DNA binding mode of the Fab fragment of a monoclonal antibody specific for cyclobutane pyrimidine dimer

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ABSTRACT
Monoclonal antibodies specific for the cyclobutane pyrimidine dimer (CPD) are widely used for detection and quantification of DNA photolesions. However, the mechanisms of antigen binding by anti-CPD antibodies are little understood. Here we report NMR analyses of antigen recognition by TDM-2, which is a mouse monoclonal antibody specific for the cis-syn-cyclobutane thymine dimer (T[c,s]T). 31P NMR and surface plasmon resonance data indicated that the epitope recognized by TDM-2 comprises hexadeoxynucleotides centered on the CPD. Chemical shift perturbations observed for TDM-2 Fab upon binding to d(T[c,s]T) and d(TAT[c,s]TAT) were examined in order to identify the binding sites for these antigen analogs. It was revealed that d(T[c,s]T) binds to the central part of the antibody-combining site, while the CPD-flanking nucleotides bind to the positively charged area of the VH domain via electrostatic interactions. By applying a novel NMR method utilizing a pair of spin-labeled DNA analogs, the orientation of DNA with respect to the antigen-binding site was determined: CPD-containing oligonucleotides bind to TDM-2 in a crooked form, draping the 3′-side of the nucleotides onto the H1 and H3 segments, with the 5′-side on the H2 and L3 segments. These data provide valuable information for antibody engineering of TDM-2.

INTRODUCTION
Irradiation of DNA with UV light produces a variety of damaged bases, which cause mutations leading to cellular transformation and cell death (1–3). Cyclobutane pyrimidine dimers (CPDs) are major photoproducts from the photo [2+2] cycloaddition of the 5,6-double bond of two adjacent pyrimidine nucleotides (Fig. 1).

It has been revealed that DNA damage in organisms is corrected by a variety of cellular repair machineries (4). In investigations of the DNA repair systems, detection and quantification of DNA photolesions have so far been achieved by the use of antibodies specific for the individual photoproducts (5–8). However, the mechanisms of antigen binding by anti-CPD antibodies is little understood, although they have been widely utilized. A detailed understanding of the binding mechanisms of anti-CPD antibodies is important not only in a proper interpretation of the binding data, but in antibody engineering aimed at improving their affinities and specificities.

It has been reported that some auto-antibodies specific for DNA have an intrinsic ability to cleave DNA, probably because they introduce strain into phosphodiester bonds upon binding (9–11). This observation gives rise to the idea that anti-CPD antibodies can be endowed with catalytic activity for selective cleavage of CPD-containing DNA if appropriate antibody engineering is applied. To realize this idea, it is obviously necessary to determine the orientation of the CPD-containing DNA with respect to the antigen-binding site and to identify amino acid residues responsible for binding to the CPD-flanking nucleotides.

Mouse monoclonal antibody TDM-2, which was established by Nikaido and co-workers by immunization with UV-irradiated DNA (6), specifically binds to DNA containing a cis-syn-cyclobutane thymine dimer (T[c,s]T). We reported that the nucleotides on both the 5′- and 3′-sides of d(T[c,s]T) enhance the affinity for TDM-2 in a sequence-independent manner (12). This suggests that not only d(T[c,s]T) but also the flanking deoxynucleotides are involved in binding to TDM-2. We also revealed that TDM-2 preferentially binds to single-stranded (ss)DNA and therefore formation of a duplex significantly precludes binding (12).

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In the present paper, in order to gain a deeper insight into the DNA recognition mechanism of TDM-2, we have performed NMR analyses of the interaction between the TDM-2 Fab and a variety of CPD-containing DNA analogs. The size of the epitope recognized by TDM-2 has been determined by 31P NMR and surface plasmon resonance (SPR) analyses. Using stable isotope-assisted NMR spectroscopy, the binding sites of d(T[c]TA) as well as of the CPD-flanking oligodeoxynucleotides have been identified. Furthermore, we have developed a novel NMR method, utilizing a pair of spin-labeled DNA analogs, to determine the orientation of DNA with respect to DNA-binding proteins. On the basis of the data thus obtained, we will discuss the mode of interaction between CPD-containing DNA and TDM-2.

MATERIALS AND METHODS

Preparation of oligonucleotides

All oligonucleotides were synthesized using phosphoroamidite units purchased from Perkin-Elmer Applied Biosystems. The cis-syn-type cyclobutane thymine dimers were inserted in oligodeoxynucleotides by coupling the cyclobutane dimer amide blocks as reported (13,14). Oligonucleotides containing biotin at the 3′-end were synthesized using Biotin-on CPG (Clontech) as the 3′-support. Phosphorylation of the 5′-position of d(T[c]TA) was performed using a chemical phosphorylation amide unit (Glen Research) on a CPG support. The 3′-phosphorothioate of d(AT[c]TA) was synthetized on a 3′-phosphate CPG (Glen Research). Oxidation of both the 5′- and 3′-dangling phosphites was carried out by treatment with tetraethylthiuram disulfide (Perkin-Elmer Applied Biosystems) for 15 min. These 5′- or 3′-phosphorothioate antigens were purified using a reverse phase column (C-18, Inertsil ODS-3, 8.0 mm i.d. × 300 mm; GL Sciences) and then desalted by Sephadex G10 chromatography (Pharmacia).

The antigen analog with either a 5′- or 3′-dangling phosphorothioate was coupled with 4-(2-iodoacetamido)TEMPO (Sigma) in a solution (210 µl) containing potassium phosphate buffer (pH 6.0) and 14% dimethylformamide as previously reported (15). The final concentrations of the antigen analog and buffer (pH 6.0) were 0.4 and 12 mM, respectively. The product was desalted by Sephadex G10 chromatography and purified by reverse phase column (C-18) chromatography. The immobilization of TEMPO was checked by measurement of electron spin resonance.

After the cis-syn-type cyclobutane thymine dimer was protected with 2-cyanoethyl and levulinyl groups (14) and deprotected in concentrated aqueous ammonia, d(T[c]TA) was purified on a reverse phase column (C-18).

Preparation of isotopically labeled Fab fragments

The hybridoma cells which produce TDM-2 (IgG2a, κ) were grown in NYSY 404 medium (Nissui) supplemented with 2% heat-inactivated FBS (JRH Biosciences) at 37°C in a humidified atmosphere of 5% CO2/95% air. The protocol for the preparation of antibodies selectively labeled with 13C- and 15N-enriched amino acids was as described (16–18), except that medium containing 2% heat-inactivated FBS was used. After cell growth, the supernatant was concentrated with a Millipore Minutan ultrafiltration system and then applied to an Affi-Gel protein A column (Bio-Rad). The purified TDM-2 antibody was reduced with dithiothreitol and carbamoylmethylated and then digested by papain as previously described (19). Fab fragments were purified using a Mono Q anion exchange column (Amersham Pharmacia Biotech) as previously described (20). Fab fragments in which either the light or heavy chain was exclusively labeled were prepared according to the protocol previously described (21), except that a Mono Q anion exchange column equilibrated with 20 mM Tris–HCl, pH 8.0, containing 6 M urea was used for separation of the heavy and light chains from each other.

SPR measurements

The binding of TDM-2 Fab to oligonucleotides containing CPD was determined by SPR measurements using a BIAcore instrument (Biacore AB) as described previously (22). All experiments were performed at 25°C. The minimal amount of DNA was immobilized on the sensor chip in order to avoid mass transport limitations. Injections of biotinylated oligonucleotides [0.015–0.032 pmol/µl in HBS (10 mM HEPES pH 7.4, 0.15 M NaCl, 3.4 mM EDTA, 0.005% Tween 20)] were repeated until the SPR signal was increased by 20–30 resonance units (RU) above the original baseline. The TDM-2 Fab solution was replaced with HBS buffer using gel filtration, then it was injected over the immobilized oligonucleotides at a flow rate of 100 µl/min over a concentration range from 200 to 800 mM. Sensorgrams were recorded and normalized to a baseline of 0 RU. An equivalent volume of diluted Fab was also injected over a non-oligonucleotide surface to serve as a blank sensorgram to allow subtraction of the bulk refractive index background. The association was monitored by measuring the rate of binding to oligonucleotide at different Fab concentrations. Dissociation of the Fab from the oligonucleotide surface was monitored after the end of the association phase. The remaining bound Fab molecules were completely removed by injecting 50–100 µl of 100 mM HCl. Kinetic rate constants were calculated using BIAevaluation 3.0 software (Biacore AB) using a single site binding model, i.e. A + B = AB. The ratio of the rate constants allowed the equilibrium constant to be calculated, Kd = koff/kon.

NMR measurements

NMR measurements of oligodeoxynucleotides. The experiments were carried out using oligodeoxynucleotides at a concentration of 0.5 mM in 420 µl of 5 mM sodium phosphate buffer, pH 7.3, containing 200 mM NaCl, 50 µM EDTA and 3 mM NaN3 in 2H2O. One-dimensional 31P NMR measurements were made on a Bruker AMX400 spectrometer at an observation frequency of 162 MHz. The probe temperature was 37°C. Spectra were recorded using a WALTZ-16 composite pulse for 1H decoupling. The free induction decay (FID) after a 40° pulse was recorded with a repetition period of 5.0 s, with 16k data points and with a spectral width of 2400 Hz. The FID was multiplied by an exponential window function prior to Fourier transformation. 31P chemical shifts were given in parts per million (p.p.m.) from the external trimethylphosphate (0.00 p.p.m.). Two-dimensional (2D) NMR spectra were recorded on a Bruker DTX400 spectrometer operating at a 1H frequency of 400 MHz with a spectral width of 4800 Hz. The solvent resonance was suppressed by selective irradiation during the relaxation delay. For total correlation spectroscopy (TOCSY) and rotating frame
Overhauser effect spectroscopy (ROESY), 16 transients of FID with 2K data points were acquired for each of the 512 \( t_1 \) points in time-proportional phase increment (TPPI) mode. The mixing times were set to 200 ms for TOCSY and to 400 ms for the ROESY measurements. \(^1\)H–\(^{31}\)P heteronuclear multiple bond correlation (HMBC) spectra were recorded with spectral widths of 2400 Hz for \(^1\)H and 500 Hz for \(^{31}\)P. For the \(^1\)H–\(^{31}\)P HMBC spectra, 16 transients of FID with 1K data points were acquired for each of the 128 \( t_1 \) points in TPPI mode. Prior to 2D Fourier transformation, the acquired data were zero filled once along the \( t_1 \) dimension and multiplied by a shifted sine square function in the \( t_2 \) dimension unless otherwise stated. Chemical shifts were given in p.p.m. from external sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

**RESULTS AND DISCUSSION**

Dependence of antigen-binding constants of TDM-2 upon DNA length

First, by BIAcore measurement, we estimated the binding constants of TDM-2 Fab for CPD-containing oligodeoxy-nucleotides of various length. The dissociation constants of TDM-2 Fab determined for d(AT[c,s]TAT), d(GTAT[c,s]TATG) and d(GTAT[c,s]TATG) were 4.2 \times 10^{-8}, 6.0 \times 10^{-10} and 5.0 \times 10^{-10} M, respectively. TDM-2 Fab has a higher affinity for d(TAT[c,s]TATG) than for d(AT[c,s]TAT), the affinity for d(GTAT[c,s]TATG) is comparable with that for d(TAT[c,s]TATG). On the basis of these data, we suggest that the epitope for TDM-2 comprises a hexadeoxynucleotide centered on the CPD. It was revealed that an increase in NaCl concentration resulted in a significant reduction in affinity of TDM-2 Fab for d(CTAT[c,s]TAAAG) (Kurira et al., unpublished observation), indicating that primarily electrostatic interactions contribute to binding.

\(^{31}\)P NMR analyses of the epitope recognized by TDM-2

We determined the epitope recognized by TDM-2 in more detail by use of \(^{31}\)P NMR spectroscopy. It has been well established that variations in the \(^{31}\)P chemical shifts of individual phosphate diesters in oligonucleotides can be attributed to differences in the torsional angles and the O–P–O bond angle in the deoxyribose phosphate backbone, while the ring current and other environmental effects, such as formation of hydrogen bonds and salt bridges, upon phosphate \(^{31}\)P chemical shifts are generally smaller than the intrinsic conformation factors.

Figure 2a and b shows the \(^{31}\)P NMR spectra of d(TAT[c,s]TAT) and d(GTAT[c,s]TATG), respectively. Assignments of the \(^{31}\)P resonances of these oligodeoxy-nucleotides were accomplished through analysis of the \(^1\)H–\(^{31}\)P HMBC, TOCSY and ROESY spectra via a sequential assignment methodology (spectra not shown). The established assignments of the \(^{31}\)P resonances are indicated in Figure 2, in which \( P_0 \) represents the ±\( 180^\circ \) phosphorus in the phosphodiester bond relative to the phosphorus at the CPD site (\( P_0 \)) (negative and positive signs correspond to the 5′- and 3′-sides,
respectively). It was confirmed that the chemical shift of the $^{31}$P resonance from P$_{0}$ is almost identical to that of the isolated d(TAT[cs]T) (data not shown). A significant downfield shift of the resonance from P$_{−1}$, which is characteristic for ssDNAs that contain d(TAT[cs]T) (25), was also observed for both of the CPD-containing oligodeoxynucleotides.

Figure 2c and d shows the $^{31}$P NMR spectra of d(TAT[cs]TAT) and d(GTAT[cs]TATG), respectively, in the presence of 1.1 molar equivalents of TDM-2 Fab. Upon complex formation with TDM-2 Fab, all of the five $^{31}$P resonances from d(TAT[cs]TAT) became significantly broader and at least four of them showed drastic changes in chemical shift. In Figure 2d, two sharp resonances were additionally observed. Comparison of Figure 2c with d allows us to conclude that P$_{−3}$ and P$_{+3}$ are scarcely involved in binding to TDM-2 and, therefore, the epitope recognized by TDM-2 comprises a hexadeoxy-nucleotide centered on the CPD. This conclusion is consistent with the SPR data.

Both d(TAT[cs]TAT) and d(GTAT[cs]TATG) gave a $^{31}$P resonance that overlaps another $^{31}$P resonance at the position of the P$_{0}$ resonance in the uncomplexed state. It has been shown that chemical shift of the $^{31}$P resonance originating from the phosphodiester bond of isolated d(TAT[cs]T) is little perturbed upon binding to TDM-2 Fab (data not shown). On inspection of these data, we conclude that the P$_{0}$ resonances from d(TAT[cs]TAT) and d(GTAT[cs]TATG) are also little perturbed upon binding to TDM-2 Fab, indicating that the backbone conformation of the CPD sites in these oligodeoxynucleotides is retained in the complexed forms with TDM-2 Fab. This automatically leads us to conclude that resonances originating from P$_{−2}$, P$_{−1}$, P$_{+1}$ and P$_{+2}$ of d(TAT[cs]TAT) and of d(TAT[cs]TATG) show drastic changes in chemical shift upon addition of TDM-2 Fab, which indicates that the deoxyribose phosphate rotamer distributions of the flanking nucleotides are significantly altered upon binding to TDM-2 Fab. The furthest upfield-shifted resonances are most broadened to the extent that it cannot be explained simply by the increased correlation time. It is possible that the CPD-flanking nucleotides exhibit conformational exchange on the protein surface.

**The antigen-binding site of TDM-2**

We attempted to identify sites in TDM-2 Fab that are responsible for binding to d(T[cs]T) and to the d(T[cs]T)-flanking nucleotides by use of stable isotope-assisted NMR spectroscopy. For this purpose, we prepared a variety of Fab analogs labeled with $^{1}$C and $^{15}$N on backbone amide groups of selected amino acid residues. Figure 3 shows, as an example, the $^{1}$H-15N HSQC spectrum of the TDM-2 Fab specifically labeled with $[^{15}$C]Trp and $[^{15}$N]Tyr. The boxed peak exhibiting a passive coupling with $^{1}$J$_{CS}$ is magnified in the inset. Protein concentration was 0.8 mM. The acquired data were zero filled once along the $t_{1}$ dimension, and multiplied by Gaussian window functions in the $t_{1}$ and $t_{2}$ dimensions.

![Figure 3.](image)

Figure 3. $^{1}$H-15N HSQC spectrum of the TDM-2 Fab labeled with $[^{1}$C]Trp and $[^{15}$N]Tyr. The boxed peak exhibiting a passive coupling with $^{1}$J$_{CS}$ is magnified in the inset. Protein concentration was 0.8 mM. The acquired data were zero filled once along the $t_{1}$ dimension, and multiplied by Gaussian window functions in the $t_{1}$ and $t_{2}$ dimensions.

As mentioned above, Tyr resonances were also unambiguously assigned by use of a variety of doubly labeled Fab analogs as indicated in Figure 3. These results led us to conclude that the missing peaks correspond to Tyr55H, Tyr79H, Tyr91H and Tyr102H. In a similar way, site-specific assignments were also performed for peaks originating from the amino acid residues other than Tyr by use of appropriately labeled Fab analogs. Upon addition of d(T[cs]T), the peaks originating from Tyr32L, Tyr94L, Tyr59H and Tyr98H were significantly perturbed in chemical shift (Fig. 4). Furthermore, peaks originating from Tyr55H, Tyr79H, Tyr91H and Tyr102H appeared in the spectrum upon addition of d(T[cs]T). The HSQC peaks from these residues in the complexed form were identified by double labeling experiments. Similar experiments were performed using the $^{1}$H-15N peaks from other amino acid residues as spectroscopic probes. Chemical shift perturbation upon binding to d(T[cs]T) was also observed for Phe33L, His34L, Trp35L, His49L, Thr51L, Trp91L, Tyr98L, Lys3H, Leu4H, Phe27H, Trp36H, Val37H and Trp103H. These perturbed residues were mapped on the three-dimensional model of TDM-2 Fab (Fig. 5a).

As mentioned above, Tyr55H, Tyr79H, Tyr91H and Tyr102H gave no detectable peak in the absence of antigen, presumably due to extreme chemical exchange broadening, but gave unambiguous peaks in the presence of d(T[cs]T). Similar phenomena were observed for Arg50L, Phe29H, Phe33H, Val35H, Met34H, Ile48H, Val69H, Arg94H and Phe99H, most of which are located in the $V_{H}$ domain. This indicates that: (i) the $V_{H}$ domain exhibits conformational multiplicity in the absence of antigen; and (ii) the pre-existing conformational multiplicity disappeared upon antigen binding. The pre-existing conformational equilibrium of the antigen-binding site and...
conformational fixation upon antigen binding of the previously flexible antigen-binding site have been reported for other antigen–antibody systems (21,29,30). The residues that show drastic line narrowing upon binding to d(T[c,s]T) were also mapped on the model TDM-2 Fv (Fig. 5a). As shown in Figure 5a, the residues whose chemical shift was perturbed upon binding to d(T[c,s]T) are concentrated in the central part of the model. Although the model might simply represent one of the possible multiple conformations at most and not consider any conformational change upon binding to d(T[c,s]T), it could at least be concluded that d(T[c,s]T) binds the central parts of the antibody-combining site.

In order to identify the sites responsible for binding to the CPD-flanking oligodeoxynucleotides, perturbations of TDM-2 Fab induced upon binding to d(TAT[c,s]TAT) were also examined. Figure 4b compares the 1H-15N HSQC spectra of TDM-2 Fab labeled with [15N]Tyr between the d(T[c,s]T)- and d(TAT[c,s]TAT)-bound forms. The peaks originating from Tyr94L, Tyr55H, Tyr59H, Tyr98H and Tyr102H exhibit significantly different chemical shifts between the d(T[c,s]T)- and d(TAT[c,s]TAT)-bound forms. Perturbations induced by d(TAT[c,s]TAT) binding were compared with those due to d(T[c,s]T) binding for the HSQC peaks originating from the other amino acid residues. The residues showing different chemical shifts between the d(T[c,s]T)- and d(TAT[c,s]TAT)-bound forms were Phe33L, Arg50L, Tyr94L, Phe27H, Phe29H, Phe33H, Met34H, Tyr55H, Tyr59H, Arg94H, Tyr98H, Phe99H and Tyr102H, most of which are located in the V H domain (Fig. 5b). On the basis of these data, we concluded that binding to the CPD-flanking nucleotides is predominantly by the V H domain.

Calculation of the surface electrostatic potential of the variable region of TDM-2 Fab indicates that the V H domain is positively charged (Fig. 5c). Taking into account the fact that interactions between TDM-2 Fab and CPD-containing oligodeoxynucleotides are significantly dependent upon the NaCl concentration (vide supra), it is conceivable that electrostatic interactions between the negatively charged phosphate groups of DNA and the positively charged V H domain of TDM-2 play major roles in complex formation.

In general, non-specific electrostatic interactions contribute significantly to binding affinities in DNA–protein interaction systems (31,32). In a model for CPD photolyase complexed with photodamaged DNA, ion pair interactions at the rim of the active site between positively charged residues on the enzyme surface and the CPD-flanking deoxyribose phosphates have been proposed (33). The contribution of electrostatic interactions has been also proved or postulated for a variety of DNA–anti-DNA antibody systems (19,34–39). Docking models of DNA and anti-DNA antibodies have thus far been based on distributions of basic amino acid residues or surface electrostatic potentials calculated for antibody-combining sites (40–42). However, it is difficult to predict an orientation of DNA bound to a protein surface solely on the basis of surface electrostatic potential.

Orientation of DNA in the antigen-binding site

In order to obtain structural information on the antigen-binding site of TDM-2 in a more quantitative way, we carried out NMR analyses by use of spin-labeled antigen analogs. It has been well established that spin-labeled ligands are useful for mapping binding sites of proteins, including Fab (43–44) and ssDNA-binding protein (45). In most previous studies, the spin-labeled ligands were designed only so as to identify the binding site itself and utilized for that purpose. Recently, Ramos and Varani have demonstrated that spin-labeled RNAs are useful for detecting long-range protein–RNA contacts (46). In the present study, we have attempted to determine the orientation of a CPD-containing oligodeoxynucleotide with respect to the antigen-binding site of TDM-2, using a pair of

![Figure 4](image-url)
spin-labeled antigen analogs in which TEMPO is covalently attached to either the 3′- or 5′-end of d(T[c,s]T) via a phosphor-thioate bond (Fig. 6). Hereafter, these spin-labeled oligodeoxynucleotides will be designated 3′-TEMPO-d(AT[c,s]T) and 5′-TEMPO-d(T[c,s]TA), respectively. It is expected that HSQC peaks originating from residues located in spatial proximity to the unpaired electron in the complex exhibit line broadening. By using the two types of spin-labeled antigen, residues located near the 5′-side and 3′-side flanking nucleotides could be separately identified and therefore the orientation of the oligodeoxynucleotide with respect to the antigen-binding site would be easily determined.

Figure 7a and b shows the 1H-15N HSQC spectra of TDM-2 Fab labeled with [15N]Phe and [15N]Tyr in the presence of 3′-TEMPO-d(AT[c,s]T) and 5′-TEMPO-d(T[c,s]TA), respectively. Assignments of the resonances that appear upon addition of ascorbic acid are indicated in the spectra. Similar experiments were made using TDM-2 analogs selectively labeled with 15N at the amide groups of Ile, Val, His, Met, Trp, Ala or Arg. The resonances from Trp91L, Tyr94L, Tyr55H and Tyr59H were affected by 5′-TEMPO-d(T[c,s]TA), whereas those from Phe29H, Phe33H, Tyr98H, Phe99H and Tyr102H were affected by 3′-TEMPO-d(AT[c,s]T). It was confirmed that the spectra of TDM-2 Fab complexed with the spin-labeled analogs (under reducing conditions) were virtually identical to those of TDM-2 Fab complexed with d(T[c,s]T), except that slight chemical shift changes were observed for the residues spatially close to the TEMPO moieties (data not shown). Therefore, these results reveal that the DNA orientation in the complex is not affected by attachment of the TEMPO moiety.

The residues perturbed by 3′-TEMPO-d(AT[c,s]T) and 5′-TEMPO-d(T[c,s]TA) were mapped on the model of TDM-2 Fv (Fig. 8). Although the 31P NMR data suggest the existence of a conformational exchange of the CPD-flanking nucleotides on the protein surface (vide supra), the residues that exhibit line broadening upon binding to each of the spin-labeled antigens are clustered in a specific area in the V_{H} domain, indicating that the orientations of CPD-containing nucleotides are fairly fixed in the antibody-combining site: both the 3′- and 5′-sides of the flanking deoxynucleotides orient towards the V_{H} domain, as predicted from the chemical shift perturbation data. It should also be noted that completely different sets of residues were perturbed by 3′-TEMPO-d(AT[c,s]T) and 5′-TEMPO-d(T[c,s]TA). On the basis of these data, along with the fact that d(T[c,s]T) binds the central parts of the antibody-combining site, we concluded that CPD-containing oligonucleotides bind TDM-2 in a crooked form (a boomerang shape), draping the 3′-side nucleotides onto the H1 and H3 segments.
and the 5'‐side nucleotides onto the H2 and L3 segments. The orientation of DNA determined in the present study is obviously different from that observed in the crystal structure of the Fab of the anti‐ssDNA auto‐antibody BV04‐01 complexed with a trinucleotide of deoxythymidylic acid, d(pT)3, which binds at the VL/VH interface, leaving the 5'‐end on the H1 and H2 segments and the 3'‐end on the L1 and L3 segments (47).

The information provided by the present NMR analyses is invaluable, especially for engineering of the regions responsible for binding to the CPD‐flanking nucleotides. For example, the affinity of TDM‐2 for CPD‐containing DNA could be improved by introducing positive charges into these regions. By use of the single chain Fv of TDM‐2 we have found that amino acid substitution of Tyr 98H by Lys, which binds the 3'‐side flanking nucleotide, results in a higher degree of affinity for d(AT[cs]T) (manuscript in preparation). It is also possible that antibodies that specifically cleave CPD‐containing DNA could be produced by attaching a DNA‐cleaving reagent to the nucleotide‐binding sites identified in the present study. Finally, the NMR methodology developed in the present study using spin‐labeled DNA analogs will be extremely useful to determine the orientation of DNA with respect to DNA‐binding proteins in general.

SUPPLEMENTARY MATERIAL
See Supplementary Material available at NAR Online.

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