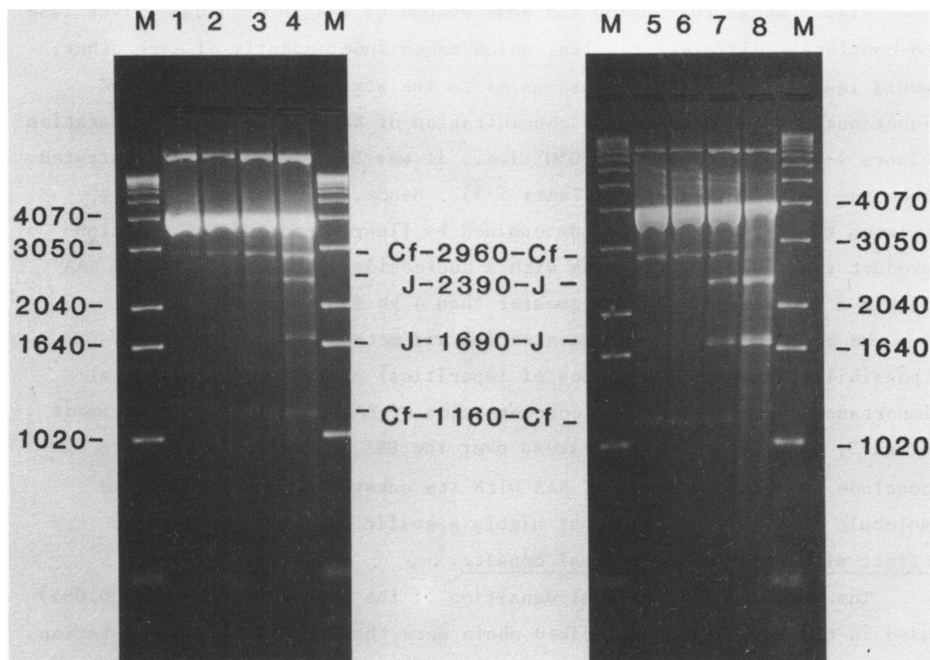


Figure 3. Effect of BAA concentration and method of preparation.
In lanes 1-4, pRW756 was treated with 1%, 2%, 4%, and 8% respectively, of a sample of BAA prepared by a harsh (50% $H_2SO_4$) hydrolysis (4), and in lanes 5-8, pRW756 was treated with 1%, 2%, 4%, and 8% respectively, of a sample of BAA prepared by the milder conditions outlined in Materials and Methods. After restriction with PvuII, the DNA was treated with 14 units of S1 nuclease for 15 minutes. M is a 1 kbp ladder (BRL), and the numbers on the extreme left and right refer to the sizes of the markers in base pairs. The numbers in the center refer to the sizes of fragments arising from reaction of BAA at the B-Z junctions (J) or at the cruciforms (Cf).

pRW756 has been shown to occur at $-\sigma = 0.04$, and results in the loss of 5.7 supercoils (13). Thus the effective superhelical density is approximately -0.045. The extrusion of the major cruciform in pBR322 has been shown to occur at a midpoint superhelical density of -0.057 (16). Thus, Z-DNA formation will shift the average superhelical density of the population back to a point at which only a small proportion of the molecules will be under a high enough torsional strain to have extruded the cruciform. The reaction of BAA with supercoiled plasmids has been shown to unwind the DNA to a small extent (5) and it may be that reaction of BAA with the B-Z junctions causes sufficient unwinding to destabilize the cruciform structure, and thus no reaction at the loop is seen.

Fig. 3 shows that using the same volume of BAA in each case gives rise to completely different results, which taken independently of each other, would lead to opposite conclusions as to the structural nature of B-Z junctions. We determined the concentration of BAA in the first preparation (lanes 1-4) to be 0.49 M ± 0.05M; i.e., it was 5-6 times less concentrated than the second preparation (lanes 5-8). Hence, a correlation exists between the concentration as determined by fluorescence of the reaction product formed by BAA reaction with a nucleoside and the reaction of BAA within a supercoiled plasmid greater than 4 kb in length.

We conclude that the concentration and method of preparation (possibility of different types of impurities) of BAA used is of vital importance. Even the lowest concentration of BAA used in this experiment (Fig. 3, lane 1) is in high excess over the DNA, and this leads us to conclude that the reaction of BAA with its substrate in a supercoiled molecule is relatively slow but highly specific.

Effect of Negative Superhelical Density.

The negative superhelical densities of the plasmids (0.060 and 0.085) used in the experiments described above were those obtained upon isolation of the plasmids from *E. coli*. Therefore, we investigated the reactions of BAA with structural features in this plasmid, in a controlled manner using the optimum conditions, as a function of superhelical density.

Topoisomer populations of pRW756 were prepared as described previously (6,23), and the average negative superhelical density of each population was calculated using a fluorescence enhancement method (23). The values obtained were corrected for the relaxation which occurs on formation of a Z helix and/or cruciforms, and these corrected values are in good agreement with those observed on chloroquine gel electrophoresis.

Fig. 4 shows the results of treatment of pRW756 with BAA as a function of negative superhelical density. As expected, the intensity of bands arising from reaction at the B-Z junctions increases with increasing superhelical density and plateaus at a value of $-\sigma = 0.070$ (0.056 corrected). At this superhelical density, bands due to reaction of BAA at one or more of the cruciforms became apparent, and in the next topoisomer family (lane 5), reaction at the cruciforms becomes predominant, such that the larger of the two fragments due to reaction at the B-Z junctions is now cleaved by S1 nuclease into two smaller fragments of approximately 1.1 and 1.3 kbp. Furthermore, it can be seen (lanes 4 and 5) that an increase in negative superhelical density produces a different length of fragment due to

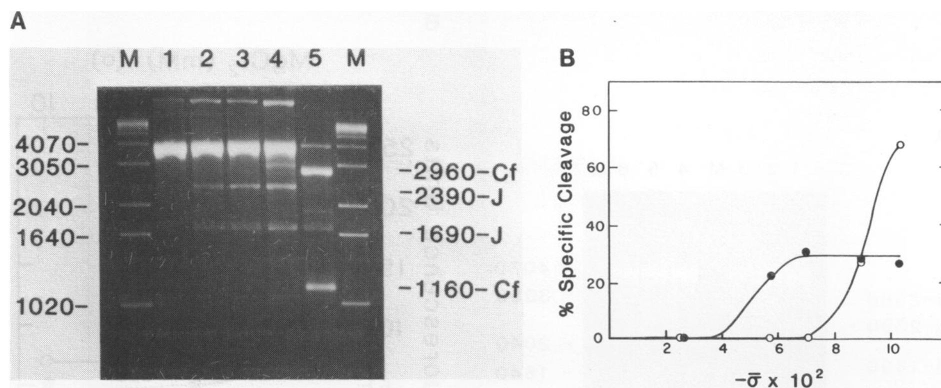


Figure 4. Effect of negative superhelical density on BAA reactive sites. Topoisomer populations of pRW756 were incubated in the presence of 2% BAA, digested with PvuII, and treated with 14 units of S1 nuclease for 15 minutes. The mean negative superhelical density in each lane was: 1) 0.026; 2) 0.057; 3) 0.070; 4) 0.089; 5) 0.103.
Panel A; Gel photograph. Panel B; Densitometric trace of data in Panel A. A photographic negative of Panel A was traced using a Bio-Rad 620 video densitometer. The increase in intensity (in per cent) of the fragment of approximately 1690 bp served to quantitate the BAA reaction at the B-Z junctions (filled circles) whereas the increase in the 2960 bp fragment quantitated the BAA reaction at the cruciforms (open circles). Superhelical densities shown are uncorrected for the transition of the $(CG)_{16}$ segment to the left-handed form.

specific reactions at the cruciforms, and this size difference is in close agreement with BAA preferentially recognizing the sub-minor cruciform (pBR322 coordinate 3123). Fig. 4 also shows this data in graphical form from densitometric quantitation of the gel photograph shown (Panel B).

These results show that when both structural features are present in the majority of the molecules (i.e., at the highest superhelical density), the loops of the cruciforms are preferred substrates for BAA. Again, it is tempting to infer that B-Z junctions thus contain fewer unpaired nucleotides than the loops of cruciforms, but since we have no knowledge at present of the effects on BAA reactions of nearest neighbor sequence interactions or the kinetics of formation of regions of single-strandedness, it is not possible to make rigorous structural statements from comparisons of this kind.

Effect of Environmental Conditions.

Changes in ionic strength, pH, temperature, etc. can have dramatic effects on DNA structure (16,17,26-29), and indeed Z-DNA was first observed as a consequence of a high concentration of sodium chloride (30-33); the B

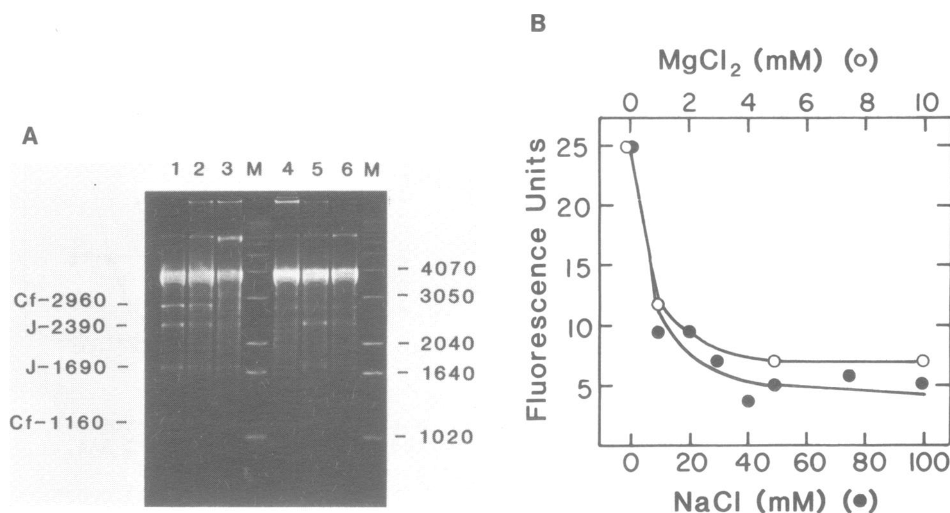


Figure 5. Effect of salt concentration on BAA reactivity.
Panel A; effect of increasing NaCl or $MgCl_2$ on the reactivity of BAA toward pRW756. Supercoiled pRW756 was treated with 2% BAA in 10 mM Tris•Cl, 0.1 mM EDTA, pH 8.0 (T.E. buffer) (lane 1), with the following salt concentrations; 10 mM NaCl (lane 2), 50 mM NaCl (lane 3), 100 mM NaCl (lane 4), 1 mM $MgCl_2$ (lane 5), 10 mM $MgCl_2$ (lane 6). After digestion with PvuII, the DNA was treated with 14 units of S1 nuclease for 15 minutes. The numbers on the right refer to the sizes of the fragments of the 1 kbp ladder (BRL), and the numbers on the left refer to the approximate sizes of fragments (in bp) arising from reaction at the B-Z junctions (J) or at the cruciforms (Cf). Panel B; effect of increasing NaCl or $MgCl_2$ on reaction of BAA with adenosine as measured by fluorescence of the reaction product (arbitrary units).

to Z transition was subsequently shown to be influenced by micromolar concentrations of certain divalent metal ions (28). Thus, it would be of considerable interest to study the effects of such environmental conditions on conformational changes in DNA, but using enzymatic probes to study this question leads to the uncertainty of whether one is effecting a change in the DNA structure, or merely changing the ability of the probe to recognize its substrate. Chemical probes (such as BAA) might surmount this problem.

We first investigated the reactivity of adenosine toward BAA over a range of salt concentrations (Fig. 5). It can be seen that the amount of reaction decreases significantly when low salt concentrations (10 mM NaCl or 1 mM $MgCl_2$) are added to the reaction mix of 10 mM Tris•Cl, 0.1 mM EDTA, pH 8.0 (T.E. buffer). Although it is still not clear whether this is due to an effect of ionic strength on the reactivity of BAA *per se*, it is evident that above certain critical salt concentrations, any further increase in ionic

strength has a minimal effect on BAA reactivity. We have successfully employed BAA as a structural probe at concentrations of up to 4 M NaCl (McLean, Lee and Wells, unpublished results). We have also investigated BAA reactivity in various electrophoretic buffer systems, restriction enzyme buffers and in S1 buffer using adenosine and poly d(A) as substrates; while the reactivity is less than that in T.E., these buffer systems are still compatible with the use of BAA (data not shown).

The effect of varying the environmental conditions on the reaction of BAA with B-Z junctions and/or cruciforms in pRW756 was investigated (Fig. 5). Lane 1 is the result of BAA reaction with pRW756 ($-\sigma = 0.060$) in T.E. followed by PvuII digestion and treatment with S1 nuclease as described in Materials and Methods. In lanes 2-4, the BAA reaction was performed in T.E. with increasing concentrations of sodium chloride as indicated in the figure legend. It can be seen that as the salt concentration increases so the amount of BAA reaction at both the B-Z junctions and the cruciform decreases, and some non-specific reaction occurs. Is this an effect of ionic strength on the DNA structures, or simply the result of less reaction of BAA as shown in Panel B. From Fig. 5B it is evident that the effects of NaCl and $MgCl_2$ on BAA reactivity are similar, notwithstanding the 10-fold difference in concentration required to produce the same effect in each case. Thus, if the decreased reaction apparent in lanes 2-4 is merely a reflection of this effect, then a similar result should be obtained by increasing the concentration of $MgCl_2$. Lane 5 shows the result of BAA reaction with pRW756 in the presence of 1 mM $MgCl_2$; it is apparent that while the reaction with the B-Z junction is relatively unaffected by this concentration, the reaction with the cruciform is significantly diminished. In lane 6, where the $MgCl_2$ concentration is now 10 mM, the bands due to reaction with B-Z junctions are also diminished in intensity. From this selective effect on different reactive sites, and from the different effects of increasing NaCl and increasing $MgCl_2$ on BAA reaction with pRW756, we infer that the observed salt effects are not due solely to a decrease in reactivity of the probe, but are also due to an influence of local ionic conditions on DNA structure. To investigate the role of the anion in this phenomenon, we substituted sodium bromide and magnesium acetate for sodium chloride and magnesium chloride in the reaction mix and found identical results to those reported above.

Thus, we feel that the use of BAA as a probe for DNA secondary structure offers several distinct advantages over the use of enzymatic

probes, in that it can be used over a wide range of conditions, and that its reactivity in any given solvent can be easily determined.

Fine Mapping of BAA Modified Bases.

In order to determine the sites of BAA reaction with the sequences in pRW756, we have developed a protocol for obtaining nucleotide level resolution of the modified bases. This was necessary to localize the reaction of the B-Z junctions since it is plausible that the dC residues in the left-handed helix may themselves react with the probe.

Our approach differs from those previously reported (24,25) in several features. Haniford and Pulleyblank (24) mapped BAA sites by exploiting the enhanced sensitivity of modified dA residues to acid-induced depurination, or the increased sensitivity of modified dC residues to cleavage by piperidine. Our method is more similar to that used previously by Kohwi-Shigematsu and Kohwi (25), in that it involved S1 nuclease treatment of 3'-end labelled DNA fragments, but we feel that our approach gives greater sensitivity and resolution of the modified sites.

After BAA reaction, restriction endonuclease cleavage and 3'-end labelling, the fragment is isolated from a native acrylamide gel and precipitated. The pellet is then redissolved in S1 nuclease buffer (40 mM sodium acetate, 50 mM NaCl, 1 mM $ZnSO_4$, pH 4.6) and digested with a high concentration of S1 nuclease for a very short time (0.3 - 1 min.). The reaction is terminated by the addition of EDTA, and the pelleted DNA is then electrophoresed on a denaturing polyacrylamide gel next to the appropriate sequencing ladder. By varying these S1 nuclease conditions (i.e., a high enzyme concentration for a short time), we aim to limit the amount of complete severance of the fragment at modified sites and thus increase the amount of single stranded nicks so as to improve the detection of minor reactive sites.

Fig. 6 shows the result of such an experiment with pRW756. The first four lanes are Maxam and Gilbert (34) sequencing reactions and the next lane is pRW756 at native superhelical density treated as outlined above. No bands are visible inside the $(CG)_{16}$ block, but a number of quite intense bands are found at the interfaces of this sequence with the vector. Thus, we conclude that BAA reacts with B-Z junctions and not the left-handed helix itself. The right-most lane is the same DNA sample, except that it was treated with 1 unit of P1 nuclease at pH 7.5 for 1 minute, instead of with S1 nuclease. Qualitatively, the result is the same except that there is slightly more exonucleolytic activity apparent than with S1 nuclease.

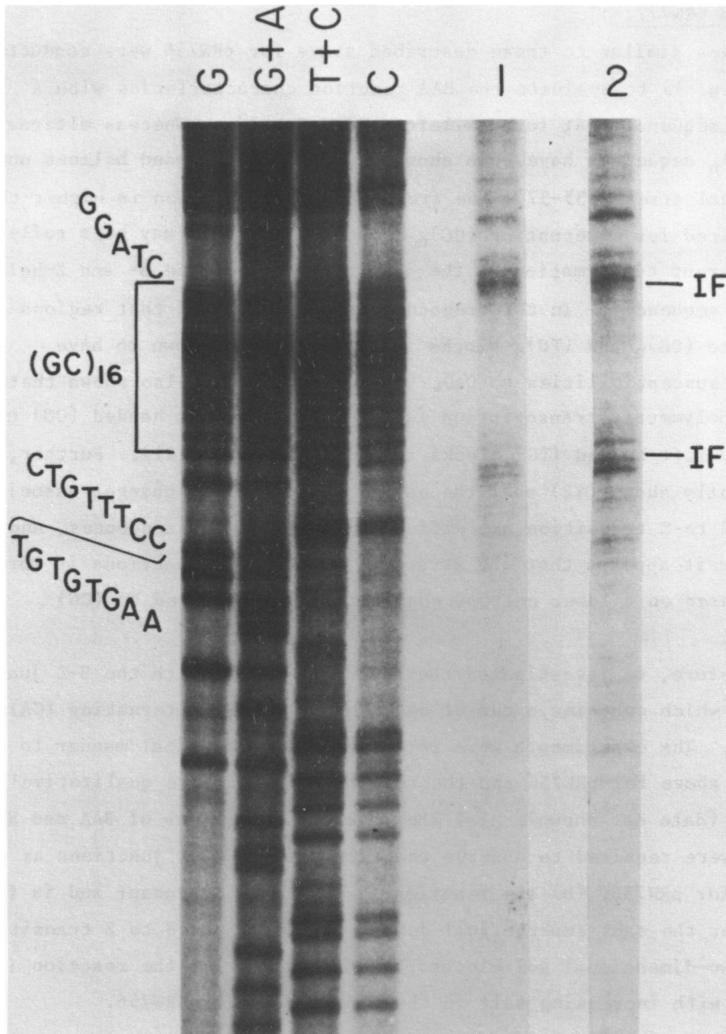


Figure 6. Fine mapping of BAA reaction at the B-Z junctions in pRW756. Reactions were performed as described in Materials and Methods. Shown on the left are Maxam and Gilbert sequencing lanes. Lane 1; Autoradiograph of sites mapped using S1 nuclease (21 units/1 min.); Lane 2; Autoradiograph of sites mapped using P1 nuclease (1 unit/1 min.). Because of 3' end labelling, the sequence of the Z-forming insert reads $(GC)_{16}$.

The sequence shown reads: 3'-AAGTGTGTCCTTTGTC$(GC)_{16}$CTAGG-5'

IF designates the DNA sequence interface between the tract of perfectly alternating $(GC)_{16}$ and the vector sequences. Studies with BAA as well as other probes (15,39,40) indicate that the interface may not necessarily be the same as a B-Z junction.

Control studies (no BAA reaction) with similar plasmids revealed the absence of specific reaction sites when treated with S1 nuclease.

Studies on pRW777.

Studies similar to those described above for pRW756 were conducted with pRW777 (Fig. 1) to evaluate the BAA reaction characteristics with a different sequence that forms a left-handed Z-helix. Whereas alternating $(TG)_n \cdot (CA)_n$ sequences have been shown to adopt left-handed helices under superhelical stress (35-37), the free energy of formation is higher than that required for alternating $(CG)_n$ sequences and this may be a reflection of a different conformation at the junctions between the B- and Z-helices for these sequences. In this regard it should be noted that regions adjacent to $(CG)_n$ and $(TG)_n$ blocks in plasmids were shown to have different susceptibilities to $OsO_4$ (38-40) and it was also shown that E. coli RNA polymerase transcription is terminated by left-handed (CG) blocks but not by left-handed (TG) blocks in one orientation (41). Further, we have recently shown (42) that the enthalpy and entropy changes associated with the B to Z transition are different for these two sequences, and in particular it appears that the structure of the (TG) junctions is more ordered based on a lower entropy change for (TG) compared to (CG) junctions.

Therefore, we investigated the reactions of BAA with the B-Z junctions in pRW777 which contains a run of 64 bp of perfectly alternating (CA)•(TG) (Fig. 1B). The experiments were performed in an identical manner to those described above for pRW756 and the results obtained were qualitatively identical (data not shown): (a) the same concentrations of BAA and S1 nuclease were required to observe reaction with the B-Z junctions as were required for pRW756; (b) the reaction is supercoil dependent and is first observed at the same superhelical density at which the B to Z transition is seen on two-dimensional gel electrophoresis (35); (c) the reaction is decreased with increasing salt in the same way as for pRW756.

Thus, no significant differences were observed between the structures of the B-Z junctions for (CG) and (TG) sequences using this probe. Also, we conclude that BAA must recognize a different structural feature in B-Z junctions than $OsO_4$, although details of this conformational preference remain to be clarified.

DISCUSSION

These results demonstrate that the method of preparation and concentration of BAA, and the reaction and analysis conditions (S1 nuclease

concentration) influence the conclusions made regarding the types of unusual DNA structures detected.

We report herein a simple method for the preparation of BAA in high yield along with a determination of the concentration of the free aldehyde in solution. CAA is less reactive towards DNA than the bromo- derivative, and this may reflect the poorer leaving group ability of chloride ion relative to bromide. Thus BAA is the reagent of choice for use as a probe for altered DNA conformations. However, CAA, being more stable over extended time periods, can be used to determine the concentration of BAA in any given preparation. CAA also reacts with B-Z junctions and cruciforms (data not shown and 48), but slower than does BAA, and this lessened reactivity is more apparent when investigating minor reactive sites such as those present in some prokaryotic promoter sequences (McLean and Wells, unpublished).

We recently reported the occurrence of unusual DNA structures in the adenovirus genome (43) as evidenced by hyperreactivity to S1 nuclease and BAA. Included in that study (43) were BAA reactions on plasmids containing direct repeat motifs that had been previously postulated to adopt slipped structures (44-47), and it was found that these sequences were not reactive towards BAA. Using the reaction conditions described above, we re-investigated these plasmids (pCol-Md, pGF3, pβ1BR16 and pHTΔ5'-186) to determine whether this non-reactivity was a consequence of the reaction conditions used previously (5,43). However, we still observed no reaction of BAA with these sequences (data not shown). This was an unexpected finding in view of the proposed structure of these direct repeat sequences. If a slipped structure is indeed formed, one would expect a gross unpairing of the primary helix which would result in a strong reaction with BAA comparable to (and possibly even stronger than) that found at the loops of cruciforms. The observed non-reactivity may imply that these structures do not in fact exist, or that there is some degree of intra- or intermolecular interaction between these unpaired regions which prohibits their reaction with BAA at neutral pH.

However, we have observed BAA reaction with the direct repeat sequences present in the joint region of herpes simplex virus type 1 (17). We believe that this segment of the genome does not form a slipped structure, but instead adopts a novel DNA conformation termed "anisomorphic DNA." In addition, we have found that simple repetitive oligopurine•oligopyrimidine

sequences exhibit a striking pattern of BAA reactivity which may indicate a different higher order structure (J.C. Hanvey, J. Klysik, D. Collier, and R.D. Wells, manuscript in preparation).

At present it is uncertain as to the degree of single-strandedness or base conformation necessary for BAA reaction with regions of otherwise double stranded DNA. Similarly we have little knowledge of how much BAA modification is necessary before S1 nuclease will recognize its substrate in the linearized molecule. Future studies on synthetic oligonucleotides containing mismatched or unpaired bases may resolve these important questions.

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