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Two New Photoaffinity Polyamines Appear To Alter the Helical Twist of DNA in Nucleosome Core Particles[†,‡]

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ABSTRACT: Two new photoaffinity derivatives of polyamines have been synthesized by the reaction of spermine or spermidine with methyl 4-azidobenzimidate. The new compounds were purified chromatographically and characterized by several methods including proton magnetic resonance spectroscopy. The spermine derivative is N^1-ABA-spermine [(azidobenzamidino)spermine], and the spermidine derivative is a mixture of N^1- and N^8-ABA-spermidine. ABA-spermine stabilizes nucleosome core particles in thermal denaturation experiments, with similar but not identical effects when compared with the parent polyamine, spermine. In circular dichroism experiments, ABA-spermine was capable of producing a B → Z transition in poly(dG-m^{5}dC) at a concentration of 30 μM, compared with 5 μM required to produce the same effect with spermine. On the other hand, ANB-spermine [(azidonitrobenzoyl)spermine; Morgan, J. E., Calkins, C. C., & Matthews, H. R. (1989) *Biochemistry 28*, 5095–5106] stabilized the B form of poly(dG-br^{5}dC). ABA-spermine is a potent inhibitor of ornithine decarboxylase from *Escherichia coli*, giving 50% inhibition at 0.12 mM, while ANB-spermine is a modest inhibitor, comparable to spermine or spermidine. Under conditions of nitrogen-limited growth, yeast take up ABA-spermine and ABA-spermidine at approximately one-third to half the rate of spermidine or spermine. In contrast, ANB-spermine was not significantly taken up. The photoaffinity polyamines were used to photoaffinity label the DNA in nucleosome core particles, and the sites of labeling were determined by exonuclease protection. All photoaffinity reagents showed both nonspecific labeling and specific sites of higher occupancy. However, the positions of the sites varied: the ANB-spermine sites confirmed those previously reported (Morgan et al., 1989); the ABA-spermine and ABA-spermidine sites were spaced at 9.8 bp intervals from the 3′ end of each DNA strand. This observation, together with the effect of spermine on the circular dichroism of DNA in nucleosome core particles, implies that polyamines alter the helical twist of DNA in nucleosome core particles. The ABA-polyamines are offered as general-purpose photoaffinity polyamine reagents.

Polyamines are small polycations found in all living cells (Tabor & Tabor, 1984). Cells may be depleted of polyamines by inhibition of their synthesis through the use of drugs or mutations (Pegg, 1988). Whenever polyamine levels are depleted, cell growth is severely inhibited or completely stopped and the cells may die (Porter & Bergeron, 1988). Ornithine decarboxylase is a very highly regulated enzyme in the polyamine synthesis pathway in mammalian cells, and its elevation is a very early response to agents that stimulate cell proliferation (Davis et al., 1988).

In spite of these indications of the essential role of polyamines in cell proliferation and other processes, very little specific information is available concerning the function(s) of polyamines in cells at the molecular level. One function emerging as essential is the modification of a protein synthesis initiation factor by the covalent addition of spermidine to a

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specific lysine residue to form deoxyhypusine which is oxidized to hypusine (Smit-McBride et al., 1989; Park et al., 1988). However, it is likely that polyamines play additional roles in cells. In particular, given their polycationic nature, polyamines are expected to bind to nucleic acids in cells, as they do in vitro (Morgan et al., 1986; Tabor & Tabor, 1984, 1976; Braunlin et al., 1982). At high concentration, polyamines can condense DNA into structures that have been characterized by electron microscopy and other methods (Sen & Crothers, 1986; Porschke, 1984). The concentrations required for this massive condensation resemble the concentrations found in some phage but appear to be higher than the concentrations to which nuclear DNA is exposed in transcriptionally active cells (Morgan, 1988).

At lower concentrations, polyamines bind to DNA, raising the stability of the DNA duplex, but do not precipitate the DNA (Morgan et al., 1986; Tabor & Tabor, 1984, 1976; Braunlin et al., 1982). The binding can be accounted for quantitatively by assuming no sequence dependence to the binding, when random sequence DNA is used (Morgan et al., 1986). However, theoretical studies have predicted that association of spermine would induce bending in specific DNA sequences so as to open the minor groove (Feuerstein et al., 1988, 1990, 1986). Although some indirect experimental evidence of polyamine-induced bending of DNA has been reported (Basu et al., 1987; Marquet & Houssier, 1988; Plum & Bloomfield, 1990; Morgan et al., 1989), direct proof of such bending has not yet been published.

In the cell, DNA is tightly associated with histones and other proteins, but estimates of the charge neutralization by histones indicate that at least 40% of the DNA phosphates remain unneutralized (Wells, 1986), so that polyamine binding to chromatin is expected to occur in cells, as it does in vitro (Morgan et al., 1987). Polyamine binding to nucleosomes and to nucleosome core particles, the basic subunit of chromatin structure, has been studied in vitro (Blankenship et al., 1987; Morgan et al., 1987). Binding of polyamines alters the conformation of the DNA in nucleosome core particles, as indicated by circular dichroism, although the nature of the alteration is uncertain (Morgan et al., 1987). Binding is primarily electrostatic and raises the thermal stability of nucleosomes or nucleosome core particles. Secondary, nonelectrostatic, effects on the binding also occur. This stabilization of chromatin is likely to occur in vivo, since the polyamine:DNA ratios used for the in vitro experiments are consistent with those expected in vivo (Morgan, 1988).

It has been proposed that nucleosome stabilization might be a dynamic effect in vivo, with polyamines helping to coil chromatin in its inactive state. The structural transition that occurs in chromatin when it becomes active would then be facilitated by the acetylation of polyamines, reducing their charge and their effect on nucleosome stability (Morgan et al., 1987). Histone acetylation is strongly correlated with the structure of transcriptionally active chromatin (Ramanathan & Smerdon, 1989; Libertini et al., 1988; Lin et al., 1989; Hebbes et al., 1988; Ausio & van Holde, 1986; Johnson et al., 1987; Waterborg & Matthews, 1983; Matthews, 1988; Allegra et al., 1987), and the enzyme that acetylates histones will also acetylate spermidine and spermine (Blankenship & Walle, 1977, 1978; Libby, 1978, 1980). Hence, histone and polyamine acetylation might act synergistically to destabilize inactive chromatin structure (Morgan et al., 1987; Blankenship & Walle, 1978).

In order to test these proposals, we are developing the use of photoaffinity polyamines for the determination of polyamine binding sites (Morgan et al., 1989). A photoaffinity reagent can be allowed to find its binding site, in the dark, and then be exposed to light, which activates the photoaffinity group which then binds covalently to nearby chemical groups, thus covalently labeling the binding site. One such polyamine, ANB-spermine, has been synthesized and used to map binding sites on nucleosome core particles (Morgan et al., 1989).

The ANB derivatization of spermine removes one of the charges, resulting in the compound resembling acetylspermine, or, more distantly, spermidine, more closely than spermine. Also, the bulky and hydrophobic ANB group has a rather long and flexible tether to the closest charged group, possibly allowing for undesired interactions by the ANB group. In recognition of these problems, we have attempted to develop other polyamine photoaffinity reagents based on diazotrifluoropropionyl (DTFP) or azidobenzamidino (ABA) derivatization. Unfortunately, DTFP-spermine is unstable, appearing to undergo an internal reaction resulting in a compound with a charge of 2+ which is not photoactivatable. In this paper we report the development of ABA-spermine and ABA-spermidine, which retain the charge of the parent compound and have a tether up to 0.5 nm shorter than the tether in ANB-spermine. We show that ANB-spermine does not promote the B $\rightarrow$ Z transition in poly(dG-m^{5}dC), whereas ABA-spermine does, and that ANB-spermine is not taken up by yeast cells, whereas ABA-spermidine and ABA-spermine are. Thus, the ABA compounds appear to be better models than ANB-spermine for polyamines in general. We have used ABA-spermine and ABA-spermidine to map their binding to DNA in nucleosome core particles, and we report the presence of specific binding sites superimposed on a background of nonspecific binding.

We present the ABA-polyamines as potential general-purpose photoaffinity polyamines that could be used to label polyamine binding sites in many other systems.

Materials and Methods

Synthesis of ABA-spermine. (Azidobenzamidino)spermine (ABA-spermine) was synthesized from methyl 4-azidobenzimidate (MABI, Pierce) and spermine tetrahydrochloride (Aldrich) (Andreasen et al., 1981). MABI (147 μmol) was dissolved in 30% acetonitrile at a concentration of 40 mM, and the pH was adjusted to 10 by using 0.5 N NaOH. From this, 3 mL was added to an equal volume of 20 mM spermine (60 μmol), pH 10, in H_2O. Thus the reaction was performed at a 2:1 molar ratio of MABI to spermine dissolved in 15% acetonitrile at pH 10. The reaction was stirred at room temperature for 24 h. Further incubation did not yield more product. The product was evaporated to 0.3 mL with a stream of N_2 gas. Pyridine, acetic acid, and 2-propanol were then added to the reaction mixture such that the final volume was 1.20 mL and the buffer consisted of pyridine/acetic acid/water/2-propanol (1:1:2:4). This mixture was injected onto a semipreparative silica HPLC column (Rainin) in 400-μL aliquots. The product was eluted with the above pyridine/acetic acid/water/2-propanol mixture at a flow rate of 3 mL/min. Fractions of 1.2 mL were collected and analyzed by thin-layer chromatography as described below. The fractions containing ABA-spermine were pooled to yield a final volume of 20 mL.

The product was further purified on a sulfopropyl-Sephadex column as follows. The acetate concentration in the pooled fractions was calculated, and an equal molar amount of NH_4OH (14.8 M) was added. The pH of the solution was adjusted to 7 with acetic acid. The sample was then diluted with H_2O such that the final NH_4OAc concentration was 0.6

M. The sample in 0.6 M NH_4OAc was loaded onto a 1 × 20 cm sulfopropyl-Sephadex column, and the column was washed with 20 mL of 0.6 M NH_4OAc and eluted with a gradient of 80 mL of 0.6 M NH_4OAc to 1.6 M NH_4OAc, followed by a wash of 20 mL of 1.6 M NH_4OAc. Again, the fractions (10 mL/fraction) from the column were characterized by thin-layer chromatography. The fractions containing ABA-spermine were pooled and diluted 2-fold with H_2O before being lyophilized. The lyophilized material was resuspended in 5 mL of H_2O.

Characterization. The thin-layer chromatography method was developed by Blankenship and Walle (1977) and modified by Morgan et al. (1989). Spots containing polyamines were further identified by use of [^{14}C]polyamines. Thus, with [^{14}C]polyamines, spermine and spermidine are UV-transparent, radioactive, and ninhydrin positive; ABA-spermine and ABA-spermidine are UV-dark, radioactive, and ninhydrin positive, MABI is UV-dark but ninhydrin negative. A small amount of an impurity that was radioactive and UV-dark but ninhydrin negative was observed. This is probably diderivatized ABA-polyamine. Mobility values, R_f, for the following compounds were determined as follows: spermine (0.45), ABA-spermine (0.55), reduced ABA-spermine (0.51), spermidine (0.59), ABA-spermidine (0.69), and MABI (0.98).

The yield of the reaction depended primarily on the age of the MABI preparation. MABI appears to be unstable and to break down during storage, perhaps to azidobenzamide (Inman et al., 1983). With fresh MABI the reaction was essentially quantitative, although a number of minor products were seen in the reaction mixture, necessitating the chromatographic purification of ABA-spermine and ABA-spermidine described above.

The extinction coefficients for ABA-spermine, ABA-spermidine, and reduced ABA-spermine were determined by a primary amine assay based on a method developed by Mokrasch (1970). The assay is performed with trinitrobenzenesulfonate and boric acid. Standard curves were prepared for spermine, acetylspermine, spermidine, and acetylspermidine. Samples of 50 μL each were mixed with 50 μL of 0.2 N H_3BO_3, pH 9.0 with NaOH, and 10 μL of 1% trinitrobenzenesulfonate. The samples were then incubated at room temperature for 20 min. Finally, 200 μL of methanol was added to each sample, and they were placed on ice to stop the reaction. The A_{420} for each sample was read, and standard curves were plotted. The extinction coefficients were estimated by first determining the concentration of the ABA compound stock solution by using the trinitrobenzenesulfonate assay. Then, the absorbance maximum of the stock solution was found from the ultraviolet spectra of the respective ABA compounds.

Photolysis of the purified ABA-spermine was performed on a 0.025 mM (1 mL) solution in cacodylate buffer. Typically, samples were exposed to 302-nm light from an Ultra Violet Products TM-36 transilluminator (7000 μW/cm^2) for 20-s intervals. The transilluminator was placed 8 cm above the stirred sample. A polystyrene filter was placed between the light source and the sample to filter out the most of the ultraviolet light below 300 nm. Aliquots taken after each 20-s interval were analyzed on a Cary 210 UV–visible spectrophotometer and by thin-layer chromatography.

The azido group of ABA-spermine was reduced to an amino group by modification of a procedure originally described by Staros et al. (1978). Dithiothreitol (100 μL of a 56 mM solution) was added to 2 mL of a 0.345 mM solution of ABA-spermine adjusted to pH 9.0 with 0.1 N NaOH. The reaction was stirred at room temperature for 30 min, and formation of the reduced ABA-spermine product was followed by thin-layer chromatography and the decrease in pH to 6.7. Thin-layer chromatography showed only one product to be formed during the reaction. Once the reaction was complete, it was diluted with 1.8 M NH_4OAc and H_2O to yield an NH_4OAc concentration of 0.8 M. This sample was loaded onto a sulfopropyl-Sephadex column, and the column was washed with 10 mL of 0.8 M NH_4OAc. The reduced ABA-spermine was eluted with a 40-mL gradient of 0.8–1.8 M NH_4OAc. The column fractions were characterized by thin-layer chromatography, and the fractions containing reduced ABA-spermine were combined. The pool fractions were diluted 2-fold with H_2O, frozen, and lyophilized. The purified reduced ABA-spermine was resuspended in 5 mL of H_2O. The concentration of reduced ABA-spermine was determined by the trinitrobenzenesulfonate assay described above.

Nuclear Magnetic Resonance Spectrum. ABA-spermine (2.7 μmol, dissolved in distilled deionized H_2O) was lyophilized. The sample was resuspended in 0.5 mL of ddH_2O, lyophilized again, and resuspended in 0.7 mL of 99.96% D_2O (Aldrich). The sample was filtered through a 0.45-μM filter (Millipore HV) and transferred to a 5-mm NMR tube (Wilmar no. 528). The pD was 7.0. The ^{1}H spectrum was determined on a GE Omega 500 Fourier transform instrument. The sample was scanned 64 times with a 6-μs pulse and an 8-kHz sweep width after a delay of 1 s.

Ornithine Decarboxylase. Ornithine decarboxylase was obtained from Sigma as a lyophilized, partly purified extract of *Escherichia coli*. L-Ornithine-*carboxy*-^{14}*C* hydrochloride (16.3 Ci/mol) was also obtained from Sigma. Enzyme activity was measured according to a modification of the published methods (Gaines et al., 1989). Microfuge tubes, 1.5 mL without lids, served as reaction vessels. Each tube was placed in a 25-mL screw-top borosilicate glass scintillation vial that contained a polypropylene well (Kontes). NCS tissue solubilizer (0.2 mL; Amersham) was placed in each well to absorb CO_2. The reaction volume was 0.1 mL, containing 25 mM Tris, 5 mM dithiothreitol, 1 mM $MgCl_2$, 0.1 mM pyridoxal 5-phosphate, 0.1 mM EDTA, pH 7.6, 0.02–0.05 unit of freshly dissolved ornithine decarboxylase, 0.6 μCi of L-ornithine-*carboxy*-^{14}*C* hydrochloride, and polyamines where indicated.

After addition of the enzyme, the vials were incubated at 37 °C for the time indicated (usually 30 min) without shaking. The vials were then placed on ice for 5 min. Each cap was removed individually, and 0.2 mL of 0.67 M HCl was quickly added to the reaction vessel, which was immediately recapped. The vials were incubated at 37 °C for 45 min and were then placed on ice for 5 min. The reaction vessel was removed, and 12 mL of scintillation fluid (Bio-Safe II from RPI or PCS from Amersham) was added. Radioactivity in the vials was measured by liquid scintillation counting.

Nucleosome Core Particles. Nucleosome core particles were prepared by micrococcal nuclease digestion of calf thymus nuclei, depletion of histone H1, digestion to core particles, and purification on sucrose gradients, as described (Morgan et al., 1989).

Saccharomyces cerevisiae. A wild-type strain of *S. cerevisiae* was maintained on complete-medium plates (2% peptone, 1% yeast extract, 2% glucose, 2% agar). Individual colonies were transferred to nitrogen-deficient minimal medium containing 0.17% yeast nitrogen base (Difco, without ammonium sulfate), 2 mg/L uracil, 2 mg/L tryptophan, 2 mg/L leucine, 2 mg/L histidine, and 2% glucose. Cultures were grown in 1 mL of nitrogen-deficient minimal medium

in a siliconized 25-mL Erlenmeyer flask. A stock culture was grown overnight at 30 °C with shaking at 200 rpm. After approximately 12 h the OD_{600} of a 1/10 dilution of the stock culture was determined on a Gilford spectrophotometer. This stock culture was then used to inoculate 1-mL nitrogen-deficient minimal medium in a siliconized 25-mL Erlenmeyer flask such that the OD_{600} = 0.157. Cell growth was monitored by checking the OD_{600} of 1/10 dilutions of the cultures at various time points. A reproducible growth curve for the yeast culture was established under these conditions. Similar cultures were grown for 12 h at 30 °C with shaking at 200 rpm to give an OD_{600} of 0.6. [^{14}C]Polyamines or [^{14}C]polyamine photoaffinity analogues (10 000 cpm) were added. The cultures were returned to 30 °C and 200 rpm and allowed to continue to grow for 3, 5, or 7 h, at which times the OD_{600} was checked and cells were harvested by centrifugation. Supernatants and pellets were removed to separate scintillation vials, the pellets were resuspended in 1-mL nitrogen-deficient minimal medium, and the samples were counted in 10 mL of PCS scintillation fluid in a scintillation counter.

Circular Dichroism Measurements. Circular dichroism (CD) spectra of polynucleotides in the presence and absence of spermine and its analogues were recorded in 50 mM NaCl and 1 mM sodium cacodylate (pH = 7.) by using an upgraded UV-5 spectropolarimeter fitted with an IBM-XT personal computer for data recording and analysis. The polyamines were added directly to the polynucleotide solution (approximately 0.15A_{260}), and samples were heated to 37 °C for 20 min in the dark and then cooled to room temperature before recording the spectra (Behe & Felsenfeld, 1981). In order to minimize the exposure to UV light, the scanning was done at the rate of 100 nm/min. At this rate the characteristic positive peak at 265 nm indicating the formation of the cross-linked product was not observed.

Mapping of Binding Sites on Nucleosome Core Particles. Nucleosome core particles with a well-defined DNA length were irradiated as described in the text, and DNA was isolated by phenol extraction. The DNA was labeled with polynucleotide kinase and [γ-^{32}P]ATP and digested with DNA polymerase/exonuclease as described by Morgan et al. (1989). The resulting DNA fragments were separated by gel electrophoresis and detected by autoradiography, as described by Morgan et al. (1989). Examples of the autoradiographs are shown in Figures 8 and 9. Some contrast has been lost in making these prints due to the wide dynamic range (very heavy labeling at one end; very faint labeling at the other) and the background due to nonspecific binding of photoaffinity polyamines, on which the specific binding is superimposed. The positions of the major sites of specific binding were determined by placing the original autoradiographs on a light box and measuring with a ruler. In order to illustrate the appearance of the bands, and their relative intensities, we scanned the autoradiographs at 500 nm using a gel scanning attachment to a Cary 210 spectrophotometer. The autoradiographs were scanned at 0.05 mm/s with a 0.2-mm slit width, 0.5-s period, and 3.5-nm bandwidth. Data were collected with a Hewlett Packard BASIC coprocessor in a personal computer at a rate of 8 readings/s. Before further analysis, the data points were averaged in groups of 4 to reduce the size of the data set. Further data processing is described below.

The scans contain data at several levels. There is labeling at each base pair, due to both specific labeling at that base pair where it occurs and nonspecific labeling due to the presence of polyamine in the charge cloud around the DNA. This provides an oscillation with a peak at each base pair

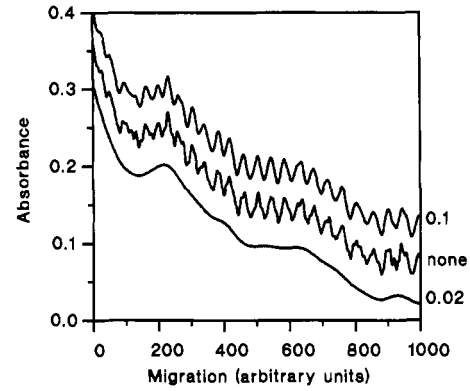

FIGURE 1: Data processing of autoradiograph scans. The central curve, labeled "none", is part of the original data obtained from the scan. The upper curve, labeled "0.1", is the same data after passing through a recursive digital filter with the filter factor set to 0.1, which removes photomultiplier "noise" and graininess in the film. The lower curve, labeled "0.02", is the same data after passing through the same filter but with the filter factor set to 0.02, which removes the fluctuations due to the individual base pairs.

position and a minimum between the base pairs. The resolution is limited by the fact that these samples are of random sequence and not a specific sequence. Thus, every base pair is represented at each position, giving a slight broadening of the peaks. Nevertheless, the base pairs show clearly (Figure 1). Superimposed on this pattern is the pattern due to specific binding of polyamine. This varies more slowly, probably representing the twist of the DNA as it wraps around the protein core (discussed in the text). Finally, there is "noise" from the photomultiplier used in the scanning and from graininess in the autoradiograph film. These three components can be separated in the frequency domain by various methods. We used a low-pass recursive digital filter whose effects are illustrated in Figure 1. The central curve in Figure 1 shows a section of the original data obtained as described in the previous paragraph. In the upper curve, these data have been passed through the digital filter with a filter factor of 0.1. Notice that this has removed "noise" but has not affected the alternation due to the base pairs. The alternation due to the base pairs is a distraction when the variations in intensity due to the helical twist of the DNA are of importance. The effect of the individual base pairs can be removed by passing the data through the digital filter with a filter factor of 0.02, the lower curve in Figure 1. Notice that the underlying variations in overall intensity have not been changed by the filter but can be seen without the distraction of the fluctuations due to individual base pairs.

The scans shown in Figure 8 and 10 have been filtered to eliminate the distractions of photomultiplier "noise" and individual base pair patterns. Since high frequencies were thus removed, every tenth point of each filtered data set was taken and used to make a smaller data set for plotting in Figures 8 and 10. Since the spacing of the base pairs varies approximately logarithmically along the gel, different filter factors, F, were used for different regions of the gel as follows: arbitrary units 1–49, F = 0.1; arbitrary units 50–197, F = 0.02; arbitrary units 198–450, F = 0.05. These scans show bands that can be seen equally clearly on the original autoradiographs but are faint or indistinguishable where contrast has been lost in the photographs of Figures 8 and 9. Band 11 is not shown on the scans illustrated, but it can be seen, especially by using tangent skimming techniques to remove very low frequency background, on data not shown. The position of band 11 was measured on original autoradiographs and is shown in Table II. We note that none of the conclusions of this paper depend

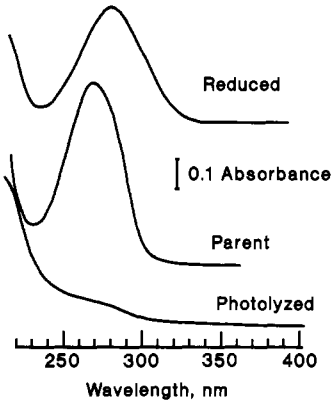

FIGURE 2: Ultraviolet spectra of ABA-spermine (labeled "Parent"), reduced ABA-spermine, and photolyzed ABA-spermine.

on the measurement of band 11.

RESULTS

Synthesis and Structure. ABA-polyamines were synthesized from the parent polyamine, spermine or spermidine, and the heterobifunctional reagent, methyl 4-azidobenzimidate, obtained from Pierce Co. Although it is no longer available from Pierce, methyl 4-azidobenzimidate can be synthesized in the laboratory (Ji, 1977; Lewis et al., 1977). The product of the reaction was purified by normal-phase HPLC and sulfopropyl-Sephadex chromatography as described under Materials and Methods. The product was analyzed by thin-layer chromatography, and in the purified samples only one spot was seen. This spot could be stained with ninhydrin, and it also absorbed ultraviolet light. When the initial polyamine was labeled with ^{14}C, the product was also labeled. Although the thin-layer chromatography showed only one spot, ABA-spermidine is expected to be a mixture of N^1-ABA-spermidine and N^8-ABA-spermidine. No attempt was made to separate the two isomers. ABA-spermine comprises a single isomer since the N^1- and N^{12}-positions are equivalent.

Figure 2 shows the ultraviolet spectra of ABA-spermine, reduced ABA-spermine, and photolyzed ABA-spermine. The extinction coefficient at 271 nm for ABA-spermine is 1.84×10^4 M^{-1} cm^{-1} and for ABA-spermidine is 1.75×10^4 M^{-1} cm^{-1}.

A sample of ABA-spermine was subjected to photolysis with 302-nm light. The photolysis was monitored by both spectral analysis and thin-layer chromatography using ninhydrin for visualization. It was found that with increasing time of exposure to ultraviolet light the 320–220-nm spectrum of the solution was altered by a decrease in the absorbance peak at 271 nm and an increase in the absorbance at wavelengths below 250 nm (Figure 2). After photolysis several products could be detected by thin-layer chromatography. The major product had a charge greater than 4+ and was ninhydrin positive and ultraviolet-dark. It was deduced to be a dimer of ABA-spermine.

ABA-spermine reacted rapidly with dithiothreitol at pH 10, requiring less than 5 min for complete reaction. After the reaction, no ABA-spermine was visible on thin-layer chromatography plates. The product, reduced ABA-spermine, was purified on a sulfopropyl-Sephadex column using an NH_4OAc gradient. The purified product produced a single spot by thin-layer chromatographic analysis, which was ninhydrin positive. Spectrophotometric analysis of the reduced ABA-spermine showed a maximum absorbance peak at 280 nm (Figure 2). The concentration of the reduced ABA-spermine was estimated by the trinitrobenzenesulfonate assay (Mokrasch, 1970) used for ABA-spermine and ABA-spermidine.

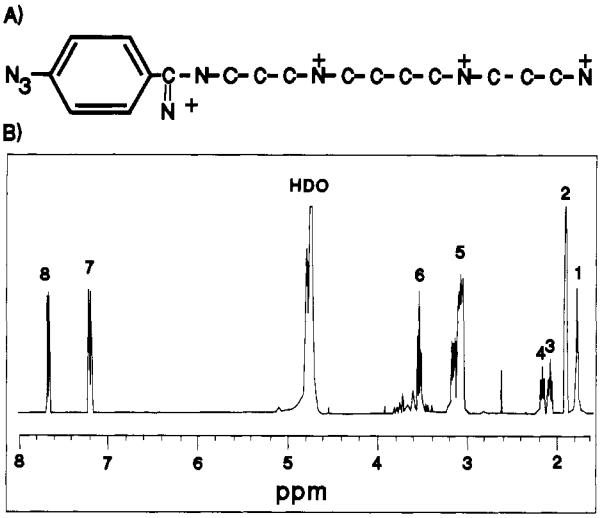

FIGURE 3: (A) Structure of ABA-spermine. Hydrogen atoms are omitted for clarity. (B) Proton nuclear magnetic resonance spectrum of ABA-spermine. The peak groups 1–8 are discussed in the text and Table I.

Table I: Assignment of NMR Resonances

peak	integration: no. of protons	chemical shift (ppm)	assignment[a]	predicted no. of protons
8	1.97	7.70	benzyl protons	2
7	1.97	7.21	benzyl protons	2
6	2.18	3.55	C1	2
5	10.96	3.02	C3, C5, C8, C10, C12	10
4	2.23	2.13	C2	2
3	2.11	2.02	C11	2
2	11.8	1.85	acetate protons	12
1	4.37	1.75	C6, C7	4

[a] The proposed structure is given in Figure 3A.

The extinction coefficient for reduced ABA-spermine was 1.06×10^4 M^{-1}.

The ^1H nuclear magnetic resonance spectrum of ABA-spermine was determined and compared with the ^1H nuclear magnetic resonance spectrum of ANB-spermine and spermine (Morgan et al., 1989). The deduced structure is shown in Figure 3A. Eight major groups of peaks in addition to HDO were identified and integrated to determine the number of protons, summarized in Table I. These groups of peaks account for the spectrum of ABA-spermine; they are numbered 1–8 in Figure 3B. Additional, much smaller peaks are present in the spectrum and presumably represent impurities in the sample. The first major peak (1.75 ppm, integrating to 4 protons) is from the 4 methylene protons in the middle of the spermine chain (the C6 and C7 protons). Peak 2 (1.94 ppm, integrating to a total of 12 protons) is from the 4 acetate counterions used during the final stages of purification. The third and fourth peak groups (2.02 and 2.13 ppm, integrating to 2 protons each) are the C11 and C2 protons, respectively. These protons split into two peak groups because the amidine linkage is close to the C2 protons. The C11 proton peak was identified by examining its chemical shift in spermine, which has a shift difference of 0.30 ppm from the C6/C7 proton peak at a pD of 5.8 (Morgan et al., 1989). In the ABA-spermine spectrum, the C6/C7 peak and the C11 peak differ by 0.27 ppm at a pD of 7.0. Peak group 5 is at 3.02 ppm and integrates to 10 protons, which corresponds to those protons neighboring the amine groups (C3, C5, C8, C10, and C12). Peak group 6 (3.55 ppm, integrating to 2 protons) is the C1 protons that neighbor the amidine linkage. Peak groups 7 and 8 (7.21 and 7.70 ppm, respectively) are the benzyl protons.

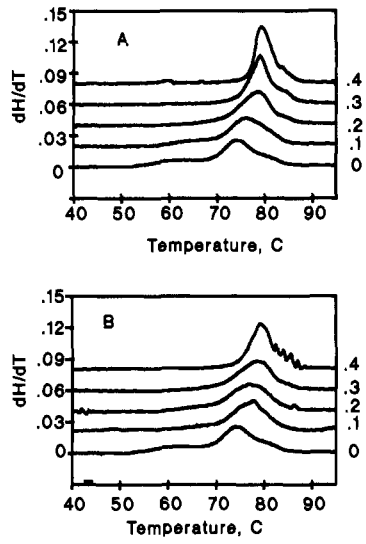

FIGURE 4: Thermal denaturation of nucleosome core particles in the presence of ABA-spermine (A) or reduced ABA-spermine (B). In both (A) and (B), the lowest curve is for nucleosome core particles alone. The higher curves have been displaced upward, for clarity. The charge ratio, i.e., the ratio of added polyamine charges to total phosphate charges on the nucleosome core particles, is shown at the right for each curve.

The finding that only 2 protons integrate at 3.55 ppm confirms the conclusion that ABA-spermine is formed by reaction of MABI with one of the primary amines of spermine. If the benzamidate group (of MABI) would have reacted with an internal amine, a total of 4 protons (2 on each side of the modified amino) would integrate at this location. Note that the impurity signal surrounding the 3.55 ppm peak may be due to such a reaction, or other side reaction(s), that may occur at a low frequency. The small peak at 2.6 ppm represents much less than 1 proton, and it was not considered when interpreting the NMR spectrum.

Properties of ABA-polyamines. Polyamines stabilize nucleosome core particles against thermal denaturation (Morgan et al., 1987). Modification of polyamines by acetylation or addition of the azidonitrobenzoyl group significantly reduces the stabilization, partly due to the loss of one charge per molecule, and partly due to the nature of the added group (Morgan et al., 1989). Figure 4 shows the thermal denaturation profiles for nucleosome core particles with ABA-spermine (Figure 4A) or reduced ABA-spermine (Figure 4B). The effects on the thermal denaturation profile include a merging of the premelt component into the main transition and a sharpening of the main transition. In the case of nucleosomes containing linker DNA and histone H1, the melting curves can be resolved into three components and the effect of added polyamine is to change the proportions of the components (Blankenship et al., 1987). The melting curves of nucleosome core particles, as used here, are more complex: the curves may still be resolved into three components, although less successfully, but the relationship between the components as polyamines are added is not as clear as it is for the larger particles (Morgan et al., 1989).

The effects on nucleosome core particles may be summarized by the change in thermal denaturation temperature for the main transition, T_m. In comparing different polyamines with one another, it is important to consider both the effect of the number of charges and the effect of various substituents. Thus, a comparison at the same molar ratio combines the effects of charge and substituents. The effects of charge may be accounted for by making comparisons between polyamines

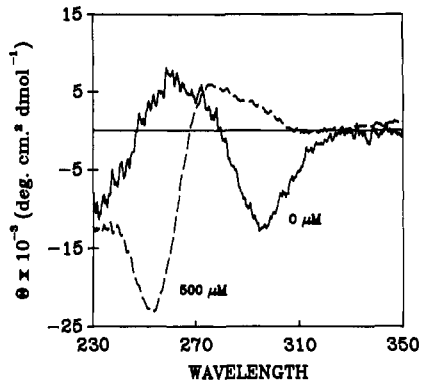

FIGURE 5: Circular dichroism spectra of poly(dG-br^{5}dC) alone (curve labeled 0 μM) or in the presence of 500 μM ANB-spermine showing the Z → B transition.

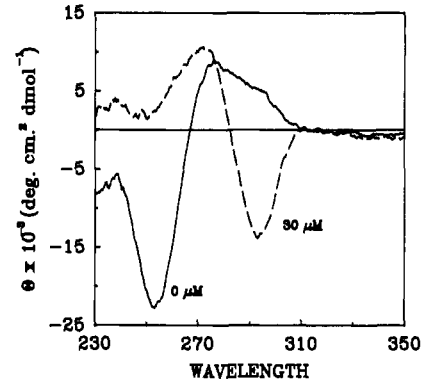

FIGURE 6: Circular dichroism spectra of poly(dG-m^{5}dC) alone (curve labeled 0 μM) or in the presence of 30 μM ABA-spermine showing the B → Z transition.

at the same "charge ratio", that is, the same ratio of added cationic charge to total DNA phosphates.

Using a constant *molar ratio* of 0.1 polyamine:DNA phosphate, spermine and spermidine raise the T_m more than any of the modified polyamines. The rank order, with values in parentheses, is spermine (80.4 °C), spermidine (80.0 °C), ABA-spermine (79.5 °C), acetylspermine (78.5 °C), and ANB-spermine (77.5 °C) (Morgan et al., 1987, 1989). The T_m with ABA-spermine is 0.9 °C below that with spermine while ANB-spermine is 1.9 °C below that with spermine. Using a constant *charge ratio* of 0.4 shows a different pattern, with spermidine and acetylspermine giving larger increases in T_m than spermine so that the rank order becomes spermidine (81.2 °C), acetylspermine (80.6 °C), spermine (80.4 °C), ABA-spermine (79.5 °C), and ANB-spermine (79.2 °C) (Morgan et al., 1987, 1989). In spite of this effect on the other triamines, ANB-spermine still has the least effect on the T_m. Overall, the thermal denaturation data indicate that ABA-spermine is closer to the parent polyamine in its effects on nucleosome core particles than is ANB-spermine, although the ability to stabilize nucleosome core particles is reduced in both cases.

Spermine, spermidine, and most of their analogues induce a B → Z transition in DNA with alternating purine–pyrimidine sequences, under favorable environmental conditions. At 50 mM NaCl most of the spermine analogues induce a complete B → Z transition of poly(dG-m^{5}dC) in the concentration range of 1–25 μM (Basu et al., 1990). The midpoint of the transition is at 2 μM for spermine (5 μM for a complete transition) and 50 μM for spermidine (Behe & Felsenfeld, 1981). At a concentration of 150 μM, ANB-spermine failed to induce this transition (data not shown). Relatively high concentrations of ANB-spermine (500 μM) even induced a

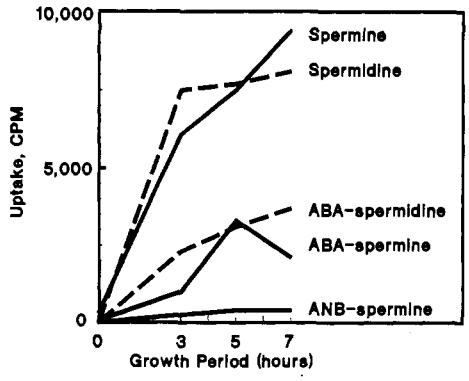

FIGURE 7: Uptake of [^{14}C]polyamines by *S. cerevisiae*. Uptake was measured at 0, 3, 5, and 7 h after addition of the labeled compound for [^{14}C]spermine, [^{14}C]spermidine, ABA-[^{14}C]spermidine, ABA-[^{14}C]spermine, or ANB-[^{14}C]spermine as indicated. The apparent drop in incorporation between 5 and 7 h for ABA-spermine was not seen in other experiments.

Z → B transition of poly(dG-br^{5}dC) (Figure 5), as inferred from the circular dichroism inversion at 240–320 nm.

On the other hand, 30 μM ABA-spermine induced a complete B → Z transition of poly(dG-m^{5}dC) under our experimental conditions (Figure 6), as inferred from the circular dichroism inversion at 240–310 nm. At similar ionic strength 5 μM spermine induces a complete B → Z transition (Basu et al., 1990; Behe & Felsenfeld, 1981). These observations show that the different side-chain modifications of spermine modify its mode of interaction with DNA or polynucleotides differently. The mode of interaction of ABA-spermine with DNA remains qualitatively similar to that of spermine with some modification of its binding affinity. On the contrary, the interaction of ANB-spermine is qualitatively different from that of spermine.

Biological Effects. The effects of the modified polyamines on ornithine decarboxylase from *E. coli* and on the uptake of polyamines into yeast cells were investigated. The reaction conditions for ornithine decarboxylase were tested, and the reaction rate was constant for 45 min. Incubation times of 30 min were used for determination of initial rates. Apparent K_M and V_{max} values were determined by varying the substrate concentration, and the values obtained were 3.8 mM and 19.6 nmol/h, as previously reported (Applebaum et al., 1977; Morris et al., 1970).

Inhibition studies were performed with the ornithine concentration kept constant, while the concentrations of polyamines were varied. At concentrations up to 1 mM, spermidine has a small (30%) stimulatory effect on ornithine decarboxylase activity and ANB-spermine has statistically insignificant effects. Spermine showed a slight stimulation at a concentration of 0.1 mM followed by inhibition at higher concentrations, up to 50% inhibition at 1.1 mM, and ABA-spermine showed inhibition at all concentrations from 0.1 mM (30% inhibition) to 1.1 mM (90% inhibition). Hence, the modification of spermine by ANB considerably reduces its effect on ornithine decarboxylase, and the modification by ABA considerably enhances the inhibitory effect. The ligands with 4 charges per molecule were both effective inhibitors while the ligands with 3 charges per molecule were not inhibitory.

A simple experiment was carried out to determine if yeast cells could take up the polyamine analogues. Preliminary experiments with [^{14}C]spermine and yeast growing in complete medium failed to demonstrate uptake. However, when the cells were grown in a nitrogen-deficient medium, uptake of [^{14}C]spermine and [^{14}C]spermidine was readily demonstrated (Figure 7). Under the same conditions, [^{14}C]ANB-spermine was not taken up. The very small increase in counts in the pellet during growth probably reflects the increase in volume of the pellets as the culture proliferates. In contrast to this negative result, [^{14}C]ABA-spermine and [^{14}C]ABA-spermidine are taken up by the culture (Figure 7). The rate of uptake is slower than that for [^{14}C]spermine and [^{14}C]spermidine. This may be partly due to partial inhibition of growth by the ABA-polyamines (data not shown). The lack of uptake of [^{14}C]ANB-spermine suggests that the uptake of [^{14}C]ABA-spermine and [^{14}C]ABA-spermidine is an active process. However, there are no data at this time to indicate if the process is competitive with the uptake of spermine or spermidine.

The data on biological effects provide preliminary evidence that ABA-spermine and ABA-spermidine have biological activity while ANB-spermine may be inert.

Binding Sites on Nucleosome Core Particles. Studies with ANB-spermine showed that it binds to nucleosome core particles at all base positions in the core particle DNA with greatly enhanced occupancy of six specific internal binding sites (Morgan et al., 1989). Similar experiments have now been carried out with ABA-polyamines to illustrate the use of these compounds as photoaffinity reagents and to test the conclusions drawn from the ANB-spermine data. The general conclusion was that polyamines bind both nonspecifically and specifically to the DNA in nucleosome core particles, and this is confirmed by the data below. However, the specific sites are in different locations on the core particle DNA.

Nucleosome core particles were mixed with ABA-spermine or ABA-spermidine or unmodified polyamines, as controls, and irradiated as described under Materials and Methods. The wavelength of irradiation was restricted to wavelengths longer than 300 nm by using a polystyrene filter with a transmission cutoff at 300 nm as measured on the Cary spectrophotometer. The time required for full activation of the ABA photoactive group was determined by measuring changes in the ultraviolet spectrum as a function of time of irradiation. The half-time was 90 s. Extensive irradiation was found to give direct photodamage to free DNA or DNA in nucleosome core particles. However, irradiation for 3 min under these conditions gave no detectable direct photodamage to either free DNA or DNA in nucleosome core particles. These conditions were used for the experiments described below.

After irradiation, the mixtures were treated with phenol to remove protein and the DNA was recovered by ethanol precipitation. The DNA was labeled at its 5′ end by polynucleotide kinase and digested from the 3′ end by T4 polymerase/exonuclease. The exonuclease stops digestion when it reaches a base modified by addition of ABA-polyamine (Morgan et al., 1989; Gale et al., 1987). Hence, the lengths of the resulting fragments indicate the positions of sites of modification, relative to the 5′ end of the DNA. The results (Figure 8) show that each of the bases of the DNA in core particles was labeled to some extent but that specific sites showed enhanced occupancy.

The following control experiments were carried out: (i) protein-free DNA irradiated in the presence of ABA-polyamines; (ii) nucleosome core particles irradiated alone; (iii) nucleosome core particles irradiated in the presence of the parent polyamine; (iv) nucleosome core particles incubated with ABA-polyamines but not irradiated. In the circumstances of polyamine binding to DNA, it is not very informative to attempt to compete the photoaffinity reagent with the natural compound. The reason is that, due to the possibility of nonspecific binding, addition of more polyamine is essentially

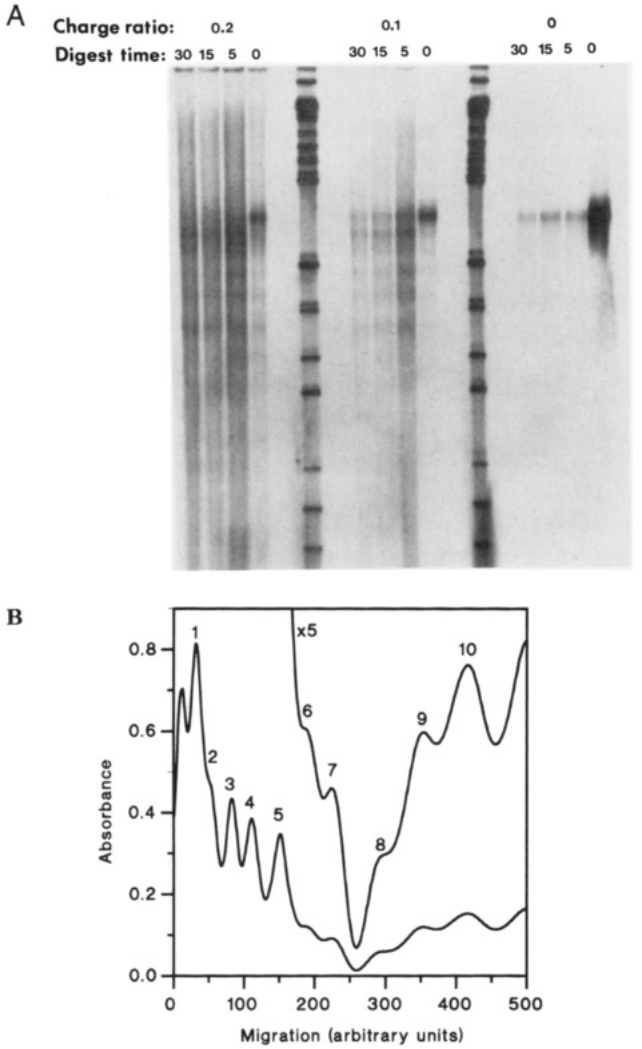

FIGURE 8: Time course digestion of DNA from photoaffinity-labeled core particles with T4 DNA polymerase/exonuclease. (A) Photograph of the autoradigraph: digestion times indicated are in min. An autoradiograph of a urea–DNA gel is shown. Charge ratio 0.2: Core particles irradiated at 302 nm for 3 min in the presence of ABA-spermine at a charge ratio of 0.2, digested for the time indicated. Charge ratio 0.1: Core particles irradiated as above except ABA-spermine was present at a 0.1 charge ratio. Charge ratio 0: Core particles with nothing added, irradiated and digested as described above. DNA markers (*Hae*III digested pBR322) separate the three groups of digestion conditions. (The sizes, in bases, of these molecular weight markers are 51, 57, 64, 80, 89, 104, 123/124, 184, 192, and larger.) (B) Scan of the autoradiograph: the lane with charge ratio 0.1 and digestion time 5 min was scanned as described under Materials and Methods. The bands due to specific polyamine binding are numbered to correspond with the rows in Table II.

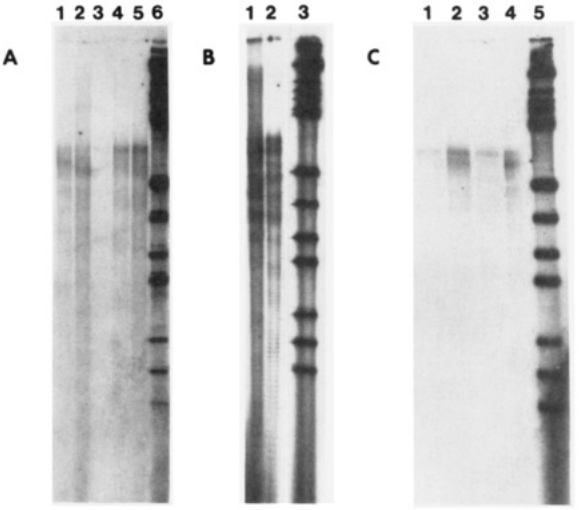

FIGURE 9: T4 DNA polymerase/exonuclease digestion of DNA from core particles. Autoradiographs of urea–DNA gels are shown. (A) UV-irradiated core particles digested for 10 min. Lane 1, core particles irradiated at 302 nm for 3 min in the presence of ABA-spermidine at a charge ratio of 0.1; lane 2, same as lane 1 except 0.3 charge ratio; lane 3, core particles irradiated alone; lane 4, core particles irradiated at 302 nm for 3 min in the presence of ANB-spermine at a charge ratio of 0.3; lane 5, same as lane 4 except irradiation was at 366 nm for 4 min; lane 6, *Hae*III digest of pBR322 (markers). (B) Comparison of ABA-spermine binding sites with pyrimidine dimers (digestion time 10 min). Lane 1, core particles irradiated at 302 nm for 3 min in the presence of ABA-spermine at a charge ratio of 0.1; lane 2, core particles alone irradiated at 302 nm for 6 min; lane 3, *Hae*III digest of pBR322 (the sizes, in bases, of these molecular weight markers are 51, 57, 64, 80, 89, 104, 123/124, 184, 192, and larger). (C) Core particles irradiated in the presence of spermine or spermidine (digestion time 30 min). Lane 1, core particles irradiated at 302 nm for 3 min in the presence of spermidine at a charge ratio of 0.1; lane 2, same conditions as lane 1 but with ABA-spermidine instead of spermidine; lane 3, same conditions as in lane 1 but with spermine (no spermidine); lane 4, same conditions as in lane 1 but with ABA-spermine (no spermidine).

additive up to the point of precipitation and very little useful competition occurs.

All the controls mentioned above showed either no labeling of the DNA, (ii)–(iv), or uniform labeling of the DNA, (i). Data obtained in the same series of experiments, but with ANB-spermine binding, show a pattern of bands superimposed on a uniform background (Figure 9). This pattern and the positions of the bands confirm results previously reported by Morgan et al. (1989) for ANB-spermine. Both ABA-spermine and ABA-spermidine gave the same type of result, namely, a pattern of bands superimposed on a uniform background. While qualitatively the same as the result with ANB-spermine, the positions of the bands due to ABA-polyamine binding are quite different.

As in the case of the ANB-spermine experiments, the full range of bands due to specific labeling is difficult to reproduce on a photograph of the autoradiograph. This is partly due to the wide dynamic range required to accommodate both the strongly labeled bands and the weakly labeled ones. It is also due to the distraction caused by labeling at all base pair positions, due to the presence of polyamine in the charge cloud of the DNA which occurs in addition to specific binding. The problem of dynamic range can be overcome by scanning the autoradiographs, and examples of these scans are shown in Figures 8 and 10. In Figure 8, for example, 7 bands can be seen in the photograph of the autoradiograph but the scan shows 10 bands, as does the original autoradiograph. Similarly, the scan of Figure 9B, lane 1, shows 10 bands although not all these can be clearly distinguished on the photograph of the autoradiograph in Figure 9B. Note that the scans in Figure 8 and 10 have been subjected to digital filtering to eliminate the distraction caused by the presence of a peak for each individual base pair. The filtering process is described briefly under Materials and Methods, and Figure 1 shows the effects of the filter. The positions of the bands were measured directly from the original autoradiographs by using transmitted light to view the bands. The positions shown in Table II are averages of data from two separate lanes of one autoradiograph, chosen because it showed the most extensive pattern of bands. Each of these lanes had an adjacent lane of pyrimidine dimers which was used as the size marker (photograph in Figure 9B; scans in Figure 10A). Similar results were obtained from other autoradiographs (not shown). Band 11 is visible on the original autoradiographs but is not shown on the scans and is not discernible on the photographs. Hence,

Table II: Positions of Blocks to Exonuclease Digestion

distance from the 5′ end (bases)				
ANB-spermine	ABA-spermine	ABA-spermidine	direct photo-damage[a]	DNase-1 cutting sites[b]
146	135	135	136	136.5
130	126	126	126	126.7
109	118	118	116	
86	108	108	106	105.9
56	99	99	95	95.0
36	87	87	84	
15	77	77	74	73.8
	68	68	65	
	58	58	53	53.2
	49	49	43	42.5
	38	38	32	
			22	22.5

[a] From Gale et al. (1987). [b] From Lutter (1979).

band 11 is only represented in Table II and Figure 10B,C. In fact, none of the conclusions of this study is affected by adding or deleting band 11 from the data set.

The major conclusion drawn from the positions of the bands is that the specific binding sites for ANB-spermine have a different spacing from those for pyrimidine dimer formation under higher UV doses (Figure 10B). The difference in spacing is difficult to see with certainty in Figure 9B where adjacent lanes show ANB-spermine binding and pyrimidine dimer formation but can be seen very clearly in the original autoradiographs and in the scans of these data shown in Figure 10A where densitometric scans of the two lanes are placed in adjacent panels. By placing a ruler over the scans, it can be clearly seen that the two panels show peaks in similar positions near the top of the gel but that the peaks move steadily "out of phase" toward the bottom of the gel. Comparison of the scans shown in Figures 8 and 10 also shows that there are reproducible differences in the intensities of the individual bands, but we have not attempted to interpret these. The widths of the specific binding sites do not appear to vary within the resolution of these measurements.

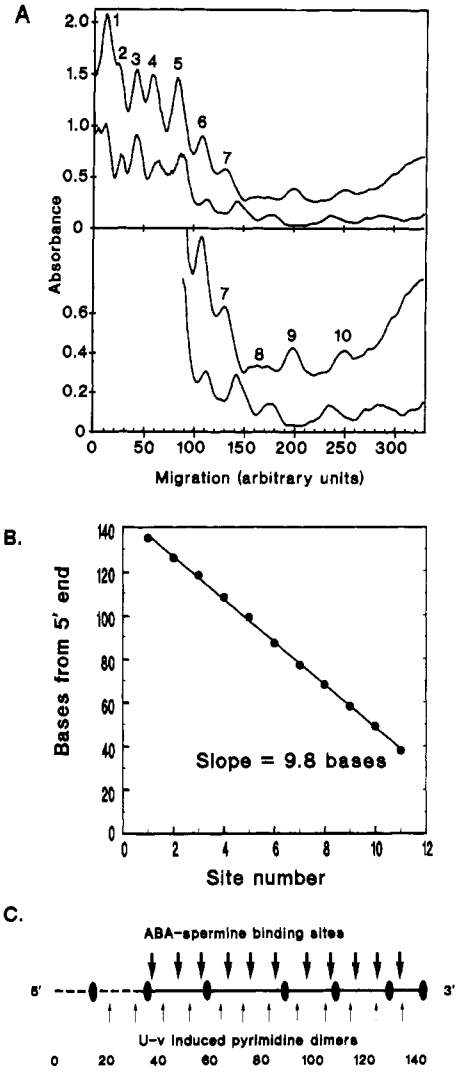

FIGURE 10: Positions of preferential binding sites for ABA-spermine or ABA-spermidine. (A) Scans of the autoradiograph shown in Figure 9B: the upper panel shows complete scans; the lower panel shows parts of the same scans at a more sensitive absorbance scale. In each panel, the upper scan is from the nucleosomes treated with ABA-spermine and the lower scan is a molecular weight marker obtained from nucleosomes treated with a much higher dose of ultraviolet light in the absence of ABA-spermine. The number above each peak corresponds with a row in Table II. (B) The sites were numbered, corresponding to the rows in Table II, and the position of each site was plotted against its number. (C) A line representing a map of the nucleosome core particle DNA with the positions of ABA-spermine and ABA-spermidine binding sites indicated by the upper arrows, the positions of direct photodamage indicated by the lower arrows, and the positions of ANB-spermine binding sites indicating by filled ellipses on the line representing the DNA. The 5′ end of the DNA is shown as a dashed line since the data for ABA-spermine and ABA-spermidine do not extend closer to the 5′ end than about 30 bp.

Discussion

This report mainly concerns the synthesis, characterization, and use of a new photoaffinity polyamine derivative, ABA-spermine. While some data are also presented on the closely related compound, ABA-spermidine, we have not studied ABA-spermidine more comprehensively at this stage since it is presumably a mixture of the N^1 and N^8 isomers. The problem of separating the isomers to give pure compounds for further studies has not yet been approached.

The primary motivation for preparing and characterizing photoaffinity derivatives of polyamines is to obtain reagents that will provide information about the behavior of polyamines that can be extrapolated to the naturally occurring polyamines (Morgan et al., 1989; Matthews et al., 1990). How do the ANB and ABA compounds compare in this regard? ABA-spermine and ABA-spermidine behave differently from ANB-spermine in the following ways: (i) effect on nucleosome stability compared with the parent polyamine; (ii) stabilization of the Z or B form of DNA secondary structure, respectively; (iii) uptake by yeast cells; (iv) binding sites on nucleosome core particles.

Morgan et al. (1987, 1989) showed that the charge of the polyamine or its derivative has a significant effect on the thermal denaturation profiles of nucleosome core particles. Thus, to compare the effect of the different substituents, ANB or ABA, each compound should be compared with an unmodified polyamine of equivalent charge. ABA-spermine reduces the T_m by 0.9 °C with respect to the T_m in the presence of spermine at a molar ratio of 0.1 (charge ratio 0.4). By comparison, ANB-spermine reduces the T_m 2.0 or 2.5 °C with respect to the T_m in the presence of spermidine at the same molar ratio or the same charge ratio, respectively. The results can be generalized as follows: the larger the hydrophobic group attached to the basic polyamine molecule, the lower the T_m. Thus, by this measure, all of the polyamine derivatives are qualitatively similar to the corresponding unmodified polyamines of equivalent charge. However, ABA-spermine is quantitatively closer to the equivalent polyamine than ANB-spermine.

ABA-spermine can cause the B → Z transition in poly(dG-m^{5}dC), like spermine, although a higher concentration of ABA-spermine is required. ANB-spermine does not cause this transition. Although the loss of one charge in ANB-spermine compared with ABA-spermine would be expected to reduce its ability to stabilize Z-DNA (Thomas & Messner, 1988, 1986; Thomas et al., 1985), this is insufficient to explain the lack of a transition with ANB-spermine and poly(dG-m^{5}dC) and the stabilization of B-DNA in experiments with poly(dG-br^{5}dC).

ABA-polyamines, unlike ANB-spermine, are taken up by yeast cells, although at a reduced rate compared with the parent polyamines. The properties of ABA-spermine and ABA-spermidine compared with ANB-spermine indicate that the ABA modification has a smaller effect on the behavior of the polyamine than does the ANB modification. ABA-spermine and ABA-spermidine are the most promising photoaffinity polyamines currently available.

As in the case of ANB-spermine, the ABA-polyamines showed both specific and nonspecific binding to DNA in nucleosome core particles. The positions of specific binding for ABA-spermine and ABA-spermidine are indistinguishable, but these sites are strikingly different from the sites previously determined for ANB-spermine (Morgan et al., 1989). Figure 9 shows that the positions of ANB-spermine binding were confirmed in the current series of experiments. The specific binding positions for ABA-spermine and ABA-spermidine correspond approximately to the positions of direct photodamage to DNA in nucleosome core particles, in the 3′ proximal region of each DNA strand (Gale et al., 1987). These are positions where the minor groove points outward and is thus somewhat widened (Morgan et al., 1989; Richmond et al., 1984). In the 5′ proximal region of the DNA, however, the binding is offset by up to 6 bases from the positions of direct photodamage, as can be seen directly by inspection of Figure 10A and as quantitated in Table II. The shift in position of the binding sites relative to the sites of direct photodamage is due to a different spacing of the sites (Figure 10B). The ABA-polyamine binding sites have an average spacing of 9.8 bp compared with 10.3 bp for the sites of direct photodamage (Gale et al., 1987).

The ABA-spermine and ABA-spermidine binding sites are symmetrically disposed about the center of the DNA. The center is at 73 bases, placing the central binding site 4 bases from the center on the 3′ side and 5 bases on the 5′ side. The next site is 14 bases away on the 3′ side and 15 bases on the 5′ side; then 26 bases and 24 bases; and then 35 bases and 35 bases, respectively.

The sites of direct photodamage are similar to those attacked by DNase-1, except that sites missing in the DNase-1 pattern are present in the photodamage sites (Gale et al., 1987; Lutter, 1979). The missing DNase-1 sites are due to shielding of the DNA by histone (Lutter, 1979; Klug et al., 1982). Shielding against photodamage is not seen. Similarly, there is no indication of missing sites in the pattern of binding of ABA-spermine, which indicates that the shielding of the DNA from DNase-1 is at least partly due to the relatively large size of the DNase-1 probe. More interesting is the 9.8-base periodicity of the ABA-spermine binding sites (Figure 10). In the case of DNase-1 and direct photodamage, the measured periodicity is 10.3–10.4 bases (Gale et al., 1987; Lutter, 1979; Klug et al., 1982). The difference is clearly seen in Table II and in Figure 10, where the ABA-spermine binding sites are approximately "in phase" with the sites of direct photodamage in the 5′ distal region of the DNA but clearly "out of phase" in the 5′ proximal region. The data suggest a smooth change from exactly "in phase" at 126 bp from the 5′ end to 6 bp "out of phase" at 49 and 36 bp from the 5′ end. The DNase-1 sites are "in phase" with the sites of direct photodamage over the whole DNA molecule (Gale et al., 1987). In the case of both the DNase-1 sites and the sites of direct photodamage, the positions of the sites are interpreted in terms of the helical conformation of the DNA as it is coiled around the histone core, giving rise to steric hindrance to DNAse-1 or tilting of the bases to make thymine dimer formation more or less likely (Gale et al., 1987; Klug et al., 1982). In the case of DNase-1 there is a 1.4–3-base stagger in the most likely positions of cutting on opposite strands of the DNA in nucleosome core particles (Lutter, 1979). Depending on the mode of binding, there could also be a stagger in the binding sites for ABA-spermine and spermidine. However, if this is a major factor, it is surprising that no difference was seen between the sites for ABA-spermine and ABA-spermidine.

The magnitude of the offset and the symmetry of the sites are such that, in the absence of a change in DNA twist, the polyamine derivatives would have to bind to opposite sides of the DNA helix at the ends of the DNA, but the same side in the middle of the DNA. This is highly unlikely. It is more likely that they are binding to the same side in all cases, but that the helical twist of the DNA is altered. Hence, we suggest that the helical twist of the DNA in the nucleosome core particle is being altered by binding of the ABA-polyamine ligands. This would account directly for the 9.8 bp periodicity. It may be significant that the DNA orientation in the nucleosome is unaltered at the site 126 bp from the 5′ end, suggesting a feature of the nucleosome may require this orientation. Thus, we propose that the change in registry of the pyrimidine dimers and the ABA-polyamine binding sites (Figure 10) is a consequence of the change in helical twist of the DNA due to the absence or presence of ABA-spermine or ABA-spermidine.

If the DNA helical twist is indeed being altered, the next question is whether this is due to the ABA group or the spermine and spermidine groups. In this regard, it was observed previously that both spermine and spermidine cause an alteration of DNA conformation in nucleosome core particles as measured by circular dichroism (Morgan et al., 1987). The nature of this change is ambiguous; it could arise from either altered DNA helical twist (MacDermott, 1985) or altered spacing of coils in the nucleosome core particle (Cowman & Fasman, 1978, 1980). Naked PM2 DNA increases in helical twist angle (i.e., less bases per turn) when modified by covalent addition of positive charges (butylamines), as measured by gel mobility. This correlates with a reduced circular dichroic peak around 280 nm, which can also be caused by high concentrations of monovalent ions (Kilkuskie et al., 1988). The reduction of circular dichroism at 280 nm is qualitatively similar to that observed with spermine and spermidine in nucleosome core particles (Morgan et al., 1987). Thus, it is plausible that the altered spacing of ABA-spermine and ABA-spermidine binding sites reflects a real effect of spermine or spermidine on nucleosome core particles. This suggests that polyamine binding to DNA in nucleosome core particles causes an increase in the average DNA helical twist angle from 34.6° to 36.7°. This helical twisting could be partly responsible for the stabilizing effect of polyamines on nucleosome core particles.

There remains the question of how the considerable difference between ANB-spermine and ABA-spermidine binding sites arises. The main difference between these two compounds

is in the length of the tether between the photoactivatable azidobenzene group and the nearest charged amino group. Possibly the longer tether (up to 0.5 nm) on the ANB group allows hydrophobic interactions with the core particle or DNA bases which are prevented on the ABA group.

Molecular mechanics calculations have predicted that polyamines would prefer to bind to the major groove of DNA, producing a bend that results in a widened minor groove (Feuerstein et al., 1990). The data presented here refer to DNA in nucleosome core particles which is not directly comparable with free DNA. Nevertheless, the binding sites on the 5′ distal region of the DNA strands do correspond with the positions of direct photodamage. These sites have been associated with a minor groove pointing out away from the protein core of the nucleosome (Gale et al., 1987). Since the superhelical coiling of the DNA will widen the minor groove where it points outward, these sites confirm the predictions from molecular mechanics (Feuerstein et al., 1990). If the DNA twist is altered, as proposed above, then all the sites may occur where the minor groove points outward. Much higher resolution structural information is needed before the exact position of the outward-facing bases can be specified precisely. In general, within the limitations of the structural information available, the data with ABA-spermine and ABA-spermidine binding are at least partly consistent with the theoretical predictions while the data with ANB-spermine are quite different.

Taken together with the other data on the comparability of the different photoaffinity polyamines with their parent polyamines, the results suggest that ABA-spermine and ABA-spermidine are indicating the positions where spermine and spermidine bind to nucleosome core particles.

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Relationship between 2'-Hydroxyls and Magnesium Binding in the Hammerhead RNA Domain: A Model for Ribozyme Catalysis†

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ABSTRACT: The use of deoxyribonucleotide substitution in RNA (mixed RNA/DNA polymers) permits an evaluation of the role of 2'-hydroxyl groups in ribozyme catalysis. Specific deoxyribonucleotide substitution at G^9 and A^{13} of the ribozyme decreases the catalytic activity (k_{cat}) of the ribozyme by factors of 14 and 20, respectively. The reduction of the reaction rate concomitant with the absence of these 2'-OHs or the 2'-OH of the substrate U^7 position can be partially compensated by increasing the Mg^{2+} concentration above 10 mM. The K_{Mg} of the all-RNA ribozyme is 5.3 mM, and the lack of either of the three influential 2'-OHs increases this value by a factor of approximately 3. These and other reaction constants for the ribozyme and the deoxy-substituted analogues have been determined by assuming a three-step mechanism. The data presented here provide the basis for the formulation of a molecular model of ribozyme activity.

The introduction of deoxyribonucleotides in specific positions of RNA molecules is made possible by an RNA synthesis scheme (Usman et al., 1987; Scaringe et al., 1990) which is compatible with the chemical synthesis procedure for DNA (Wu et al., 1989). We expect deoxyribonucleotide incorporation in polyribonucleotide chains to have minimal structural consequences, since unlike site-directed mutagenesis, where one nitrogen base is replaced by another, neither base-pairing nor base-stacking, the principle determinants of nucleic acid structure, should be affected. Deoxyribonucleotide substitution (i.e., modification of the sugar moiety) in RNA thus offers a unique and incisive tool in the study of the functional and structural role of 2'-hydroxyls in RNA. Using these mixed RNA/DNA polymers, we are exploring structure–function relationships in the self-cleaving activity associated with the RNA hammerhead domain (Perreault et al., 1990). Hammerhead catalysis is characterized by the cleavage of a specific phosphodiester bond producing 5'-hydroxyl and 2',3'-cyclic phosphate products (Uhlenbeck, 1987). As shown in Figure 1, this domain can be partitioned into 2 RNA fragments which allows the formalism of defining a ribozyme fragment of 35 nucleotides and a substrate fragment of 14 nucleotides (Gerlach & Haseloff, 1988; Koizumi et al., 1988; Forster et al., 1988; Jeffries & Symons, 1989). We have previously established that the 2'-OH adjacent to the scissile phosphate of the substrate at position C^8 (Figure 1) is the nucleophile in the reaction (Perreault et al., 1990) and that this is the only essential 2'-OH in the substrate fragment, even though absence of the 2'-OH of U^7 has a negative effect on the reaction rate (Yang et al., 1990).

As for the ribozyme fragment itself, no single 2'-OH is essential in the reaction, in spite of the fact that the 2'-OH of G^9 and at least one other 2'-OH of the central core strongly affect catalytic efficiency (Perreault et al., 1990). We now report the identification of a second influential hydroxyl in the ribozyme fragment, that of A^{13}. Furthermore, our data show that the 2-OHs of the ribozyme G^9 and A^{13} and the substrate U^7 are implicated in a low-affinity binding of the Mg^{2+} cofactor to a specific site in the substrate/ribozyme complex. These data allow the formulation of a structural model of ribozyme activity.

Materials and Methods

Synthesis and Purification of DNA/RNA Mixed Polymers. All oligonucleotides were synthesized on an automated oligonucleotide synthesizer (Milligen/Biosearch and Pharmacia) and deprotected as described elsewhere (Perreault et al., 1990;

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