Photoaffinity polyamines: sequence-specific interactions with DNA

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ABSTRACT

ANB-spermine is a photoaffinity analog of the naturally-occurring polyamine, acetylspermine. ANB-spermine was used to determine its binding sites on naked double stranded DNA, at the nucleotide level, using a modification of the primer extension technique. A total of 1,275 nucleotides was examined in 5 sequences of DNA from *Saccharomyces cerevisiae*. Binding sites were non-random. The primary determinant of binding was the presence of a thymidine residue. Secondary determinants appeared to depend on the secondary structure of the DNA, with runs of thymidines providing unusually poor binding sites while TA and, especially, TATA providing the strongest binding sites. The 'TATA element' upstream of the URA3 gene from *S. cerevisiae* was the strongest binding site. The data indicate that ANB-spermine binding to DNA is a probe for DNA secondary structure and suggest a role for polyamines in regulating the structure of chromatin *in vivo*.

INTRODUCTION

The primary mode of interaction of polyamines with DNA is electrostatic, as recognized by many investigators (1,2). In a detailed study of the interaction of polyamines with DNA, using thermal denaturation, polyamines significantly broadened the thermal denaturation curve of calf thymus DNA as if there were sequence-specific binding. However, it proved possible to account quantitatively for the broadening by assuming only rapid exchange between binding sites and different association coefficients for single stranded and double stranded DNA. The broadening was, in fact, used to determine the two association coefficients, using either an excluded sites model (3) or an independent sites model. Hence, there was no evidence for sequence-specific binding at this level of resolution (4).

Polyamine binding to DNA is, nevertheless, dependent on the DNA secondary structure, as shown by the stabilization of Z-DNA by polyamines (5–7). In those cases where the resolution permits the localization of spermine in crystals of tRNA or oligodeoxyribonucleotides, spermine molecules have been found to be specifically located in the crystal structure (e.g. (8)). Molecular mechanics calculations predict that spermine will bind preferentially where DNA has a tendency to bend with the minor groove on the outside (9). On the other hand, proton magnetic

resonance data on the interaction of polyamines with DNA in solution do not support precise localization of polyamines on specific DNA sequences (10). A more general approach to the question of polyamine binding sites on DNA is limited by the general problem with polyamines, namely the rapid reversibility of their binding and the difficulty of separating polyamine-specific binding from counter-ion condensation.

Photoaffinity polyamines were developed recently as a new approach to determining the locations of polyamines (11). The first derivative, ANB-spermine, was used to map binding sites on isolated nucleosome core particles and two additional derivatives, ABA-spermine and ABA-spermidine, have subsequently been synthesized and used for the same purpose (11,12). In both cases, specific binding sites were observed, superimposed on a background of non-specific binding. However, the positions of the specific sites were different between the ANB-spermine sites on the one hand and the ABA-polyamine sites on the other. In the case of the ABA-compounds, the data appeared to show a change in the helical twist of the DNA in nucleosome core particles due to the polyamine (12). In all cases, control experiments with 'random sequence' deproteinized nucleosome core particle DNA showed only the non-specific binding. Hence, the photoaffinity polyamines are sensitive to the conformation of the DNA and/or the presence of proteins.

Before proceeding to analyze photoaffinity polyamine binding sites on specific regions of chromatin, it was necessary to investigate whether the DNA, itself, contained preferential binding sites for photoaffinity polyamines. Nucleosome core particle DNA is sufficiently random that no conclusions could be drawn about sequence-specific binding from the studies mentioned above. Our chosen model for chromatin structure is the 2,629 bp yeast plasmid, TRURAP, developed by Thoma from the TRP1ARS1 plasmid originally prepared by Zakian and Scott (13,14). The DNA sequence is known and the chromatin structure has been mapped by Thoma and his colleagues (14). We have used this sequence, cloned into pUC19, to map at the nucleotide level the positions of binding sites for ANB-spermine.

MATERIALS AND METHODS

Enzymes and chemicals

N-(5-azido-2-nitrobenzoyl)-N-oxysuccinimide was obtained from Pierce. Spermine and spermidine were obtained from Aldrich

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and Fluka AG. Other reagents were obtained from Sigma, Fisher and USB Corporation. Restriction endonucleases were obtained from GIBCO BRL and New England Biolabs. *Thermus aquaticus* DNA polymerase was obtained from Pharmacia-LKB. Sequenase Version 2.0 DNA polymerase and Sequenase reagent kit were obtained from USB Corporation. Polynucleotide kinase was obtained from New England Biolabs. $[\alpha-^{32}P]$ dATP (3000Ci/mmol) was obtained from Du Pont NEN and $[\gamma-^{32}P]$ ATP (3000Ci/mmol) from Amersham.

Synthesis of (azidonitrobenzoyl) spermine

(Azidonitrobenzoyl) spermine (ANB-spermine) was synthesized from N-(5-azido-2-nitrobenzoyl)-N-oxysuccinimide and spermine (11). The ANB-spermine was stored in the dark at 4°C. The structure of ANB-spermine is depicted in Fig. 1a.

Preparation of template DNA

Plasmid pUC-R1 was constructed by inserting the 2619-bp EcoRI fragment of yeast plasmid DNA (TRURAP) (14) into the EcoRI site of a pUC19 vector. The supercoiled plasmid (pUC-R1) DNA was isolated from *E. coli* as described (15). The RNA-free plasmid DNA was digested with an appropriate restriction enzyme as described below, extracted with phenol/chloroform and precipitated with sodium acetate and ethanol. The DNA was dissolved in TE (10 mM Tris.Cl, pH 8.0; 1 mM EDTA) at a concentration of 1 to 0.5 mg/ml.

Synthesis and purification of oligodeoxyribonucleotides

Oligodeoxyribonucleotides were synthesized at the scale of 0.5 μmole on a Systec 1450 DNA synthesizer at the Protein Structure Laboratory of this school. Oligodeoxyribonucleotides were deprotected and cleaved from the support. The deblocked oligodeoxyribonucleotides were purified by using 20% polyacrylamide gels (19:1 cross-linking) with 7M urea in TBE buffer (89 mM Tris.borate, 2 mM EDTA, pH8.3) (16). The full length oligodeoxyribonucleotides were recovered by electroeluting from the gel in 40 mM Tris.acetate, 2 mM EDTA, pH 8.0 and lyophilized. The purified oligodeoxyribonucleotides were dissolved in sterile water. The purity of the oligodeoxyribonucleotides was checked by electrophoresis after 5'-end $[^{32}P]$ labeling.

Photoaffinity labeling of DNA

The RNA-free supercoiled plasmid pUC-R1 was digested with a restriction enzyme. The enzyme used and the length of the fragment were chosen for each primer as follows: primer 1, EcoRV, 4662 bp; primer 2, HindIII, 3250 bp; primer 3, BglI, 5305 bp; primer 4, PvuII, 2941 bp; primer 5, PvuII + StuI, 856 bp and 1368 bp (Figure 1b).

The digested DNA was mixed with either ANB-spermine or spermidine-(HCl)₃ at a charge ratio of 0.3 in TE (pH 8.0) and allowed to equilibrate at room temperature. Charge ratio is defined as the total added cation positive charges divided by the total DNA phosphate negative charges (17). The mixture of DNA and ANB-spermine was spotted on a piece of Parafilm and irradiated by using a 366-nm, 750 μW/cm² light (Ultraviolet Products UVL-56 Blak-Ray) for 40 sec. The light source was placed directly over the Parafilm. The irradiated DNA was stored at −20°C. No direct photodamage to the DNA is observed under these conditions (11).

Primer extension assay

Primer extensions on irradiated DNA samples were performed by the following methods.

1) The ultra-violet-irradiated DNA (0.3−0.5 pmole) was mixed with a primer (2−4 pmoles) in Taq sequencing buffer (50 mM Tris.Cl, pH8.5, 30 mM MgCl₂) with a total volume of 10 μl. The reaction mixture was boiled for 5 min, to denature the double-stranded DNA, and incubated at 40−45°C for 20 min for annealing the primer. The annealed template was then chilled on ice. 2 μl of labeling mix (10 μM dGTP; 5μM dCTP; 5μM dTTP), 5·Ci of $[\alpha-^{32}P]$ dATP (10·Ci/μl) and 2 unit of Taq DNA polymerase (1 U/μl, diluted in 10 mM Tris.Cl, pH8.5; 0.1 mM EDTA; 0.45% Tween 20; 0.45% Nonidet P-40) (18) were added, and the reaction mixture was left at room temperature for 1 min. A mix of dATP, dCTP, dGTP, and dTTP (6 mM each) was added immediately such that each deoxyribonucleoside triphosphate was at a final concentration of 400μM. The extension reaction was allowed to proceed for 10 min at 70°C and terminated by adding 4 μl of formamide-loading dye mix (91% deionized formamide; 20 mM EDTA, pH8.0; 0.05%-w/v xylene cyanol; 0.05%-w/v bromophenol blue).

2) The irradiated DNA (0.43 pmoles) was mixed with 4 pmoles of the ^{32}P-end-labeled primer (Promega Protocols and Applications Guide) in Sequenase buffer (40 mM Tris.Cl, pH7.5, 20 mM MgCl₂, 50 mM NaCl). The mixture was boiled for 5 min, incubated at 45°C for 20 min, and left at room temperature. 1 μl of 0.1 M dithiothreitol (DTT), 2 μl of sterile water, and 2 μl of diluted Sequenase version 2.0 DNA polymerase (1:6 diluted in a buffer of 10 mM Tris.Cl, pH7.5; 5 mM DTT; 0.5 mg/ml BSA) was added, and incubated for 1 min at room temperature. A mix of four kinds of dNTP (6 mM each) was added to a final concentration of 400μM, and the extension proceeded at 45°C for 10 min.

DNA sequencing and sequencing gels

DNA sequence standards were produced using Sequenase version 2.0 DNA polymerase on the double-stranded plasmid (pUC-R1) DNA with chain-terminating dideoxyribonucleotides (19). The procedure was modified when the ^{32}P-end-labeled primer was used for DNA sequencing (20). The samples for DNA sequencing and primer extension were analyzed by electrophoresis in either 6% or 8% polyacrylamide gels (30×36×0.04 cm) (19:1 cross-linking) with 7 M urea in TBE buffer (21). All samples were boiled for 3 min, and then chilled in an ice-bath just before being loaded on a sequencing gel. The sample irradiated in the presence of ANB-spermine (6 μl) was loaded next to a set of four sequencing reactions (2 μl each). The sample irradiated in the presence of spermidine was used as a primer extension control. Samples were electrophoresed for 4 to 6 h at 40 Watts. Dried gels were exposed to Kodak XAR-5 film (35×43 cm) for 5 to 48 hours at room temperature.

RESULTS

The photoaffinity polyamine, ANB-spermine, contains an azidonitrobenzoyl group linked to spermine, in which the aryl azide can be activated by ultra-violet light. ANB-spermine was allowed to interact with DNA in the dark and then illuminated. The chemically active azido-group reacted immediately with a neighboring group and resulted in covalent modifications on nucleic acids, as shown previously (11). The positions of covalent

binding on DNA were determined by an adaptation of the technique of mapping by primer extension. Mapping by primer extension is an accurate procedure for locating the 5' end of a single-stranded nucleic acid molecule. The essential requirement is an oligodeoxyribonucleotide (the primer) that will bind to a complementary sequence on the DNA (the template) and provide a primer for a DNA polymerase. The polymerase is then used to replicate the part of the template between the primer and the 5' end of the template. Replication terminates at the physical end of the template so that the length of the synthesized strand gives the distance of the 5' end of the template from the primer site. We reasoned that the addition of a photoaffinity polyamine to a nucleotide of the DNA would cause the polymerase to stop or pause at the site of modification. This is analogous to the pause observed in the passage of exonuclease along a DNA strand when a photoaffinity polyamine modification is encountered (11,22). When such primer extension reactions were carried out with unmodified DNA, no pausing or stopping of the polymerase was seen, unless the DNA was cut, by restriction enzyme digestion, in the region of interest, when a complete stop at the expected site of termination was seen. On the other hand, DNA that had been modified by photoaffinity labelling showed multiple sites where the polymerase stopped or paused (Figure 2). These sites are interpreted as the sites of photoaffinity modification.

The DNA sequences that we examined were part of a plasmid, pUC-R1, that contains two yeast sequences cloned in pUC19. The yeast DNA was the TRP1ARS1 EcoRI fragment with the URA3 Hind III fragment inserted in the Hind III site near the 5' end of the TRP1 gene. Five short sequences (16 to 18 bases long) located in different regions of this plasmid were selected as primers and purchased or synthesized. The physical map of pUC-R1 and the positions of the primers are shown in figure 1b. As polyamine binding may be different on relaxed or supercoiled DNA (23), we used linear double-stranded DNA produced by restriction digestions for photoaffinity labeling.

Similar primer extension techniques have been used to map the positions of DNA damage induced by ultra-violet irradiation

(24). While the irradiation conditions used here are rather mild, it is clearly essential to rule out the possibility that direct damage by ultraviolet irradiation could cause some of the observed polymerase pause sites. Direct photodamage to DNA by ultra-violet irradiation was checked with control experiments in which the DNA was irradiated in the presence or the absence of spermidine in place of the ANB-spermine. The results show that no polymerase pause sites were created in DNA under the conditions used for photoaffinity labeling, either in the presence or absence of spermidine. Similarly, the addition of ANB-spermine without irradiation generated no polymerase pause sites. The spermidine control was carried out in parallel with photoaffinity labeling for every primer extension experiment reported here.

Nucleotide-specificity for binding of ANB-spermine to DNA

The mixture of linear DNA fragments was irradiated with 366-nm ultra-violet light in the presence of ANB-spermine at a charge ratio of 0.3 (molar ratio of ANB-spermine to DNA phosphate is 0.1). Two kinds of DNA polymerase were used to map the binding sites of ANB-spermine on different DNA templates. Binding sites more than 100 bp from the primer site were determined using Taq DNA polymerase with labeling during extension with $[\alpha\text{-}^{32}P]$ dATP. Sites close to the primers were determined using Sequenase DNA polymerase with an end-labeled primer. The use of different template lengths indicates that the data are not dependent on the length of the template.

Multiple pause sites were observed for each DNA sequence studied. The sites were not randomly distributed over the DNA but showed a striking and reproducible specificity (Fig. 2). The majority of the pause sites were aligned with the positions of adenosine-terminated bands in the DNA sequence standards. Hence, we deduce that the final nucleotide recognized by the polymerase was a thymidine residue. Further passage of the polymerase is then prevented either by a modification of the thymidine residue itself or by a modification of its 3' neighbor. We conclude that at least part of the ANB-spermine binds to the
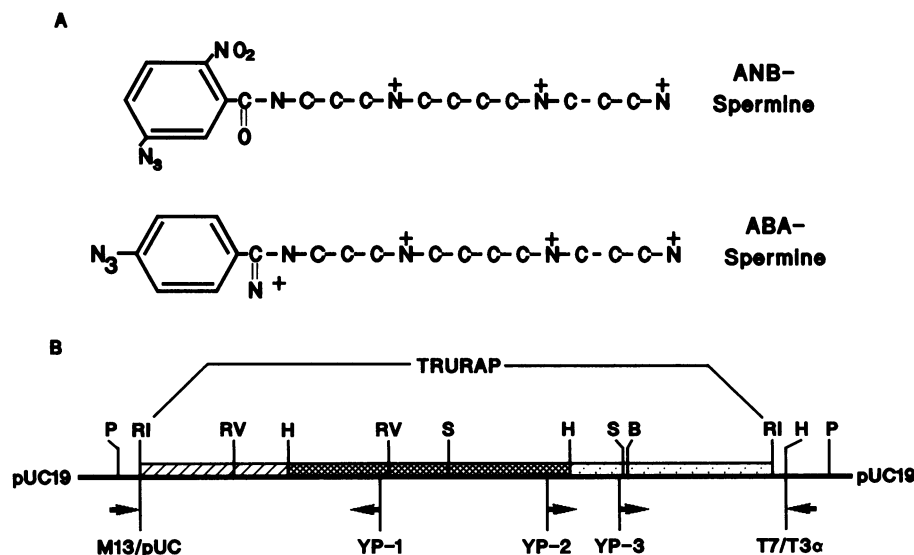

Figure 1. A. Photoaffinity polyamine structures: ANB-spermine and ABA-spermine. Hydrogen atoms are omitted for clarity. B. Physical map of plasmid pUC-R1 and positions of five primers. Restriction sites used are indicated as follows: Bgl II—B, Eco RI—RI, Eco RV—RV, Hind III—H, Pvu II—P, and Stu I—S. Five primers are M13/pUC, T7/T3α, YP-1, YP-2, and YP-3. The striped box denotes yeast TRP1 sequence; the crossed box, yeast URA3 sequence: and the spotted box, yeast ARS1 region. Arrow directions indicate the orientation of DNA synthesis in primer extension.

thymidine residue but it is not clear which portion of the DNA reacts with the activated aryl nitrene of ANB-spermine. It is difficult to conclude that the pyrimidine ring of thymine itself was modified, except possibly on the methyl group, since
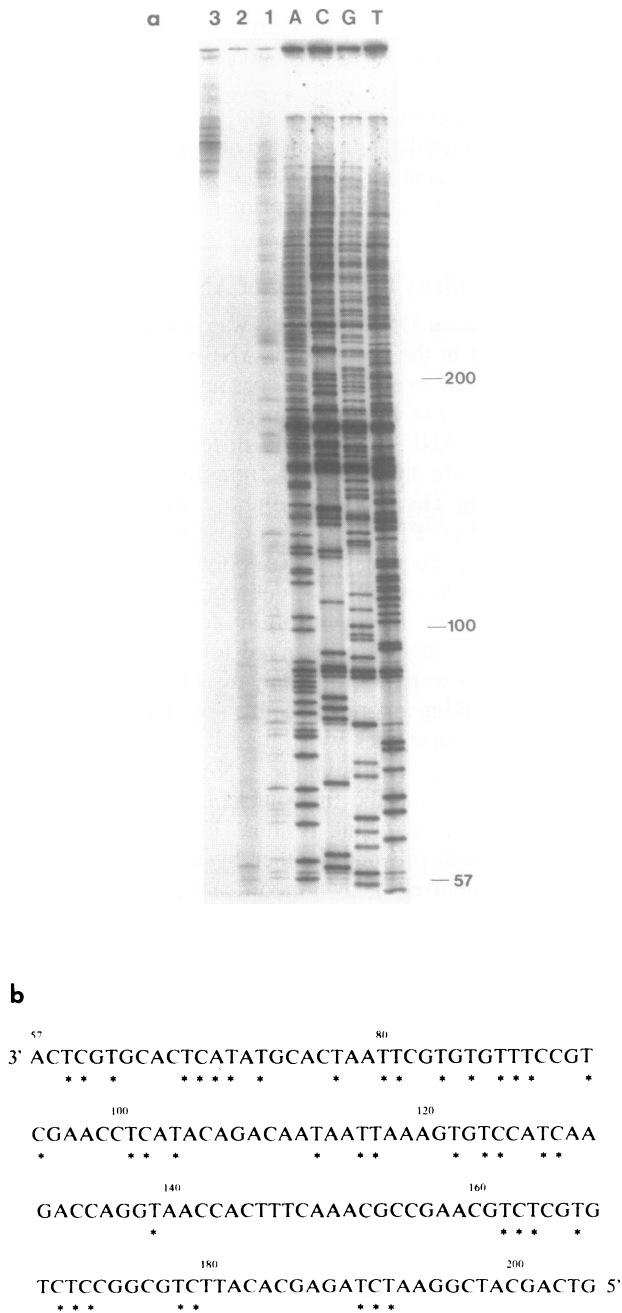

modification would probably prevent the polymerase from placing an A in the complementary position. It is more likely that modification occurs on the sugar-phosphate linker between the thymine and the next nucleotide, or possibly on the next nucleotide itself (i.e. the 3' neighbor).

Although the vast majority of the pause sites were at the position of thymidine residues, some pause sites were observed at cytidine residues and, very rarely indeed, at purine residues. Conversely, there were many thymidine residues that did not cause pausing. Where a pause site occurred, the intensity of the band on the gel varied in a reproducible manner from site to site, probably reflecting different occupancies of those sites or different reactivities of those sites with the ANB-spermine.

The role of reactivity was tested by using a different photoaffinity reagent, with a very similar azidobenzene photoactivateable group. This reagent is ABA-spermine and the structure is shown in Figure 1a. With nucleosome core particles, ABA-spermine binds to specific sites at 9.8 bp intervals along the DNA while ANB-spermine binds to a smaller set of sites generally different from those bound by ABA-spermine (12). If the specificity of binding of ANB-spermine to DNA were due to differing reactivities of different bases and environments in protein-free DNA, then the pattern of pause sites generated by the two ligands should appear similar. Figure 2 shows that ABA-spermine also generates specific pause sites, but they are in quite different positions from those generated by ANB-spermine on the same DNA sequence. Hence, we conclude that the specificity of the pause sites reflects binding sites for the ligand.

For the purposes of further analysis, each pause site was assigned an intensity value between 1 and 4. These numbers were

b

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57                                          80
3' ACTCGTGCACTCATATGCACTAATTCGTGTGTTTCCGT
   * * *    * * * * *    *   *   * *  *  * * *    *

        100                    120
   CGAACCTCATACAGACAATAATTAAAGTGTCCATCAA
   *      * * *        *  * *     * * *   * *

        140                    160
   GACCAGGTAACCACTTTCAAACGCCGAACGTCTCGTG
        *                            * * *    *

        180                    200
   TCTCCGGCGTCTTACACGAGATCTAAGGCTACGACTG 5'
   * * *        * *              * * *
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Figure 2. Mapping of binding sites of ANB-spermine on linear pUC-R1 DNA. a. Supercoiled pUC-R1 was linearized by restriction digestion (Pvu II and Stu I). The 1368-bp fragment was used as template for primer extension. DNA molecules were UV-irradiated in the presence of either ANB-spermine (lane 1), or ABA-spermine (lane 2), or spermidine (lane 3) at a charge ratio of 0.3 in TE buffer (10 mM Tris.Cl, 0.1 mM EDTA, pH 8.0). Primer extensions were carried out using Taq DNA polymerase with [γ-^{32}P] dATP incorporation. Lanes A, C, G, and T are sequence standards produced by sequenase version 2.0 DNA polymerase on double-stranded pUC-R1. The sequence given is of the synthesized strand. The numbers labeled next to the DNA standards are nucleotide positions from the primer M13/pUC. b. The partial sequence of the 1368-bp PvuII-StuI fragment of pUC-R1. Asterisks underneath the sequence indicate pause sites for DNA polymerase on DNA modified by ANB-spermine.

Table 1. Strength of ANB-spermine binding sites at each base

	Number					Percentage				
*	0	1	2	3	4	0	1	2	3	4
Base										
A	367	6	1	1	0	98	2	0	0	0
C	235	33	16	3	0	82	11	6	1	0
G	198	6	0	0	0	97	3	0	0	0
T	122	120	138	28	1	30	29	34	7	0

* The numbers, 0 to 4, in this row represent arbitrary levels of binding strength, as deduced from the intensity of the pause sites. Thus, for example, 367 or 98% of the A residues showed zero binding (no pausing) and 6 or 2% of the A residues showed weak binding (weak pause site).

Table 2. Nearest neighbors to ANB-binding sites at thymidine residues

	Number					Percentage				
	0	1	2	3	4	0	1	2	3	4
Sequence*										
A**T**	29	39	32	7	0	27	36	30	7	0
C**T**	15	13	42	11	0	19	16	52	14	0
G**T**	14	16	26	6	1	22	25	41	10	2
T**T**	64	52	37	4	0	41	33	24	3	0
TA	11	29	35	16	1	12	32	38	17	1
TC	36	24	20	2	0	44	29	24	2	0
TG	14	22	37	5	0	18	28	47	6	0
TT	61	45	46	5	0	39	29	29	3	0

* Each T residue appears twice, once with its 5' neighbor (upper 4 rows) and once with its 3' neighbor (lower 4 rows). The T at which the pause site occurs is in underlined bold type.

assigned subjectively and simply represent weak, average, strong and exceptionally strong pause sites. More detailed quantitation of the intensities of the bands was not attempted. A short computer program was written to construct a data-base of the information in which each nucleotide in the DNA sequences studied was assigned a value between 0 and 4, inclusive, to represent the existence and intensity of a pause site at that position. The number of nucleotides examined was 1,275. Analysis of this data showed that of the 409 thymidine residues, 287 (70%) were pause sites; in contrast, of the 375 adenosine residues, only 8 (2%) were pause sites (Table 1).

Sequences around pause sites

Since the strength of the pause site varied from thymidine to thymidine, the presence of a thymidine is not a sufficient condition for binding ANB-spermine. The effect of nearest neighbors was investigated by tabulating the binding at thymidines with each of the 4 possible 3' neighbors and each of the 4 possible 5' neighbors. Table 2 shows that the identity of the nearest neighbor is less important than the presence of a thymidine at the pause site. Nevertheless, it is clear that some neighbors are preferred and others are avoided. In particular, a 5' thymidine or 3' thymidine or cytidine are avoided: only about 60% of the thymidines in these environments were pause sites compared with 88% for the most preferred sequence, a 3' adenosine (Table 2).

The relative lack of pause sites for nearest neighbor thymidines suggested that runs of thymidine residues might be particularly avoided. This was tested by tabulating the strength of pause sites in all the runs of thymidines in the data set. Table 3 shows the results. The first row shows data for a T with non-T residues on both sides. As expected, this is a favorable situation for generating a pause site, with 82% of these sites generating a pause compared with only 70% for T residues in general. For runs of two and only two Ts, the percentage generating a pause site drops to 75% for both the 5' and the 3' member of the run. There is almost no difference in the strength of binding to the 5' T compared with the 3' T in these pairs of Ts. As the runs get longer, the number of pause sites generated, on average, falls to 59% for 3 and only 3 Ts.

The number of runs of greater than 3 Ts is small; hence the data for these longer runs must be interpreted with care. Runs

of 4 and only 4 Ts show similar lack of binding at the ends of the run, but the two central Ts show frequent binding comparable with that of single Ts. Binding at the 3' T of a run of 4 Ts is particularly weak with only 1 out of 14 showing even average binding. There are 3 runs of 5 and only 5 Ts in this dataset and they show a striking preponderance of no pause sites or weak pause sites. There are no runs of 6 and only 6 Ts but the one run of 7 and only 7 Ts shows an average pause site at the 5' T of the run but no pausing at the remaining Ts (not shown in the table). The only other run is of 12 Ts and no pausing was observed within this run as shown in Figure 3. Hence, in general, ANB-spermine tends to bind weakly or not at all to T residues in poly T tracts.

Perhaps the most interesting observation in Table 2 is the preference for TA sequences: 88% of TA sequences generate a pause site compared with 65% for all other T residues. We looked at the pause sites generated by TATA sequences and found 6 TATAs plus one TATATATA. All these sequences generated a pause site at each T and 7 of these 16 pause sites (31%) were strong or exceptionally strong compared with only 6% strong or exceptionally strong for all other Ts. The TATATATA sequence is shown in Figure 4; it is the 'TATA element' for the URA3 gene (25) and is the most strongly labeled sequence in

Table 3. Pause sites in poly(T) tracts

| Sequence* | Number Of Pause Sites Of Specified Intensity | | | | |
---	0	1	2	3	4
XTX	30	46	70	20	1
XTTX	11	12	19	2	0
XTTX	11	14	16	3	0
XTTTX	9	6	4	2	0
XTTTX	8	6	6	1	0
XTTTX	9	8	4	0	0
XTTTTX	6	3	5	0	0
XTTTTX	2	5	7	0	0
XTTTTX	3	9	2	0	0
XTTTTX	7	6	1	0	0
XTTTTTX	1	1	1	0	0
XTTTTTX	1	1	1	0	0
XTTTTTX	2	1	0	0	0
XTTTTTX	2	1	0	0	0
XTTTTTX	2	1	0	0	0

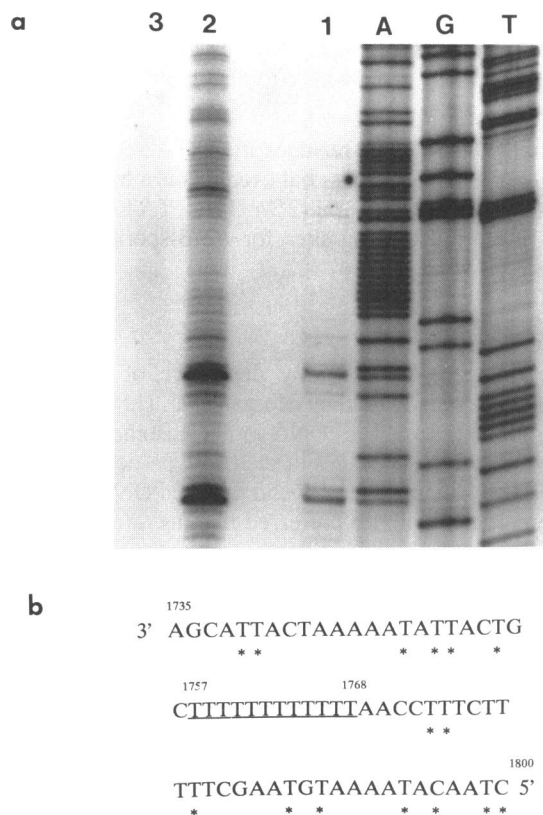* X represents either A,G or C. The pause site is in underlined bold type.

Figure 3. Interactions of ANB-spermine with the A-tract structure in solution. a. The pUC-R1 DNA was digested with a restriction enzyme (Pvu II) and irradiated in the presence of either ANB-spermine (lanes 1, 2) or spermidine (3) in TE buffer. Primer extensions were performed using either a ^{32}P-end-labeled oligonucleotide (YP-1) with sequenase DNA polymerase (1) or Taq DNA polymerase (lanes 2,3) with [γ-^{32}P] dATP incorporation. DNA sequencing lanes were labeled A, G, and T. b. DNA sequence surrounding the poly(dT)$_{12}$ (A-tract) in the ARS1 region of pUC-R1. The poly(dT)$_{12}$ sequence is underlined. Asterisks underneath the sequence indicate pause sites for DNA polymerase on DNA modified by ANB-spermine.
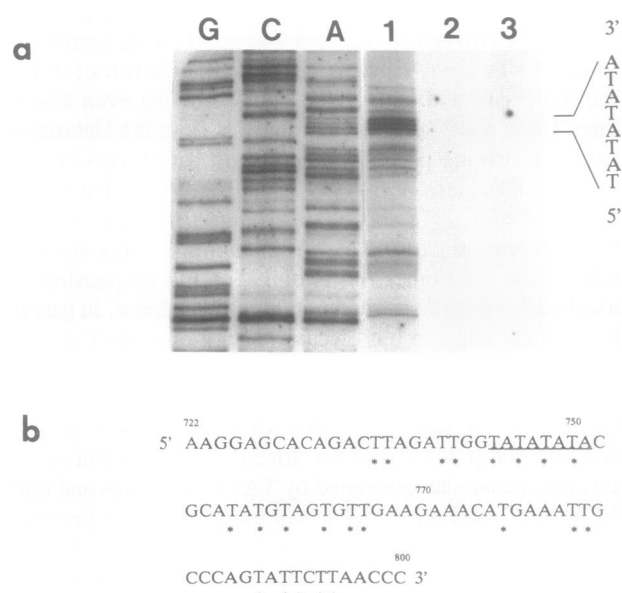
Figure 4. Interactions of ANB-spermine with the 'TATA' element in the 5' control region of the yeast URA3 gene. a. Lanes 1 to 3 are primer extensions. The DNA was irradiated with either ANB-spermine (lane 1), or ABA-spermine (lane 2), or spermidine (lane 3). DNA sequencing lanes were labeled A, G and C. b. DNA sequence surrounding 'TATA' element in the 5' control region of URA3 gene. The 'TATA' sequence is underlined. Asterisks underneath the sequence indicate pause sites for DNA polymerase on DNA modified by ANB-spermine.

the dataset. Of the 16 A residues in TATA sequences, only 2 (12%) generated pause sites, but even this is a higher frequency than for A residues in general (2%). Thus, TATA sequences are strongly preferred binding sites for ANB-spermine.

DISCUSSION

ANB-spermine, a photoaffinity derivative of the polyamine spermine, was initially used by Morgan et al. (11) to map binding sites on 'random sequence' DNA in nucleosome core particles. Although the DNA sequences in such core particles are not truly random (26), ANB-spermine bound to naked DNA isolated from the core particles with no preferred binding sites. Thus, it was inferred that the preferred binding sites seen for binding of ANB-spermine to nucleosome core particles were due to either the perturbation of the DNA structure by the proteins or to a direct effect of the protein part of the nucleosome. The experiments reported in the current paper show that DNA primary and secondary structure are major factors in determining the binding of ANB-spermine to DNA.

In the study of polyamines, it is of central importance to distinguish effects that are due primarily to non-specific functions of these poly cations from effects that require the specific molecular features of the polyamines. At low resolution, the effects of polyamines on DNA appear to be largely electrostatic and non-specific. However, recent studies have suggested that there are specific effects in addition to the non-specific electrostatic interactions. The most dramatic of these is the stabilization of left-handed DNA (Z-DNA) by spermine and spermidine (5–7) but other effects have been noted as well (e.g. (11)). Theoretical studies have indicated that polyamine binding to DNA would be sensitive to secondary structure of the DNA

(27) The dependence on secondary structure shown by ANB-spermine (this paper) implies that polyamine binding, in general, is sensitive to a variety of DNA structural variations.

Virtually all the binding sites observed for ANB-spermine on the 1,275 bases studied cause pausing of DNA polymerase at a pyrimidine residue. Since the complementary purine is added by the polymerase, it is unlikely that the template is modified directly on the pyrimidine base. Since there is no obvious pattern to the next base downstream on the template, we conclude that the base that is recognized by ANB-spermine is the pyrimidine but that the actual modification occurs either on the adjacent base, or on the sugar-phosphate backbone of the DNA.

The question arises as to whether the specificity observed is due to the polyamine part of the ANB-spermine or to the photoaffinity group. As an approach to this question, we carried out some limited experiments with another photoaffinity polyamine, ABA-spermine. This molecule has a similar photoaffinity group, an azide, and it shows sequence specificity of binding. However, the specificity is different from that observed with ANB-spermine (Figure 2). We conclude that reactivity to the photoactivated azide is not the major factor determining binding of these photoaffinity polyamines to DNA. This is also consistent with the suggestion, above, that the site of modification of DNA might be the sugar-phosphate backbone, whose primary structure is not sequence-dependent. ANB-spermine and ABA-spermine do differ in the way in which the photoaffinity group is linked to the polyamine. In particular, the polyamine part is considerably different since the terminal charge is lost in ANB-spermine but retained in ABA-spermine. This makes ANB-spermine an analog of acetylspermine while ABA-spermine is more akin to spermine itself. There are also differences in the benzyl group that links the azido group to the polyamine moiety. In ANB-spermine, the azido group is on the 3' carbon of the benzene ring while in ABA-spermine it is on the 4' carbon; in addition, ANB-spermine includes a nitro group on the ring. We cannot tell to what extent the sequence specificities of ANB-spermine and ABA-spermine are due to differences in the polyamine part or the linker benzene ring.

Data reported previously by others indicates that at least some of the sequence specificity shown here for ANB-spermine may also be shared by spermine itself. In particular, Basu et al. (28) showed different changes in imino proton exchange rates when spermine bound to poly(dG-dC) compared with poly(dA-dT). Plum and Bloomfield (29) observed differences in the effects of spermidine on imino proton exchange rates between poly[dA]·poly[dT] and the alternating copolymer d[AT]$_{15}$·d[AT]$_{15}$. Marquet & Houssier (30) found different binding modes of spermine to poly(dG-dC) as compared to polynucleotides containing A-T bp. Binding to A-T bp appeared to be asymmetric and to induce bending in the DNA. Stewart (31) found differences in ethidium displacement between poly(dA-dT) and poly (dG-dC). None of these published experiments show such a pronounced sequence specificity as is found in our experiments. However, the new data reflects binding in 'real' DNA sequences, rather than the homopolymeric or alternating copolymer sequences used in the previous studies. The effects of competition between sites in a 'real' DNA sequence may enhance the sequence specificity effects.

Since binding to particular T residues varies over a wide range, there must be an effect of other residues on the binding of ANB-spermine to DNA. The study of the nearest neighbor residues shows some specificity but nothing as striking as the primary

requirement for a pyrimidine, particularly T, at the binding site. This suggests that secondary structure, rather than primary structure *per se*, might be the major subsidiary factor determining binding. This conclusion is reinforced by a comparison of binding to alternating poly d(AT)·d(AT) sequences with binding to homopolymer sequences, poly dA·T. Binding to T residues in the alternating sequences is greatly enhanced over binding to the T residues in the homopolymer sequences. The structure of homopolymeric dA·T sequences is very different from that of most sequences in the B-DNA conformation, both in the crystal form (32) and in solution (33–36). In fact, phased runs of poly dA·T give rise to intrinsically bent DNA (37). The presence of bifurcated hydrogen bonds in the crystal structure of d(CGCA-AAAAAGCG) and its complement suggests that a minimum of three consecutive As would be necessary and sufficient to form the specific poly dA·T structure (32). This is consistent with the data in Table 3: 54% or 45% of the T residues in XTX or XTTX sequences have medium or strong binding while only 27% of T residues in XTTTX or XTTTTX sequences have medium or strong binding. From the point of view of ANB-spermine, the structure of poly dA·T sequences is characterized by ordered water within the minor groove and a narrowing of that minor groove (e.g. (38)). These conditions clearly lead to an inhibition of binding of ANB-spermine.

Runs of alternating poly(dAT·dAT) also have a characteristic structure (39–43) with an alternating helical twist between the TpA steps and the ApT steps. The most striking example in our data set is the 'TATA box' region of the URA3 gene. Thoma (14) showed that this sequence is exceptionally sensitive to micrococcal nuclease, both as naked DNA and when it is complexed into chromatin in the TRURAP plasmid in yeast. Thus, there is an unusual structure present which is also associated with a nucleosome-free region in chromatin. Figure 4 shows that ANB-spermine can also recognize and label this structure.

It has previously been proposed (17) that acetylation of polyamines might be involved, together with histone acetylation, in the formation and/or maintenance of active chromatin. If the preference of ANB-spermine for the TATA element is shared by spermine or spermidine, then these polyamines would mask the TATA element in inactive chromatin. Acetylation would then weaken the interaction of the polyamines with the TATA element, enhancing the binding of competing transcription factors. This is consistent with the suggestion from X-ray crystallographic studies that spermine and DNA form complexes that are both site-specific and dynamic (44). In addition, it has been suggested that polyamines might bind to DNA and displace basic proteins (45,46). In this case, binding of polyamines to the TATA element would inhibit the binding of histones and the subsequent formation of nucleosomes, keeping the TATA element nucleosome-free, as observed (14). It will clearly be of interest to see if the specific binding to the TATA element can be generalized to other TATA elements and hence if the above hypothesis can be generalized.

The use of photoaffinity polyamines to map polyamine binding sites was introduced by Morgan et al. (11) and binding sites on nucleosome core particles *in vitro* have been mapped (11,12). Of particular interest is the observation that ABA-polyamines appear to change the helical twist of DNA in the nucleosome core particle (12), which is consistent with the changes in circular dichroism of nucleosome core particles in the presence of unmodified polyamines (17). Thus, these photoaffinity polyamines are providing insights into the structure of chromatin at the nucleosome level. In this paper, the use of photoaffinity

polyamines has been extended to the nucleotide level, with the mapping of binding sites for ANB-spermine to several DNA sequences. The data suggest that polyamines may bind particularly strongly to particular DNA sequences, e.g. the TATA element, and thus play an important and dynamic role in chromatin structure and function.

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