# Sequence-Directed Single Strand Cleavage of DNA by a Netropsin−Flavin Hybrid Molecule†

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ABSTRACT: In an attempt to obtain sequence specific DNA-cleaving molecules, we have synthesized a series of hybrid minor groove binders composed of a photoactiveable isoalloxazine (flavin) chromophore linked through a polymethylenic chain to a bis-pyrrolecarboxamide moiety related to netropsin. Like netropsin, the hybrid derivatives preferentially bind to A+T-rich sequences. Activation of the flavin chromophore by visible light results in the appearance of single strand breaks in the vicinity of the DNA binding site. We have further investigated the cleavage affinity properties of one of these compounds referred to as netropsin−flavin (Net-Fla) and considered as representative of the series. Net-Fla cleaves only one strand at a specific locus downstream of 5′-AAAT-3′, upstream of 5′-TAAA-3′ and on either side of a 5′-AAAA-3′ sequence. Net-Fla cleaves both strands downstream to 5′-AATT-3′. This makes the properties of Net-Fla similar to that of a restriction endonuclease and provides additional insight into establishing the rules for the readout of B-DNA helix by non-nucleotidic compounds. Using molecular modeling, we show that Net-Fla binds to an asymmetric site in one orientation. The values of the energetic minima lie in the same order as expected from the cleavage patterns, which suggests that the oriented cleavage is a consequence of a sequence-oriented binding of Net-Fla in the DNA minor groove.

The design of sequence specific cleaving molecules for double helical DNA requires the linkage of a DNA-cleaving moiety to a sequence specific DNA binding molecule. Targeted cleavage of double helical polynucleotides by either complementary oligonucleotides (Moser & Dervan, 1987; François *et al.*, 1988) or poly(pyrrolecarboxamide) derivatives coupled to metal-chelating agents generating oxy radicals (Schultz *et al.*, 1982; Youngquist & Dervan, 1985) has been attempted by several groups. It was found that these functionalized hybrid molecules may act as artificial nucleases and can serve as models for the design of molecules of pharmacological interest (Dervan, 1986). Along this line, we have synthesized molecules (Figure 1) composed of a bis-pyrrolecarboxamide moiety related to netropsin linked to a photoactiveable isoalloxazine (flavin) moiety, generating oxy radicals upon light irradiation (Herfeld *et al.*, 1994). These hybrid molecules preferentially bind to A+T-rich regions in the DNA minor groove as expected from the netropsin-like moiety (Zimmer, 1975) and in the presence of molecular oxygen cleave pBR322 DNA at discrete sites upon irradiation by visible light. In order to further investigate the sequence-reading properties of these molecules, one of them referred to as netropsin−flavin (Net-Fla, Figure 1) was used to analyze the DNA cleavage patterns

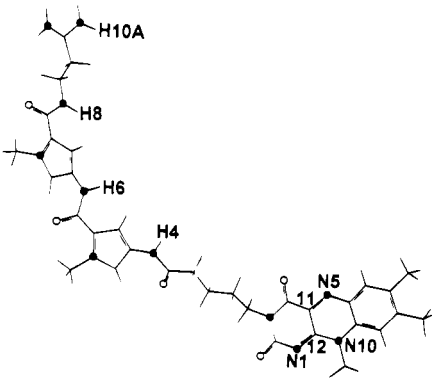


FIGURE 1: Plane projection of the three-dimensional structure of the hybrid molecule Net-Fla (nitrogen, full circles; oxygen, empty circles; carbon and hydrogen atoms are not specifically distinguished). Net-Fla is made of a netropsin moiety (to the left) and a flavin moiety (to the right) joined by an aliphatic tether. The netropsin moiety is made of a propionamidinium and two *N*-methylpyrroles joined by carboxamide links. The four atoms N1, C12, C11, and N5 are implicated in the oxidoreduction process of the flavin moiety. The inner part of the crescent-shaped Net-Fla interacts with the DNA minor groove. Some atoms have their names for easy reference.

on 5′-end-labeled DNA fragments containing a binding site of four A or T bases in various sequence combinations. As Patel (1979) showed, an asymmetrical drug such as netropsin should bind to a self-complementary DNA duplex whose dynamic interconversion can be observable at the NMR time scale in two ways (Patel & Shapiro, 1985; Leupin *et al.*, 1986; Klevit *et al.*, 1986). This flip−flop inversion may have its influence during the nucleation of crystals. Narrow groove binders can crystallize in the isomorphous lattice

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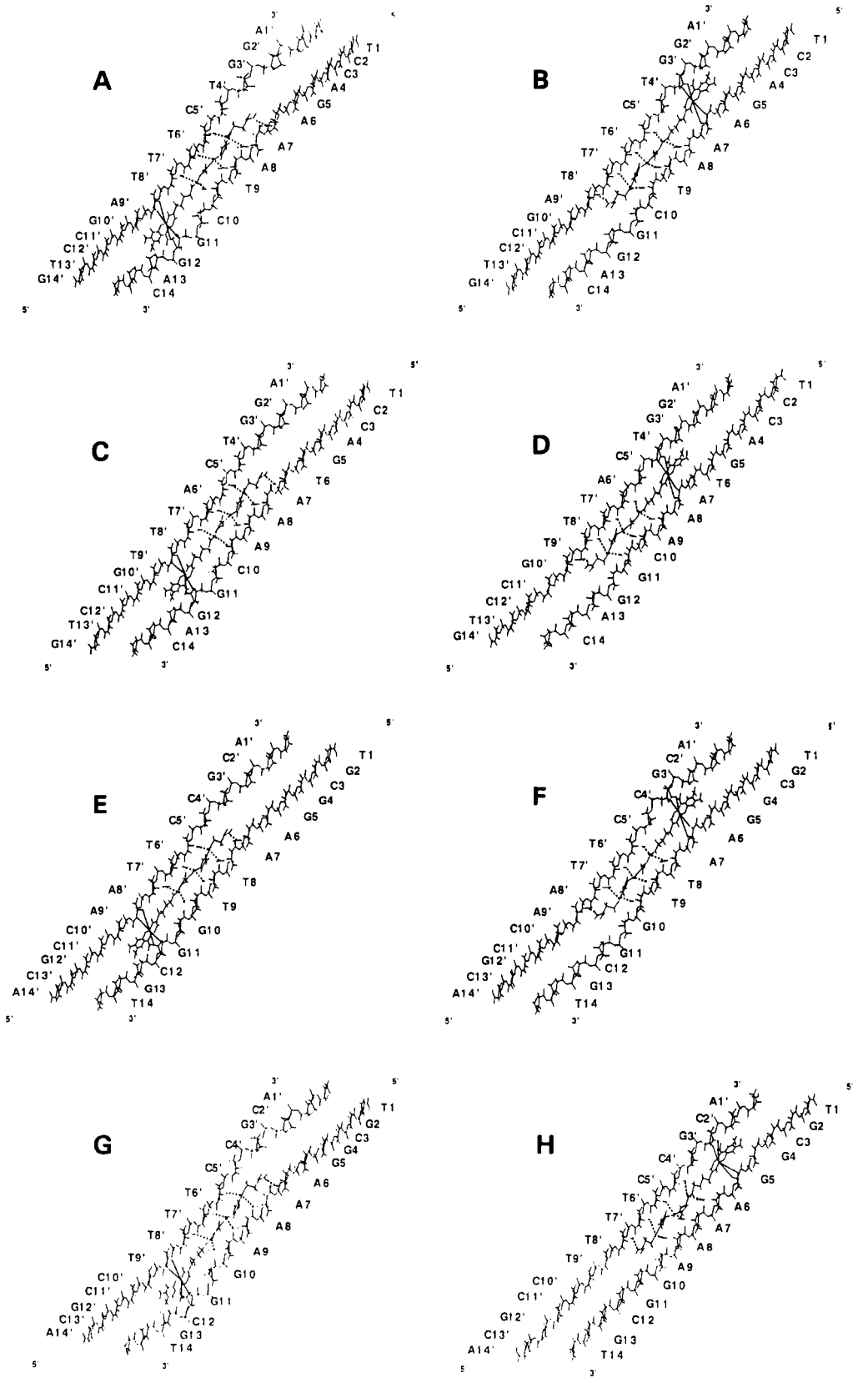


FIGURE 2: Cylindrical projection of the fragments studied in the molecular modeling: (A) sequence net1 in the case of direct binding, (B) sequence net1 in the case of reverse binding, (C) sequence net2 in the case of direct binding, (D) sequence net2 in the case of reverse binding, (E) sequence net3 in the case of direct binding, (F) sequence net3 in the case of reverse binding, (G) sequence net4 in the case of direct binding, and (H) sequence net4 in the case of reverse binding. Bases have been removed for clarity except for the atoms involved in hydrogen bonds, N3 of adenine and O2 of thymine/cytosine which are materialized by two half-segments belonging to the bases. Dashed lines represent hydrogen bonds. Thick lines represent distances between the C11 atom of the flavin and the H1′ or H4′ atoms belonging to the nucleotides facing the flavin on the sugar−phosphate backbone of the DNA.

either in one unique orientation (Kopka *et al.*, 1985) or in 2-fold disordered structures (Coll *et al.*, 1989; Tabernero *et al.*, 1993). As a consequence of these data, the narrow groove binders are expected to bind to self-complementary

Table 1: Sequences of the 42-Base Pair Fragments Used Here[a]

| Name | Sequence (5'→3') | Size (bp) |
|---|---|---|
| net1 | AAGAGATCAAGAGTCCAGAAATCGGACTGCCATGAGCCATGA | 42 |
| net2 | AAGAGATCAAGAGTCCAGTAAACGGACTGCCATGAGCCATGA | 42 |
| net3 | AGGACTCCGAGCCGTGCGGAATTGGCGTGCCGAGCCTCAGGA | 42 |
| net4 | AGGACTCCGAGCCGTGCGGAAAAGGCGTGCCGAGCCTCAGGA | 42 |

[a] The binding sites are underlined.

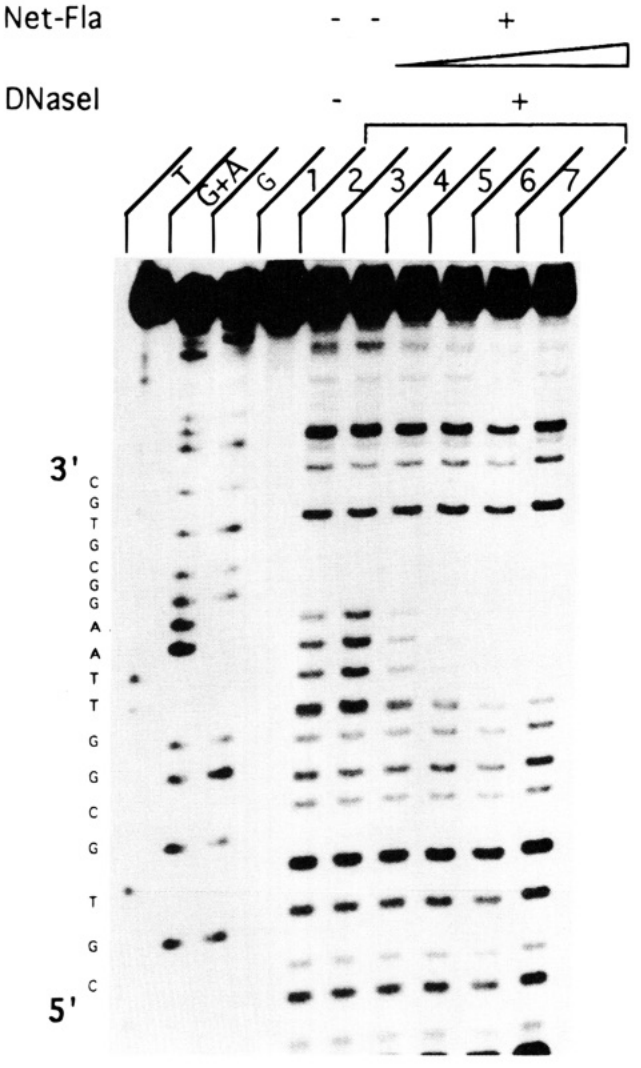


FIGURE 3: Footprinting assay using DNase I in the presence of Net-Fla at 4 °C. The 42-base pair fragment was 5′ labeled and annealed to the complementary strand. The 42-mer target was incubated for 30 min at 4 °C in the presence of Net-Fla and submitted to DNase I digestion for 2 min at 4 °C. Lanes: 1, target fragment; 2, digestion without Net-Fla; 3−7, increasing concentrations of Net-Fla (1, 2.5, 5, 7.5, and 10 $\mu$M); G, G+A, and T, sequences.

double helices in either orientation with similar probabilities.

In this work, we investigated the binding of Net-Fla to asymmetric DNA sequences. In this way, we hoped to find a single, unique binding orientation for the Net-Fla molecule that would enable a corresponding unique DNA cleavage locus to be obtained. We use molecular mechanics to correlate the experimental cleavage patterns to the energies of the DNA Net-Fla complex predicted in either orientation and in many positions along the narrow groove.

## MATERIALS AND METHODS

*Chemicals.* The 3-[1-methyl-4-[1-methyl-4-[[4-(7,8,10-trimethylisoalloxazin-3-yl)butyl]carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]propionamidinium chloride (Net-Fla) was synthesized as previously described (Herfeld *et al.*, 1994). Stock solutions were prepared in dimethyl sulfoxide (DMSO) before subsequent dilution with water.

*Footprinting Experiments.* A 0.1 $\mu$g portion of target oligonucleotide was labeled with polynucleotide kinase and annealed to the complementary strand in 40 $\mu$l. DNase I footprinting was performed in buffer containing 50 mM Tris-HCl (pH 7.0), 15 mM NaCl, 25 mM $MgCl_2$, 10 mM $MnCl_2$, the target DNA (10 nM), and additional aspecific unlabeled DNA (60 ng). In the standard assay, Net-Fla was added to the reaction mixture and incubated at 4 °C for 30 min. Digestion was started by addition of DNase I (3 units/mL) and stopped after 2 min by addition of ethylenediaminetetraacetic acid (EDTA) (10 mM), sodium acetate (0.3 M), and carrier tRNA (5 $\mu$g). Products of reaction were subsequently precipitated with ethanol, dried, and resuspended in formamide/EDTA gel-loading buffer. The cleavage products were loaded on 18% denaturing gel and visualized by autoradiography.

*Oligonucleotide Sequencing.* Modified Maxam−Gilbert sequencing reactions were used to generate G, G+A, and T ladders of end-labeled DNA (Williamson & Celander, 1990). For G reactions, 9 $\mu$L of end-labeled DNA in TE was incubated for 10 min at room temperature with 1 $\mu$L of 1/100 dimethyl sulfate in water. For G+A reaction, 9 $\mu$L of labeled DNA in TE was mixed with 1 $\mu$L of 1 M piperidine formate (pH 2.0) and incubated for 5 min at 65 °C. For T reactions, 9 $\mu$L of labeled DNA was heated to 90 °C for 2 min, cooled quickly to room temperature, mixed with 1 $\mu$L of 3 mM $KMnO_4$, and incubated for 7 min at room temperature, and the reaction was finally quenched with 1 $\mu$L of allyl alcohol. The three reaction mixtures were then treated for 15 min with pyrrolidine (1 M) at 90 °C, dried, and resuspended in formamide loading buffer.

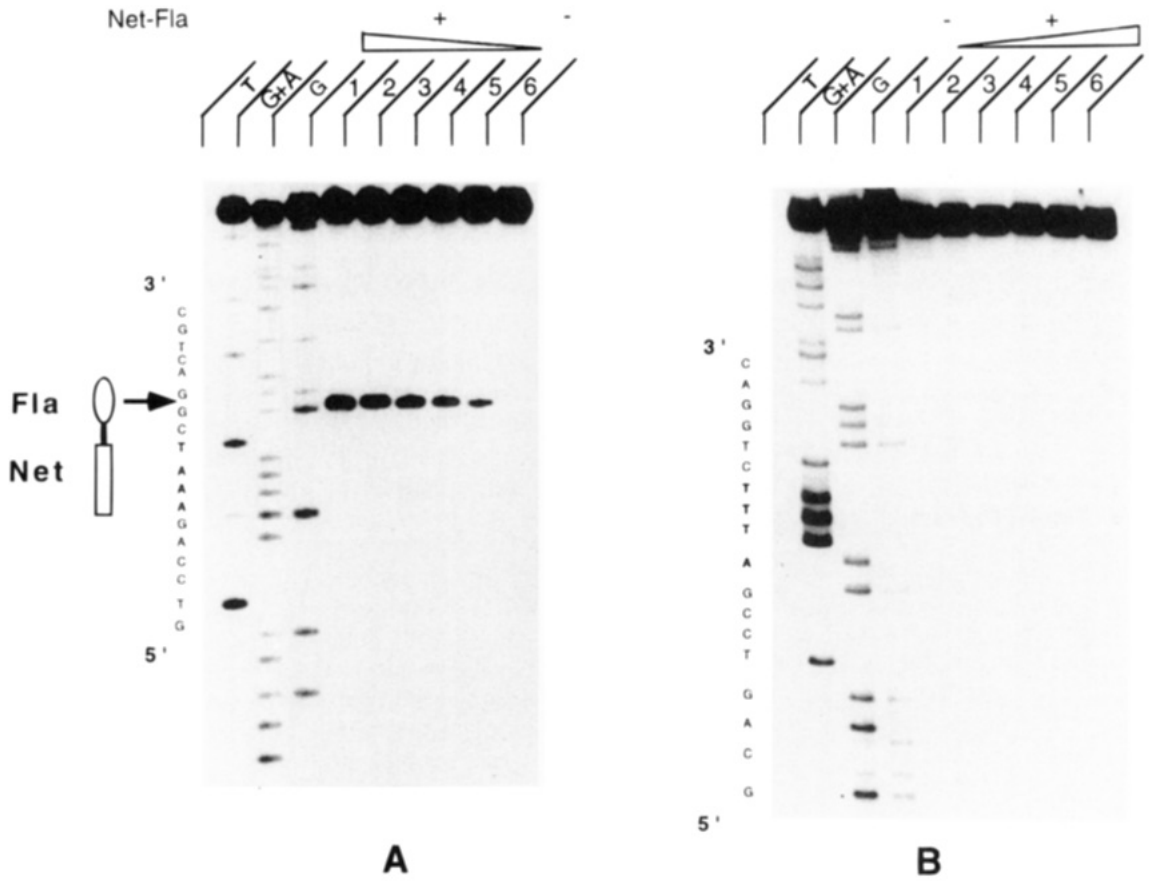


FIGURE 4: Site specific cleavage reactions induced in the 42-mer by Net-Fla. After irradiation at 4 °C and pH 8.0 for 30 min, the sample was electrophoresed on a denaturing polyacrylamide gel. (A) Cleavage reactions on the $^{32}$P-labeled net1 oligonucleotide containing the 5′-AAAT-3′ sequence by Net-Fla. Lanes: 1−5, decreasing concentrations of Net-Fla (10, 7.5, 5, 2.5, and 1 $\mu$M); 6, target fragment; G, G+A, and T, sequences. (B) Cleavage reactions on the net1 oligonucleotide complementary strand in the same conditions. Lanes: 1, fragment without Net-Fla; 2−6, increasing concentrations of Net-Fla (1, 2.5, 5, 7.5, and 10 $\mu$M).

*DNA Strand Breakage.* Strand breakage by Net-Fla was monitored in phosphate buffer (0.05 M and pH 8.0) containing 2.5 mM EDTA under the same conditions of footprinting. The mixtures were irradiated at 4 °C for 30 min using an incident light from a polychromatic lamp at an energy close to 1.5 W·m$^{-2}$. The sequences of the 42-base pair fragments containing specific sites of cleavage are described in Table 1.

*Reference Frames and Ligand Docking.* The DNA fragments actually modeled were restricted to pieces of 14 base pairs containing in their center the 5′-AAAT-3′ (net1), 5′-TAAA-3′ (net2), 5′-AATT-3′ (net3), and 5′-AAAA-3′ (net4) sequences (Figure 2). In the starting conformation, the DNA fragments were in the canonical B form (Arnott *et al.*, 1976) with a helical twist of 34.1° and a helical rise of 3.34 Å. Both the direct and the reverse complexes have been simulated (Figure 2). The starting conformations for the netropsin moiety were built as close as possible to the crystal structures (Kopka *et al.*, 1985; Coll *et al.*, 1989). The docking of the rest of the drug (second moiety, linker and flavin) was performed using our programs MORCAD (Le Bret *et al.*, 1991) and OCL (Gabarro-Arpa *et al.*, 1992) so that the flavin fits tightly in the narrow minor groove, its methyl groups pointing out. An orthonormal reference frame was assigned to the AT-rich double-stranded tracts 5′-AAAT-3′, 5′-TAAA-3′, 5′-AATT-3′, or 5′-AAAA-3′ forming the binding site of netropsin. The origin of the frame is the center of mass of the four C6/C8 atoms of the two central A·T base pairs. Its Z-axis is the helical axis of the A+T-rich tract built using OCL according to Rosenberg *et al.* (1976). Its *X*-axis points toward the narrow groove. Relative to such a reference, the center of mass of the two pyrroles and of its central peptide link of the netropsin in its known crystallographic conformations has cylindrical coordinates ($\theta$, $z$) close to zero. Net-Fla was moved through helicoidal displacements to 76 positions ($-70° < \theta < 150°$ and $-6$ Å $< z < 15$ Å for direct-binding models and $-150° < \theta < 70°$ and $-15$ Å $< z < 6$ Å for reverse-binding models) lying regularly in the minor groove of the DNA.

*Force Field.* We used the all-atom AMBER force field (Weiner *et al.*, 1986) in the same way as described by Mouscadet *et al.* (1994). The partial net charges on the pyrrolecarboxamide moieties and on the flavine residue have been computed through the program QUEST of the AMBER package (Weiner & Kollman, 1981; Pearlman *et al.*, 1991) using the minimal basis set STO-3G. The force constants of the dihedrals around the flavin were set to 50 kcal·mol$^{-1}$ to reduce its warping out of planarity.

*Minimizations.* The starting conformations were then submitted to a quasi Newtonian minimizer (Le Bret *et al.*, 1991) until the energy gradient was less than 0.1 kcal/Å. The minimization typically took 300−350 iteration cycles, which is roughly 3 times less than required by a conjugate gradient algorithm. The generated conformations were displayed on the workstations using the programs developed in this laboratory: MORCAD (Le Bret *et al.*, 1991) and CHART.

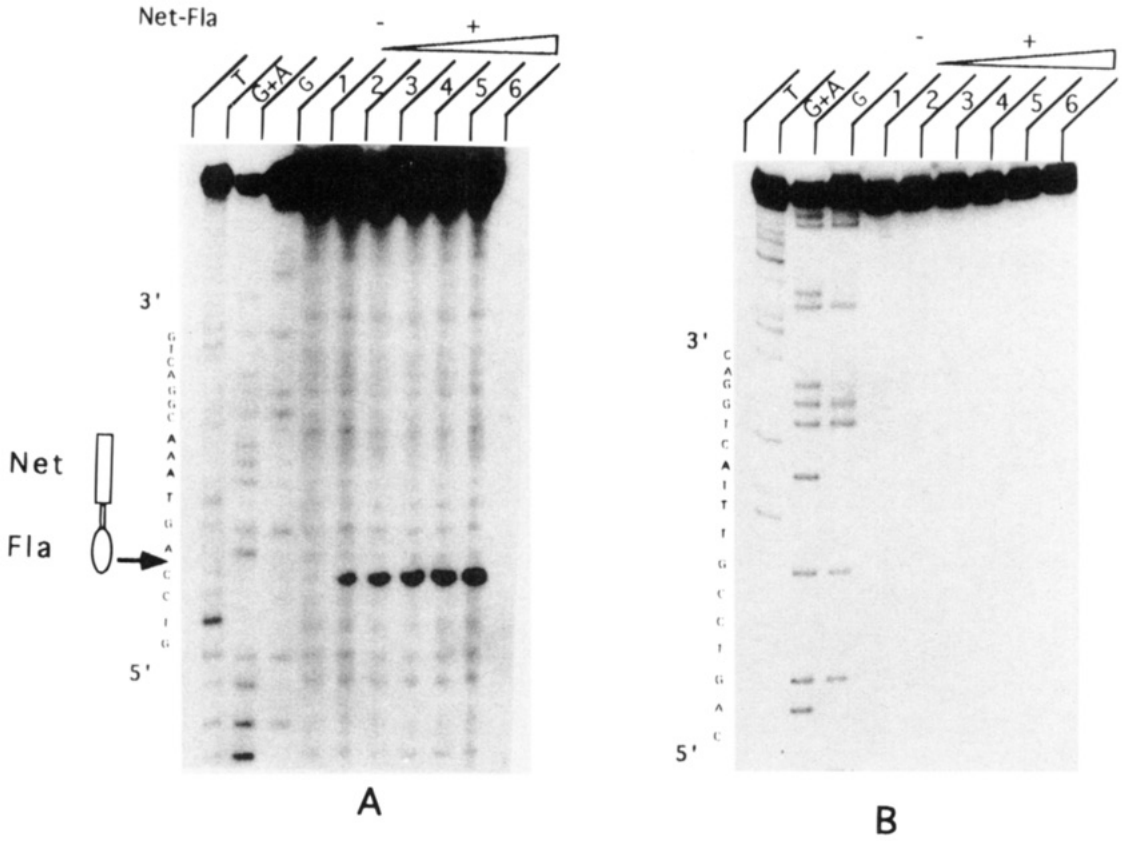


FIGURE 5: Site specific cleavage reactions induced in the 42-mer by Net-Fla. After irradiation at 4 °C and pH 8.0 for 30 min, the sample was electrophoresed on a denaturing polyacrylamide gel. (A) Cleavage reactions on the $^{32}$P-labeled net2 oligonucleotide containing the 5′-TAAA-3′ sequence by Net-Fla. Lanes: 1, target fragment; 2−6, increasing concentrations of Net-Fla (1, 2.5, 5, 7.5, and 10 $\mu$M); G, G+A, and T, sequences. (B) Cleavage reactions on the net2 oligonucleotide complementary strand in the same conditions. Lanes: 1, target fragment; 2−6, increasing concentrations of Net-Fla (1, 2.5, 5, 7.5, and 10 $\mu$M); G, G+A, and T, sequences.

## RESULTS

*Net-Fla Selectively Binds to A+T-Rich DNA Sites.* We first examined the possibility that the hybrid molecule Net-Fla binds preferentially to A+T-rich sequences. To facilitate this analysis, a 42-base pair fragment was constructed so that it contained the target binding site 5′-TTAA-3′ embedded within an essentially random sequence. Typical DNase I-induced cleavage patterns for this fragment are presented in Figure 3. Experiments performed in the presence of Net-Fla (from 1 to 10 $\mu$M) show a very strong protection against cleavage at the position of the 5′-TTAA-3′ target site. We note, however, that the adjacent 5′-GGCG-3′ sequence also appears to be efficiently protected. A plausible explanation for this may be the protection of the DNA from digestion by the DNase I itself. As inferred from structural studies (Suck & Oefner, 1986), this region would correspond to the binding site of the enzyme whose 3′ → 5′ processing activity is stalled by the presence of the ligand bound to the target site.

*DNA Cleavage Pattern Analysis.* Previous results have shown that, at micromolar concentrations and upon irradiation by visible light, the hybrid molecule Net-Fla cleaves supercoiled (form I) pBR322 DNA, yielding nicked circular DNA (form II). Further treatment by nuclease S1, which cleaves the opposite strand facing the Net-Fla-induced nick, revealed that the strand breaks preferentially occurred at the vicinity of A·T-rich sequences (Herfeld *et al.*, 1994). In order to characterize the strand-breaking process in terms of the selectivity of binding and cleavage, we investigated the cleavage patterns of DNA fragments containing a four-base pair AT-rich sequence corresponding to the canonical binding site of the netropsin moiety. The sequences investigated were 5′-AAAT-3′, 5′-TAAA-3′, 5′-AATT-3′, and 5′-AAAA-3′. The location of the cleavage sites for the Net-Fla molecule was determined on $^{32}$P end-labeled fragments after resolution of the reaction products by sequence gel electrophoresis. No cleavage was observed on either strand in the absence of irradiation, nor upon irradiation of the DNA in the absence of Net-Fla. However, after irradiation for 30 min in the presence of the hybrid molecule, a distinct cleavage pattern was obtained for the plus (+) strand containing the 5′-AAAT-3′ site, as shown in Figure 4A as a function of the concentration of the ligand. The first striking feature is the occurrence of a single cleavage site downstream of the binding site. This indicates that the ligand had bound in a single orientation only, with the activeable chromophore lying opposite to the adenines of the binding site, two base pairs downstream of the A+T-rich sequence. Under similar experimental conditions, no detectable cleavage was observed on the opposite strand (Figure 4B). In order to assess the sequence dependence on ligand orientation, we performed a second set of experiments, where in this case the sequence of the binding site was inverted (5′-TAAA-3′). This DNA fragment was again cleaved efficiently (Figure 5), but the cleavage locus, now positioned two base pairs upstream of the A+T-rich sequence, indicated that the ligand had bound in the opposite orientation than it had to 5′-AAAT-3′. These results clearly demonstrate the capacity of the ligand to discern the sequence of the binding site. We surmised that the factor directing this sequence dependence might be the

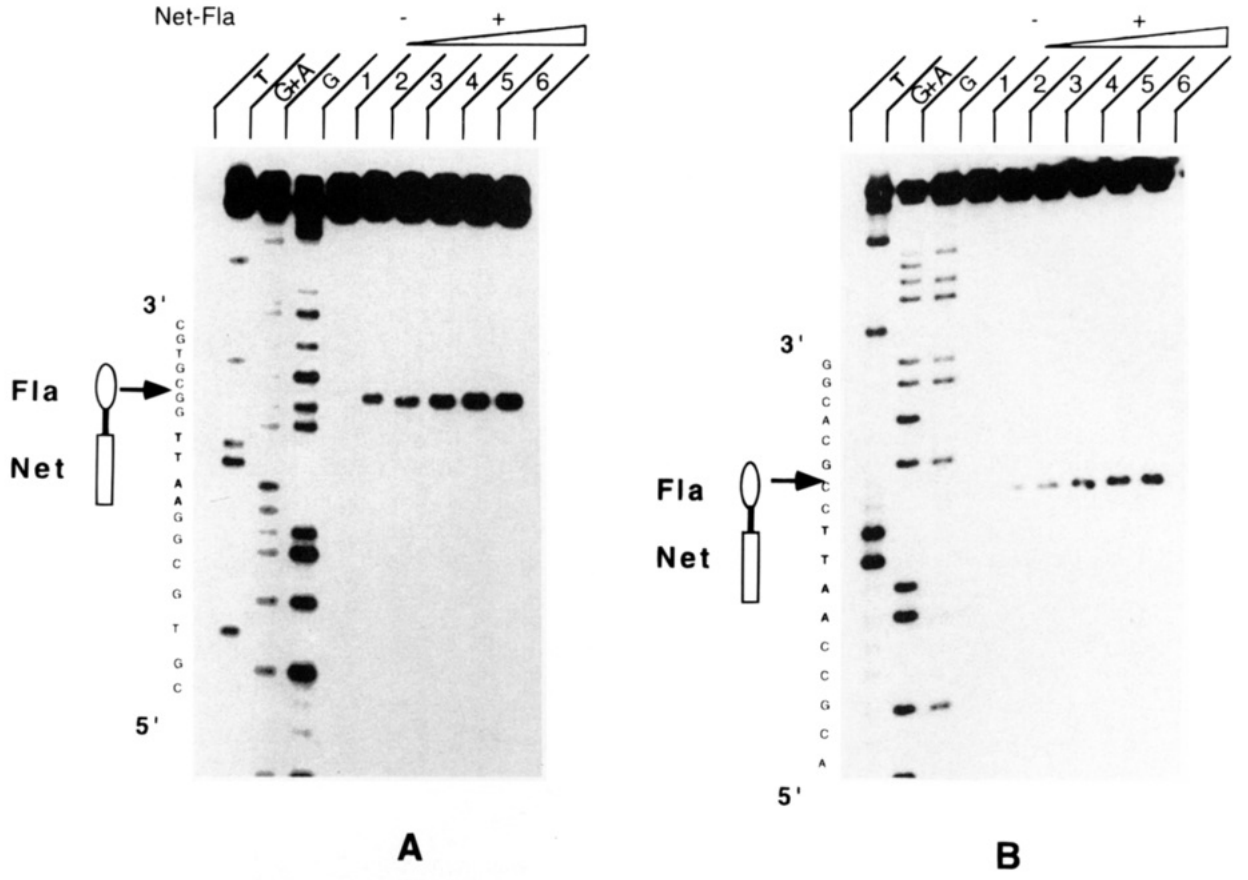


FIGURE 6: Site specific cleavage reactions induced in the 42-mer by Net-Fla. After irradiation at 4 °C and pH 8.0 for 30 min, the sample was electrophoresed on a denaturing polyacrylamide gel. (A) Cleavage reactions on the $^{32}$P-labeled net4 oligonucleotide containing the 5′-AATT-3′ sequence by the Net-Fla. Lanes: 1, target fragment; 2−6, increasing concentrations of Net-Fla (1, 2.5, 5, 7.5, and 10 $\mu$M); G, G+A, and T, sequences. (B) Cleavage reactions on the net3 oligonucleotide complementary strand in the same conditions. Lanes: 1, target fragment; 2−6, increasing concentrations of Net-Fla (1, 2.5, 5, 7.5, and 10 $\mu$M); G, G+A, and T, sequences.

two adjacent adenines that begin or end the four A+T binding sites. In this sense, Net-Fla would be seen to cleave the strand at a downstream location when the AA dinucleotide of the binding site is located in the 5′ position but, conversely, at the upsteam location when located at the 3′ position. To test this hypothesis, we studied the location of the cleavage at a 5′-AATT-3′ binding site (Figure 6). As predicted, the DNA was cleaved downstream of the binding site (Figure 6A). However, in this case, due to the palindromic symmetry of the 5′-AATT-3′ site, cleavage was also obtained on the opposite strand, but similarly located downstream of the AA dinucleotide (Figure 6B). It is of interest to notice that, with such symmetric sequences, double strand cleavage may occur by two sequential single strand cleavages of opposite orientation. Finally, using the homogeneous 5′-AAAA-3′ sequence as the target site (Figure 7), Net-Fla produced a symmetric cleavage pattern located on the A-bearing strand only, on both sides of the binding site which reflects the two possible orientations of the ligand on this particular sequence.

*Minimized Conformations.* The binding of Net-Fla in either direction on 14-base pair fragments extracted from the net1, net2, net3, and net4 sequences has been studied using molecular mechanics. The 14-base pair fragments used in the molecular modeling and the eight best minimized conformations are shown in a cylindrical projection in Figure 2. Energetical values show that net1 is more stable in the direct binding mode than in the reverse one, net2 is more stable in the reverse binding mode than in the direct one, and net3 and net4 can adopt both direct- and reverse-binding conformations (Table 2).

## DISCUSSION

We have demonstrated that the hybrid compound Net-Fla can introduce single strand breakages in a double-standed DNA fragment upon photoactivation of the flavin chromophore. This approach offers several advantages over previously described chemical cleavage systems, such as the EDTA−Fe(II) system. Firstly, unlike the former systems, the cleavage may be directed in a sequence dependent manner to a specific site on a predefined strand bearing the target binding site for neotropsin. Secondly, the requirement for a chemical-reducing agent to obtain cleavage is avoided by incorporation of the photoactiveable chromophore. The cleavage may be modulated simply by altering the intensity or duration of irradiation or by cellular enzymes capable of reducing the flavin moiety. The occurrence of a single cleavage event on one strand only suggests that the photoactiveable chromophore should lie in a defined position with respect to the phosphodiester backbone. In all the cases studied, the predicted orientation of the binding of Net-Fla compares well to the experimental data.

The position of the flavin moiety near its DNA cleavage locus can directly explain the cleavage. For instance, the flavin moiety is near G11 of sequence net1 (Figure 2A), and the flavin moiety is near G11 and C4′ on the sequence net3 containing the palindromic site 5′-AATT-3′ (Figure 2E,F).

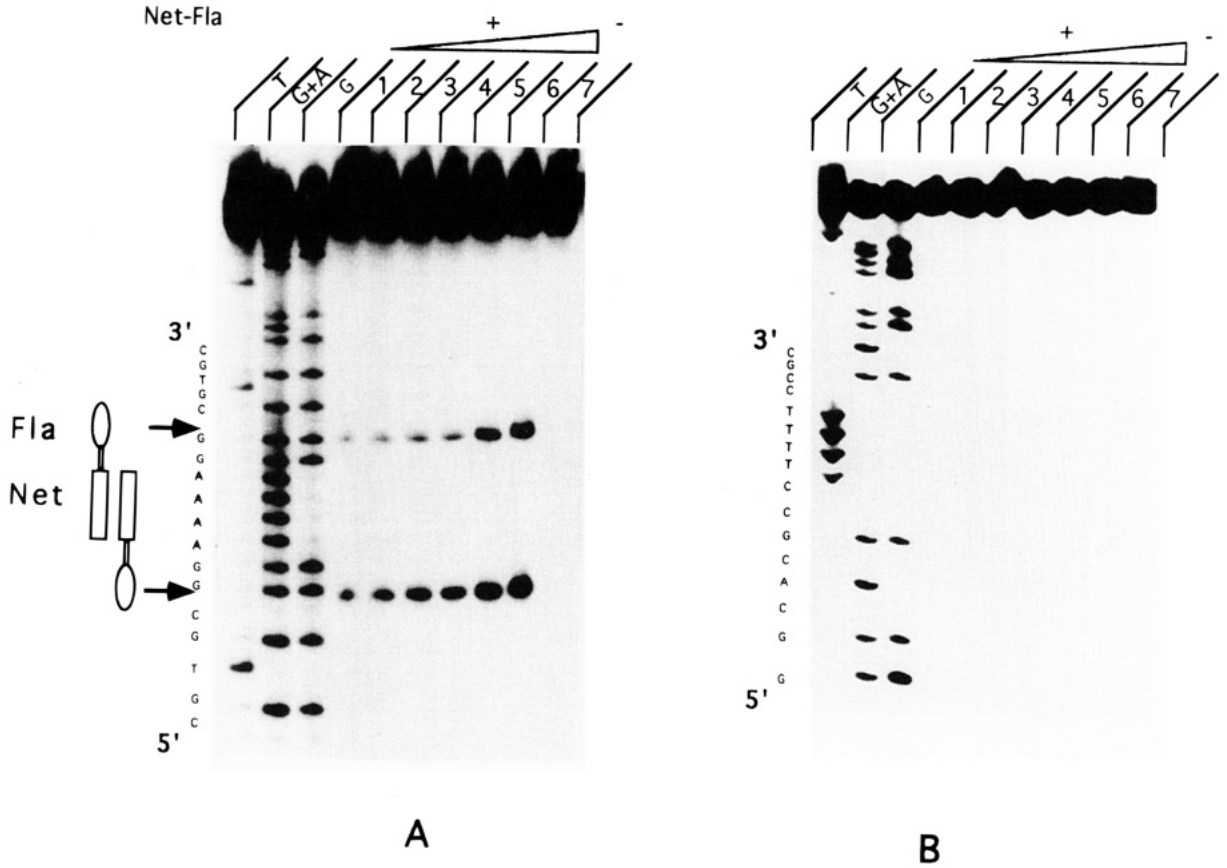


FIGURE 7: Site specific cleavage reactions induced in the 42-mer by Net-Fla. After irradiation at 4 °C and pH 8.0 for 30 min, the sample was electrophoresed on a denaturing polyacrylamide gel. (A) Cleavage reactions on the $^{32}$P-labeled net3 oligonucleotide containing the 5′-AAAA-3′ sequence by Net-Fla. Lanes: 1−6, increasing concentrations of Net-Fla (1, 2.5, 5, 7.5, 10, and 12.5 $\mu$M); 7, target fragment; G, G+A, and T, sequences. (B) Cleavage reactions on the net4 oligonucleotide complementary strand in the same conditions. Lanes: 1−6, increasing concentrations of Net-Fla (1, 2.5, 5, 7.5, 10, and 12.5 $\mu$M); 7, target fragment; G, G+A, and T, sequences.

Table 2: Energy Minima Characteristics (kcal/mol) for the Binding of Net-Fla on the 14-Base Pair Fragments Corresponding to Models Shown in Figure 2

| sequence | direct binding | reverse binding |
|---|---|---|
| net1 | −222.0 | −220.7 |
| net2 | −218.4 | −219.9 |
| net3 | −229.0 | −229.5 |
| net4 | −229.9 | −229.7 |

Further dynamical studies will explain the stability and deformations of all complexes.

The C−H bond cleavage in the DNA-breaking process depends on the DNA cleaver used. For oxido reduction process, the major routes of DNA sugar attack seem to be H1′ and H4′. For example, in the case of iron−bleomycin, the major target is C4′−H (Hecht, 1986; Stubbe & Kozarich, 1987), whereas the bis(*o*-phenanthroline)copper attacks at C1′−H (Sigman, 1990; Krishnamoorthy *et al.*, 1988). The oxido reduction process of flavines includes three redox states: quinone (oxidized), semiquinone (free radical), and hydroquinone (reduced) (Yoshimura, 1977). In this process, the four atoms of the conjugate system N1−C12−C11−N5 shown on Figure 1 are implicated and the free electron of the semiquinone is delocalized. As shown in Figure 2, the flavin moiety sits a similar distance from both strands so that the cleavage on a single strand is not simply correlated with some geometrical parameters. More sophisticated techniques using quantum mechanics may be needed to elucidate the exact mechanism of the cleavage.

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