Location of Spermine and Other Polyamines on DNA As Revealed by Photoaffinity Cleavage with Polyaminobenzenediazonium Salts†

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ABSTRACT: Although polyamines interact strongly with nucleic acids, X-ray and NMR studies have not revealed much structural information about spermine–DNA complexes. Therefore, it was of interest to look at the binding of polyamines to ^{32}P-labeled DNA restriction fragments by sequencing gel electrophoresis of the photoaffinity cleavage products induced by polyaminobenzenediazonium salts. The shift of cleavage patterns observed on opposite strands as well as competition experiments with distamycin shows polyamines to be located in the *minor groove of B-DNA* and to depend on the nucleic acid polymorphism, jumping to the major groove in the A-form. The sequence selectivities of various polycations (spermine, putrescine, and cobalt(III) hexaammine) are similar and *slightly favor A,T-rich regions*. Taken together, these results show that polycations which are not point charges are guided by the electronegative potential along the nucleic acid and suggest *fast crawling of the polyamine within the minor groove*, due to individual NH_2^+ jumping between multiple equidistant and isoenergetic bidentate hydrogen-bonding sites. Such a picture could be the clue to the unexpected NMR and to the frequently silent X-ray behavior of polyamines when bound to DNA.

A great deal of work has been devoted to the naturally occurring polyamines, owing to some major function(s) expected on the basis of their wide and abundant distribution and of a strong regulation of turnover and acetylation [for reviews, see Tabor and Tabor (1984), Pegg (1986), and Schuber (1989)]. Since the first physicochemical evidence of interactions between polyamines and DNA (Tabor, 1962), nucleic acids have been considered to be among their prime targets, and complexation has been studied at the molecular level by numerous techniques. Although thermodynamic measurements show the association with double-stranded DNA (Braunlin et al., 1982; Morgan et al., 1986) and RNA (Sakai & Cohen, 1976) to be quite strong, and despite the well-known stabilizing effect and the common use of spermine in the crystallization of nucleic acids, structural information on the complexes is very scarcely documented. Spermine has been localized in only a few single-crystal X-ray structures of oligodeoxynucleotides, one belonging to the A-DNA (Jain et al., 1989) and one to the Z-DNA (Gessner et al., 1989) family, two in anticancer drug–DNA complexes (Frederick et al., 1990), and less precisely in the structure of an oligonucleotide of the B family (Drew & Dickerson, 1981), and in $tRNA^{Phe}$ (Quigley et al., 1978) and $tRNA^{Asp}$ (Dumas, 1986). Solution studies performed with NMR relaxation techniques highlight the major dynamic changes brought about by polyamines on DNA (Braunlin & Bloomfield, 1988), but NOE studies also failed to give structural information, apparently due to the fast diffusion of the cation within the complex (Wemmer et al., 1985). Such poor experimental structural data have stimulated much theoretical work (Zakrzewska & Pullman, 1986; Feuerstein et al., 1986, 1990, and references therein) since Tsuboi and Liquori's first models (Tsuboi, 1964; Liquori et al., 1967); however, emerging pictures are controversial with respect to groove preference as well as binding sites (phosphate versus bases) or sequence selectivity. Thus, a decade after Cohen's News and Views article in *Nature*, "What do the polyamines do?" (Cohen, 1978), the simple question "Where do the polyamines go?" on their most ubiquitous B-DNA target still remains open.

We recently synthesized a series of polyaminobenzenediazonium salts able to photocleave DNA at nanomolar concentrations (Behr, 1989); these compounds have been used as photoaffinity probes for RNA structure and interaction with proteins (Garcia et al., 1990). In the present study, we make use of a similar approach on a DNA restriction fragment in order to give an answer to the above question with respect to groove nature, base sequence, and polyamine structure. Furthermore, we show with distamycin as an example that the specific advantages of sperminediazonium make it a valuable footprinting reagent.

Materials and Methods

The *p*-diazonioanilides of L-ornithine (PuN_2^{3+}) and 5-carboxy-L-spermine ($SperN_2^{5+}$) were prepared as previously described (Behr, 1989). The plasmid pBR322 was from Boehringer Manheim, and all enzymes were from BRL. The radioactive nucleotides [α-^{32}P]TTP and [γ-^{32}P]ATP, both at 3000 Ci/mmol, were from Amersham.

Preparation of End-Labeled DNA Fragments. The plasmid pBR322 was first digested with *Eco*RI and then labeled either at its 3′ or at its 5′ end; 3′-end labeling was performed with [α-^{32}P]TTP, dATP, and the Klenow fragment of DNA polymerase I. For 5′-end labeling, DNA fragments were first dephosphorylated with alkaline phosphatase and then phosphorylated with [γ-^{32}P]ATP and phage T4 polynucleotide kinase (Maniatis et al., 1982). A second enzymatic digest with *Taq*I yielded two end-labeled fragments of 343 and 24 base pairs. The 343-base-pair fragment was isolated by electrophoresis on a 1/30 cross-linked 8% polyacrylamide gel.

Photochemical Cleavage. The photochemical reaction was performed at room temperature in 10 mM sodium acetate buffer, pH 6 (buffers containing nucleophiles reacted with the probes: half-life in Tris, pH 7.6, was 3 min; in phosphate, pH

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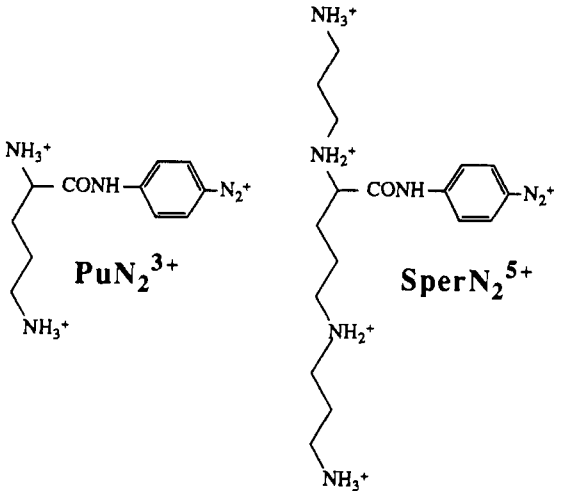

FIGURE 1: Structures of the *p*-diazonioanilides of L-ornithine (PuN_2^{3+}) and 5-carboxy-L-spermine ($SperN_2^{5+}$).

7.3, 12 min; in Mes, pH 6.1, 5 min; and in acetate, pH 6, >3 days). General conditions were as follows: 1 μM (base pair) labeled restriction fragment (>3000 cpm), 0.1 mM (base pair) calf thymus DNA, and 2 μM $SperN_2^{5+}$ or 20 μM PuN_2^{3+} in a total volume of 10 μL. For samples containing distamycin, the buffered DNA solution was incubated with the drug (1 and 10 μM) for 20 min at room temperature. Samples were immediately illuminated with a slide projector after probe addition.

Analysis of Cleavage Positions. Equal amounts of radioactivity for each sample were loaded on a denaturing 1/20 cross-linked 8% polyacrylamide gel; DNA sequencing was carried out as usual (Maxam & Gilbert, 1980). Electrophoresis was carried out at 1400 V for 100 min, and gels were autoradiographed at −80 °C with intensifying screens. The autoradiograms were scanned on a Shimatzu CS-930 densitometer.

Results and Discussion

In order to leave intact the primary and secondary ammonium groups of putrescine and spermine, which are expected to be responsible for DNA binding, the nucleic acid reactive moiety was linked to the polyamine through a carbonyl group [PuN_2^{3+} and $SperN_2^{5+}$, see structures in Figure 1, were synthesized from ornithine in a five-step procedure; see Behr (1989)]. However, the attached benzenediazonium group bears some structural similarity to part of the structure of berenil and other phthalanilides thought to prefer the DNA minor groove (Zimmer & Wähnert, 1986), which may interfere with the natural polyamine's preference. Competition experiments between PuN_2^{3+}, $SperN_2^{5+}$, and their corresponding natural analogues have shown that probes and polyamines bind to DNA (Behr, 1989) and RNA (Garcia et al., 1990) with similar strength and sequence selectivity (see also below); the only perturbation brought about by attachment of the photoreactive group could be a p*K* lowering of the proximal amine function. On the other hand, possible van der Waals interactions with the minor-groove walls or unlikely intercalation between base pairs are expected to perturb the electronic absorption and ^1H NMR spectra of the highly dipolar benzenediazonium chromophore; no such effects were observed when excess DNA was added to $SperN_2^{5+}$ in the dark (Behr, 1989).

p-Anilido diazonium salt solutions are stable for months in the absence of strong nucleophiles, provided they are kept in the dark. When illuminated with soft 320–400-nm UV light (sunlight or a slide projector), they instantaneously induce single-strand breaks in nucleic acids with high quantum and chemical yields. The reactive species is probably the short-lived benzene carbocation generated by photochemical nitrogen abstraction since the corresponding phenol is the sole compound detected by NMR [Behr, 1989; for a discussion of the mechanism, see Garcia et al. (1990)]. Thus the reactive species is not diffusible, and due to restricted conformational freedom along the *CH–CO–NH–Ar linker, it is altogether very likely that $SperN_2^{5+}$ cuts DNA at spermine-binding sites. Figure 2 shows the effects of $SperN_2^{5+}$ on a 5′- and a 3′-^{32}P-labeled *Eco*RI/*Taq*I 343 base-pair restriction fragment of pBR322, as revealed by denaturing polyacrylamide gel electrophoresis. Several interesting features emerge on careful examination of this autoradiogram.

First of all, for the sequencing method to reveal true binding sites on *intact* B-DNA, care must be taken that the probe cuts each restriction fragment once at most, and this is probably the case as long as the intact fragment remains in large excess. The short photogenerated fragments do not migrate with anomalous mobility in the sequencing gels, suggesting that the polyamine is not covalently bound to either the 5′ or 3′ newly formed ends. In our experimental conditions (10 mM buffer and 0.1 mM (base pair) DNA), micromolar $SperN_2^{5+}$ concentrations (and less when omitting the carrier DNA) are sufficient to give a cleavage pattern, highlighting the strong polycation–polyanion interactions. This pattern is similar, at somewhat higher probe concentration, in 100 mM NaCl or in the presence of competing spermine (data not shown).

With respect to the gross shape of the double-stranded nucleic acid, major-groove, phosphate strand, and minor-groove binding sites may be distinguished. Groove preference may be assessed by comparison of cleavage patterns on opposite strands (Taylor et al., 1984). The 3′- and 5′-end-labeled fragments exhibit similar wavelike patterns (lanes 4 and 5 in Figure 2a; see also Figure 2b) except for a systematic 2–3-base-pair shift of the 5′-end-labeled fragment toward heavier fragments. Such a similarity rules out phosphate strand binding, and the shift's polarity shows that *spermine lies in the minor groove of B-DNA.* Indeed, if the photogenerated electrophile reacts with the deoxyribose–phosphate backbone from within the minor groove (Figure 3), the closest cleavage sites on opposite strands are 2–3 base pairs apart, and the right-handed nature of the double helix leads to longer 5′-labeled fragments (the reverse would hold if it bound to the major groove). Furthermore, in the presence of 1–10 μM distamycin, an antibiotic known to bind to the minor groove of A,T-rich regions (Van Dyke et al., 1982; Kopka et al., 1985, and references therein), cleavage by 2 μM sperminediazonium is completely inhibited in these regions (Figure 4), showing again that spermine binds exclusively to the minor groove at micromolar concentrations. This finding contrasts with a crystal-structure study in which a spermine molecule was tentatively localized in the major groove of an oligonucleotide belonging to the B-family [Drew & Dickerson, 1981; the polyamine was not reported in a subsequent rerefinement by Westhof (1987)]. Of course, preferential minor-groove binding does not exclude additional major-groove or phosphate binding at higher concentrations.

Quite interestingly, the shift between cleavage positions on opposite strands also reveals the location of polyamines in an A-form double-stranded nucleic acid. Indeed, previous work [Figure 4 in Garcia et al. (1990)] shows that PuN_2^{2+} and $SperN_2^{5+}$ map a diffuse binding site along the contiguous anticodon and D stems of $tRNA^{Asp}$. The strongest cleavage

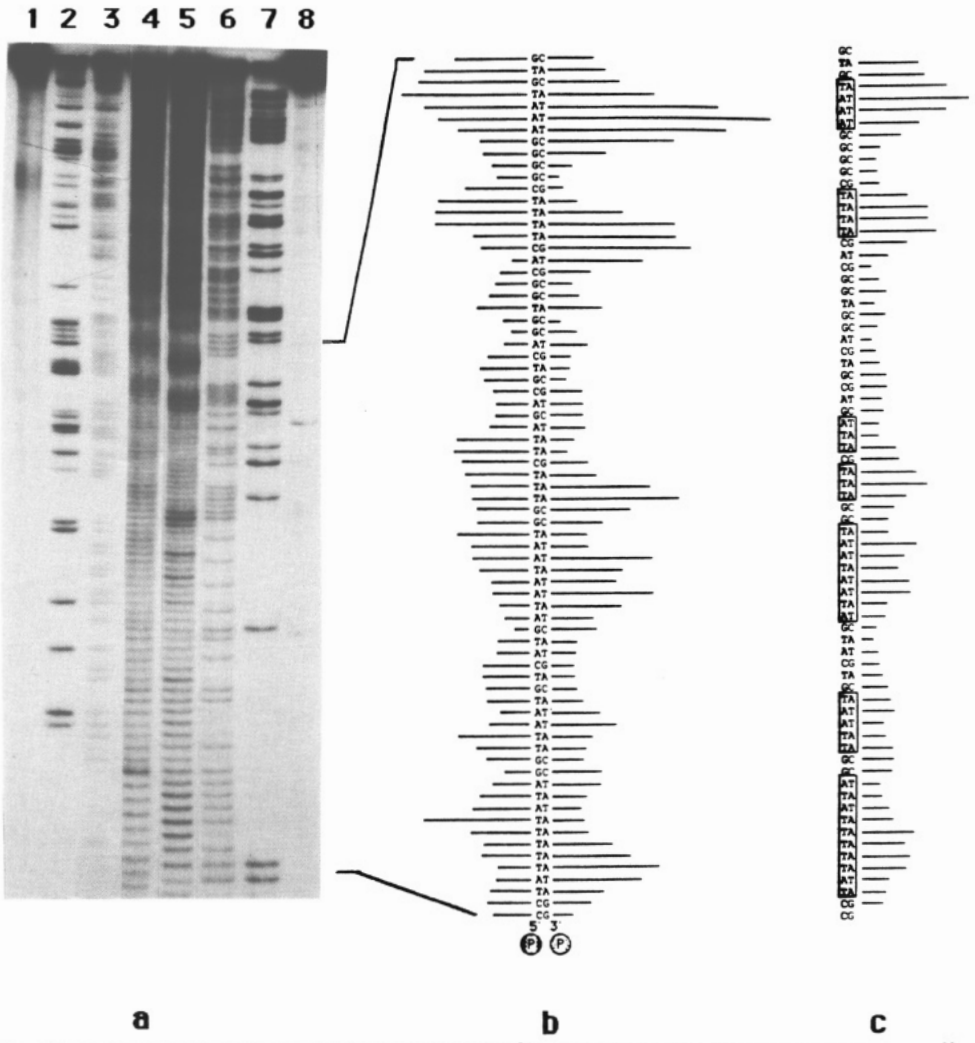

FIGURE 2: (a) Denaturating gel electrophoresis autoradiogram of $SperN_2^{5+}$-induced strand breaks on a 343-base-pair ^{32}P-labeled restriction fragment [0.1 mM (base pair) calf thymus DNA]: lanes 1–4, 5′ labeling; lanes 5–8, 3′ labeling; lanes 1 and 8, controls; lanes 2, 3 and 7, 6, Maxam–Gilbert G and G+A reactions, respectively, lanes 4 and 5, 2 μM photoprobe. (b) Densitometric scan intensities of the lower parts of lanes 4 and 5 reported on the corresponding sequence showing the 2–3-base-pair shift of the 5′-labeled strand toward longer fragments; in the upper part, signals become increasingly higher due to peak overlap. (c) Spermine sequence selectivity obtained by shifting the 5′ spectrum one base pair lower and the 3′ spectrum one base pair higher and then averaging intensities.

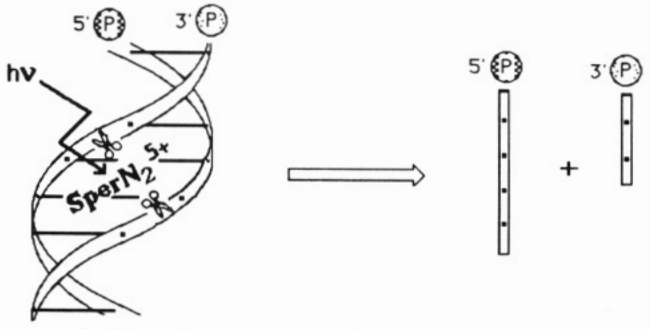

FIGURE 3: Schematic representation of a minor-groove attack, leading to ca. 2 base pairs heavier 5′-labeled fragments.

is centered around P41 on one strand, and the corresponding opposite strand cut is at P24 (and not P31), showing that in this case *polycations probably lie in the deep major groove of the A-form RNA*, in agreement with former X-ray work (Quigley et al., 1978; Jain et al., 1989). This minor-to-major-groove jump of spermine relative to the B/A polymorphism was predicted on the basis of theoretical simulations (Zakrzewska & Pullman, 1986).

Assuming that the sequencing gel band intensities reflect the occupation of each site, the observed wavelike patterns are due to sequence-modulated affinity (Figure 2c). Although binding is weaker for PuN_2^{3+} than for $SperN_2^{5+}$ (about 10 times higher concentration is necessary to give comparable band intensities; data not shown), *both polyamines display the same approximately 2- to 3-fold preference for A,T-rich regions* [spermine has been reported previously to have weak or no base-pair selectivity (Hirschman et al., 1967; Morgan et al., 1986) or G,C selectivity (Igarashi et al., 1982; Stewart, 1988)]. Yet the definite A,T selectivity found here represents only a 0.7 kcal/mol difference in binding energies, which amounts only to 10% of the total energy (this may account for the discrepancies observed between experiments performed in different conditions and sometimes with homopolymeric DNA's of noncanonical B structure). Competition experiments done with $Co(NH_3)_6^{3+}$, another $N-H^{\delta+}$ delocalized polycation, support similar conclusions (data not shown). Thus, *for a given charge, there seems to be no strong constraint on the polyamine structure or on the DNA sequence for complexation to occur*. The first point is also supported by the structure-independent increase in DNA-melting temperature observed in the presence of various spermidine analogues with the structure $^+H_3N(CH_2)_3NH_2^+(CH_2)_nNH_3^+$, provided $n > 2$ (Thomas & Bloomfield, 1984). On the nucleic acid side, the small selectivity observed may arise from a significantly

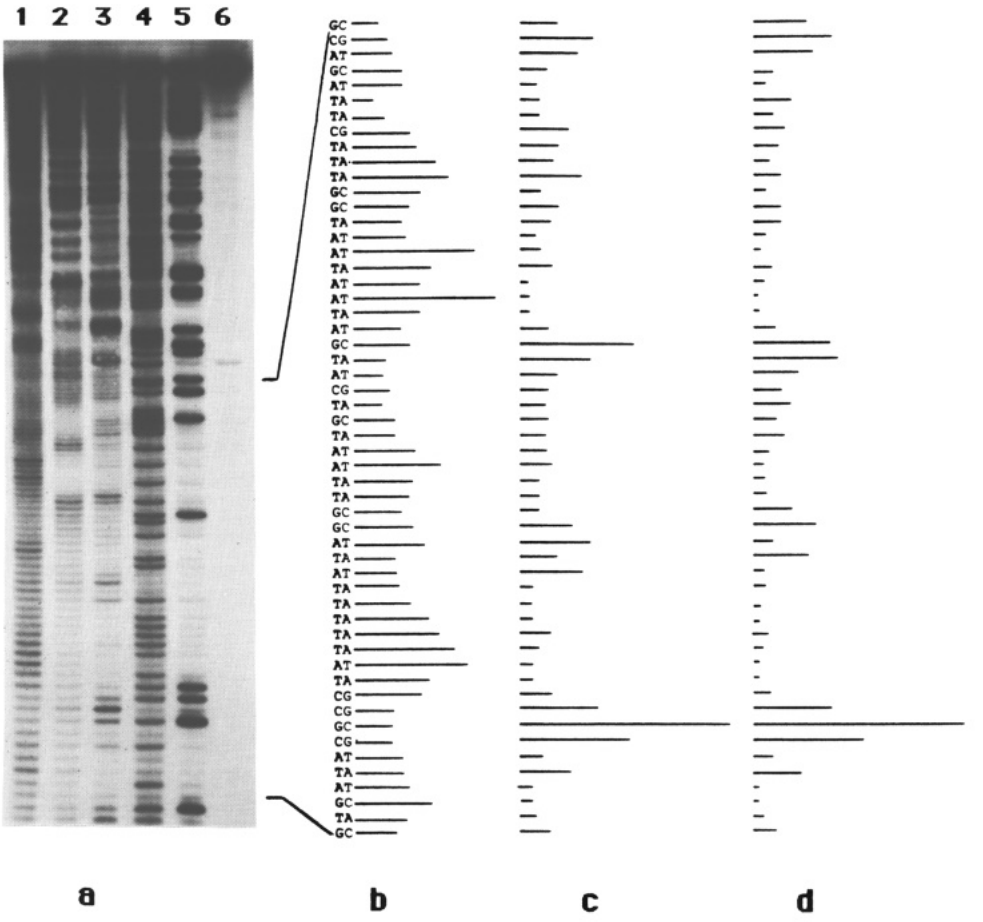

FIGURE 4: (a) Footprinting of distamycin binding by $SperN_2^{5+}$ on the 3′-labeled fragment (conditions as in Figure 2): lanes 1–3, 2 μM photoprobe; lanes 2 and 3, 1 and 10 μM distamycin, respectively; lanes 4 and 5, Maxam–Gilbert G+A and G reactions, respectively; lane 6, control. (b–d) Densitometric scan intensities of lanes 1 and 3 and the ratio of scan c over scan b, highlighting the drug's selectivity; the actual binding sequences are shifted one base pair higher due to 3′ detection (see Figures 2c and 3).

narrower minor groove in A,T-rich regions than in mixed sequences, leading to closer phosphate strands. Indeed, this is the only characteristic deviation that is common to all structures containing four or more successive A,T base pairs analyzed so far (Yoon et al., 1988); two base pairs are not sufficient to induce the groove narrowing, and no structure has been solved to date with three consecutive A,T pairs. From the limited data available here (Figure 2c), it seems that the homopolymeric TTT sequence is better in doing so than 5′-TTA.

Taken together, strong minor-groove and weak A,T preferences demonstrate that polyamines are guided by the electronegative potential along the nucleic acid, which is highest on the groove's floor (Zakrzewska & Pullman, 1986), lined with the two hydrogen-bond donors O2(pyrimidine) and N3(purine) for each base pair. Cross-strand bidentate hydrogen bonds between the donor atoms of two successive base pairs and a water molecule have been found in the structure of an A,T-rich oligonucleotide (Drew & Dickerson, 1981). Similarly, the bidentate ammonium groups of polyamines could form $O(2)_{i-1}$–HNH^+–$N(3)_j$ or $O(2)_j$ bonds (Figure 5), and molecular models show the distance between consecutive sites to be compatible with propyl and longer spacings in the polyamine, in agreement with experiment (Thomas & Bloomfield, 1984). Other bidentate binding sites of compatible distance from $O(2)_{i-1}$–HNH^+–$N(3)_j$ or $O(2)_j$ include intrastrand $O(4')_{i-1}$–HNH^+–$N(3)_i$ or $O(2)_i$ and $O(4')_{j+2}$–HNH^+–$N(3)_{j+1}$ or $O(2)_{j+1}$ (Figure 5), which may be used by the polyamine as well, especially as a bypass in regions where the NH_2 group of guanine is protruding from the groove's floor. NH_2^+ intersite crossing would break only one hydrogen bond at a time, and thus *the polyamine's behavior could be seen as a fast "crawling" along the groove, with ammonium groups jumping to multiple equidistant and isoenergetic binding sites, hence the low activation energy for molecular motion.* Such a picture could be the clue to the apparent independence of spermine motion with respect to that of the nucleic acid (Wemmer et al., 1985), despite a dissociation constant in the micromolar range. Indeed, as pointed out by

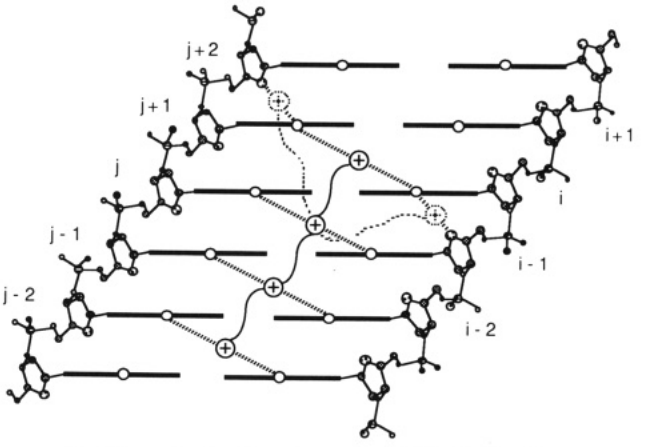

FIGURE 5: Schematic drawing of spermine binding in the minor groove of B-DNA. Nucleic bases are seen through their N(3) and O(2) pointing edges (O); the polyamine's ammonium groups (+) can jump from the bottom N(3),O(2) to the edges O(4′),N(3)/O(2), allowing fast crawling motion without decomplexation.

Wemmer et al. on the basis of dynamic NMR measurements, this surprising behavior "could arise either from very rapid diffusion along the DNA duplex between specific tight-binding sites or from delocalized interactions with no discrete binding sites". On the other hand, the well-documented polyamine-induced DNA-melting temperature increase may be related to the formation of a zipperlike NH_2^+ interstrand hydrogen-bonding network. Further along this line, some of the electron-density peaks found in the structures of B-type double-stranded oligonucleotides, which have been interpreted to be water molecules hydrating the minor groove [Privé et al., 1987; Cruse et al., 1986; see also Westhof (1988)], may in fact be localized ammonium groups of spermine molecules with invisible carbon skeletons averaged over space or time by variable positioning in the crystal.

The strong affinity but weak sequence selectivity of $SperN_2^{5+}$ toward DNA add to its straightforward use to photochemically induce instantaneous strand breaks and make it an easy-to-use footprinting agent. As an example, distamycin binding is clearly revealed by the diazonium salt, when both are at micromolar concentrations (Figure 4). The patterns generated by sperminediazonium photoaffinity cleavage and distamycin footprinting (lanes 1 and 2 in Figure 4) are like a photograph and its negative, due to similar site preference. At 10 μM drug, cleavages at the borders of the drug-binding sites are even more pronounced than those of spermine alone; the reason for this may be an antibiotic-induced modification of the minor groove in its neighborhood or may be simply the consequence of an apparent increase in photoprobe concentration for the sites remaining drug-free. Distamycin is known to have a minimum binding site of four A,T base pairs (Pelton & Wemmer, 1990, and references therein), and indeed all of these sites are mapped to one-base resolution. However, the selectivity seems not to be as strong as inferred from work on oligonucleotides with a given sequence, since in the concentration range used here 5'-TGAT is well mapped too.

Forthcoming work with spermine derivatives will be aimed at understanding the role of polyamines in DNA structural transitions (B to A or Z, triple helixes) or at taking advantage of the carboxylate anchor group to introduce new functions into the molecule, such as lipospermines able to coat plasmid DNA with a lipid layer thus facilitating gene transfer (Behr et al., 1989).

ACKNOWLEDGMENTS

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Registry No. PuN_2^{3+}, 132622-01-6; $SperN_2^{5+}$, 132644-44-1; $Co(NH_3)_6^{3+}$, 14695-95-5; putrescine, 110-60-1; spermine, 71-44-3.

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