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**GENES: STRUCTURE AND  
REGULATION:**  
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# Generation of Sequence-specific, High Affinity Anti-DNA Antibodies\*

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By taking advantage of the extreme stability of a protein-DNA complex, we have obtained two highly specific monoclonal antibodies against a predetermined palindromic DNA sequence corresponding to the binding site of the E2 transcriptional regulator of the human papillomavirus (HPV-16). The purified univalent antibody fragments bind to a double-stranded DNA oligonucleotide corresponding to the E2 binding site in solution with dissociation constants in the low and subnanomolar range. This affinity matches that of the natural DNA binding domain and is severalfold higher than the affinity of a homologous bovine E2 C-terminal domain (BPV-1) for the same DNA. These antibodies discriminate effectively among a number of double- and single-stranded synthetic DNAs with factors ranging from 125- to 20,000-fold the dissociation constant of the specific DNA sequence used in the immunogenic protein-DNA complex. Moreover, they are capable of fine specificity tuning, since they both bind less tightly to another HPV-16 E2 binding site, differing in only 1 base pair in a noncontact flexible region. Beyond the relevance of obtaining a specific anti-DNA response, these results provide a first glance at how DNA as an antigen is recognized specifically by an antibody. The accuracy of the spectroscopic method used for the binding analysis suggests that a detailed mechanistic analysis is attainable.

Unveiling the molecular rules for protein-DNA recognition is a necessary step for the understanding of gene function and regulation. A large number of proteins and cognate DNA sequences displaying a large variety of natural structures and recognition modes have been and are being identified as involved in physiological and pathological mechanisms (1, 2). In addition, the ability to design new DNA binding activities constitutes a major scientific challenge with technological applications such as control of gene function, gene therapy, genome research, and diagnostics.

Antibodies that bind to DNA are a hallmark of the autoimmune disease in systemic lupus erythematosus (3), but these are not specific to particular sequences of single- or double-stranded DNA, or at least the putative specific sequences that elicit them have not been yet identified. Although a number of natural anti-DNA antibodies have been described, DNA is

known to be a poor immunogen (4), and it has been virtually impossible to generate antibodies against a specific DNA sequence to date. DNA binding antibodies were obtained using phage display technology, but these bound to repetitive, non-specific sequences (5). A chimeric sequence-specific DNA binding antibody was engineered by incorporating the DNA binding domain of a transcription factor into the CDR3 of the heavy chain (HCDR3) from a recombinant Fab molecule (6). This elegant engineering approach can be further exploited through antibody display in phage, but it cannot take advantage of the natural diversity of antibody repertoires. Other approaches for obtaining novel DNA binding activities arise from the combination of phage display technology in a zinc finger framework (7); some of these chimeras bind DNA in the low nanomolar range as judged by electrophoretic methods (8). A major goal for generating sequence specific anti-DNA activities is the intracellular expression of the resulting protein for control of gene function (9).

Can antibodies be raised against specific and predetermined DNA sequences? If so, how might such antibodies interact with the DNA compared with natural DNA binders? In an attempt to answer these questions, we used a natural protein-DNA complex, the DNA binding domain of the human papillomavirus E2 protein (E2C) bound to its target DNA (10), as immunogen. This domain shows a particular dimeric  $\beta$ -barrel topology consisting of an eight-stranded (four per subunit)  $\beta$ -barrel, with major DNA binding and minor  $\alpha$ -helices, packed against opposite sides of the barrel (11). Based on the large stability of the E2C-DNA complex (12), we used it as the antigen to produce a specific anti-DNA response against this key viral regulatory DNA binding site. We characterize two newly obtained sequence-specific anti-DNA antibodies (a-DNABs)<sup>1</sup> using an accurate spectroscopic method in solution.

## MATERIALS AND METHODS

**Anti-DNA Monoclonal Antibodies**—The C-terminal DNA binding domain of the E2 protein from human papillomavirus strain-16 was expressed in BL21-DE3 *Escherichia coli* cells as described previously (13). The sequence of the A chain of the synthetic oligonucleotide is 5'-GTAACCGAAATCGGTTGA-3', corresponding to E2 site 35 in the HPV-16 genome, where the underlined sequence indicates the consensus E2 binding site. A protein-DNA complex was formed by mixing E2C and DNA at a 1:1 ratio. Details of the immunization will be described elsewhere. Briefly, BALB/c mice were immunized intraperitoneally with 25  $\mu$ g of protein-DNA 1:1 complex emulsified in MPL® + TDM Adjuvant System (Sigma). Second and third boosters were administered intraperitoneally at 20-day intervals using similar doses. The immune response elicited was monitored by indirect ELISA using the 18-bp oligonucleotide and the protein-DNA complexes as antigens, as

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<sup>1</sup> The abbreviations used are: a-DNAB(s), anti-DNA antibody(s); bp, base pair(s); ELISA, enzyme-linked immunosorbent assay; MOPS, morpholinepropanesulfonic acid; EMSA, electrophoretic mobility shift assay; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate.

described below. Final boosting involved an intraperitoneal injection of 20  $\mu$ g of protein-DNA 1:1 complex dissolved in TBS (25 mM Tris, 150 mM NaCl, pH 7.4) 4 days prior to the somatic cell hybridization. Splenocytes obtained from the immunized mouse with the highest anti-DNA titer were fused with a NSO mouse plasmacytoma cell line following established techniques (14). For the selection of anti-DNA antibodies we used the site 35 double-stranded oligonucleotide as antigen (1  $\mu$ g/well in TBS). Of 92 culture supernatants screened after the fusion, eight displayed anti-site 35 DNA reactivity. Two hybridomas producing anti-DNA monoclonal antibodies (ED-10 and ED-84) were expanded and isotyped as IgG1 by ELISA. Ascites fluids were produced and IgGs and their derived Fab fragments prepared following standard procedures (15). An additional gel chromatography step was included to ensure that only univalent and highly purified Fab fragments were present. The variable regions of both antibodies were cloned (16) and according to their  $V_H$  sequences, they belong to the J558 superfamily, shared by several pathogenic autoimmune anti-DNA antibodies (17) (Fig. 1B).

**Electrophoretic Mobility Assays**—A site 35 DNA solution of 3  $\mu$ M was incubated with the different proteins at different ratios, and the concentrations were determined from the respective molar extinction coefficients. A 6% acrylamide native gel was run in 0.1 M MOPS/Imidazol, pH 6.5, at 5 V  $\text{cm}^{-1}$  and the bands visualized by standard ethidium bromide staining. Overall, the EMSA experiments in our simplified buffer conditions (0.2 M NaCl, 25 mM Bis-Tris HCl, pH 7.0, 1 mM dithiothreitol, and 10% glycerol for loading) appear to be dissociating, and relative affinities cannot be inferred from these type of experiments (18). Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA).

**Quantitative ELISA Binding**—A quantitative ELISA assay for the determination of the  $K_D$  was carried out as described previously (19). Briefly, ELISA plates were coated with streptavidin (1  $\mu$ g/well in TBS) for 60 min. After blocking with 1% BSA in TBS (BSA/TBS), 5 ng/well of a 5'-biotinylated site 35 DNA in BSA/TBS was added to the plate and further incubated for 15 min. ED-10 or ED-84 IgGs were incubated at 0.8 nM concentration with different concentrations of site 35 oligonucleotide solution in BSA/TBS. The 300- $\mu$ l sample was incubated in microcentrifuge tubes for 30 min at room temperature. The entire mixture was transferred to the biotinylated DNA-coated ELISA plates for 60 min and subsequently washed three times with TBS. The retention of a-DNAb IgGs in the solid phase, representing the degree of binding to the biotinylated specific DNA, was developed using an anti-IgG peroxidase-conjugated polyclonal antibody. For each separate tube, the same mixture was incubated in the absence of biotinylated oligonucleotide as a blank. The resulting  $A$  at 492 nm value was used to calculate the antibody bound fraction,  $v$ . The data were linearized according to Klotz:  $1/v$  versus  $1/Ag$  concentration (19).  $K_D$  values were obtained from the slope of the plot.

**Fluorescence Binding Experiments**—Fluorescence spectra were carried out using an Aminco Bowman Series 2 instrument. Excitation wavelength was fixed at 295 nm and the emission registered from 310 to 410 nm. Buffer base lines were subtracted. Tryptophan fluorescence titrations were carried out with excitation at 295 nm, monitoring the emission at 340 nm. The proteins were incubated in TBS in 3.0 ml volumes at  $25 \pm 0.1$   $^{\circ}\text{C}$ , with protein concentration ranging from 5 to 100 nM, depending on the sequence used (see Table I and Fig. 4). DNA oligonucleotides were gradually added, and the tryptophan fluorescence was measured after 5 min equilibration at each point. The maximum dilution was 10%, and the fluorescence was corrected accordingly. The data were fitted to a simple binding model where both protein and DNA concentrations are considered (20).

## RESULTS

**Generation of High Affinity Anti-DNA Antibodies**—Using the HPV16-E2C-site 35 DNA complex as immunogen, we obtained a set of monoclonal antibodies against the E2C protein as well as antibodies that reacted against the free or E2C bound DNA but not with the protein (Fig. 1 and see "Materials and Methods"). We focused our studies on two selected a-DNABs, ED-10 and ED-84, which showed reactivity in ELISA assays toward the HPV-16 site 35 double-stranded DNA oligonucleotide either free or bound to the HPV-16 E2C domain (Fig. 1A). These antibodies also recognize the DNA when bound to the bovine (BPV-1) E2C domain.

We cloned the variable regions of the two a-DNABs antibodies, and the amino acid sequence analysis indicates hypermu-

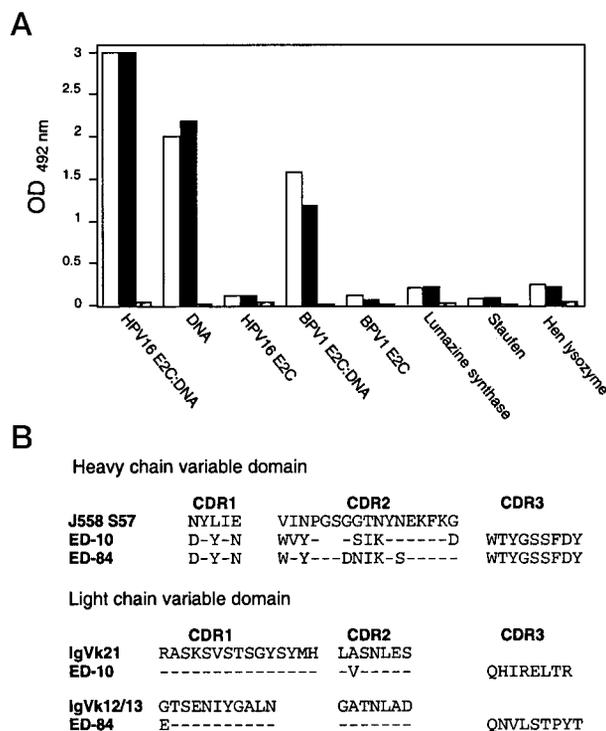
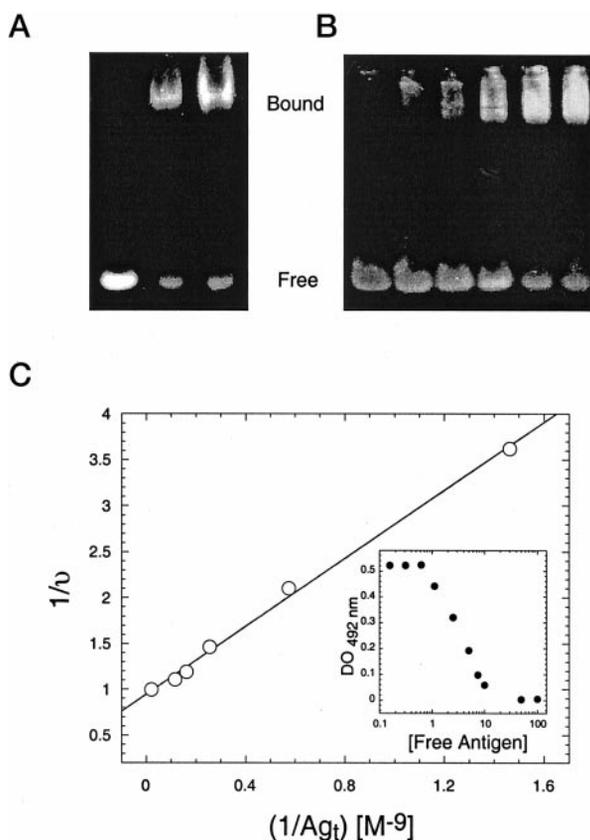


FIG. 1. Characterization of two anti-DNA monoclonal antibodies. A, ELISA reactivity of ED-10 (open bars) and ED-84 (filled bars) anti-DNA IgGs toward the different antigens, where DNA refers to E2 binding site 35 in HPV-16 genome (see above). Serum from a non-immunized mouse was used as a negative control (shaded bars). The anti-DNA IgGs did not react toward a panel of control protein antigens, and none of the specific free or E2C-bound DNA antigens were recognized by a control antibody. B, comparison of the amino acid sequences of the  $V_H$  and  $V_L$  CDR regions of the newly obtained a-DNABs with their corresponding germ line sequences (17, 25, 26).  $V_H$  germ line sequences analysis indicates they belong to the J558 family, used by several pathogenic autoimmune anti-DNA antibodies (17). They present a total HCDR3 homology and hypermutation, suggesting a marked antigen-driven selection.

tation in the  $V_H$  domains. In addition, the surprisingly identical CDR3s of the  $V_H$  domain suggest a strong antigen-driven selection (Fig. 1B). These CDR3s show no sequence homology to any antibody described in data banks. Contrary to previously reported for germ line gene J558-derived anti-DNA antibodies, there is not an evident predominance of positively charged residues in the a-DNABs as the main source for binding affinity as discussed elsewhere (21). Both a-DNABs have different  $V_L$  chains, explaining at least in part the fine differences in specificity.

**Analysis of DNA Binding by the Antibodies**—The interaction of the a-DNABs with the E2 site 35 oligonucleotide was preliminary analyzed by an EMSA. No shift, and therefore no detectable binding, appeared when the EMSA was performed using a radioactively phosphorylated oligonucleotide (not shown). Thus, we used an unmodified oligonucleotide and developed the bands using ethidium bromide staining. Fig. 2A shows the result of such a binding experiment where the two Fabs, ED-10 and ED-84, produced a band shift as expected from the initial ELISA assays. The absence of a band shift using radioactively phosphorylated oligonucleotide strongly suggest that DNA binding by both antibodies is sensitive to the phosphorylation at the 5' terminus of the oligonucleotide (see below). Incremental additions of a-DNAB to the free DNA produce a gradual band shift to the antibody-bound form as shown in Fig. 2B. However, no reliable quantitative binding analysis would be possible (see "Materials and Methods").

The binding was quantitatively analyzed by competition

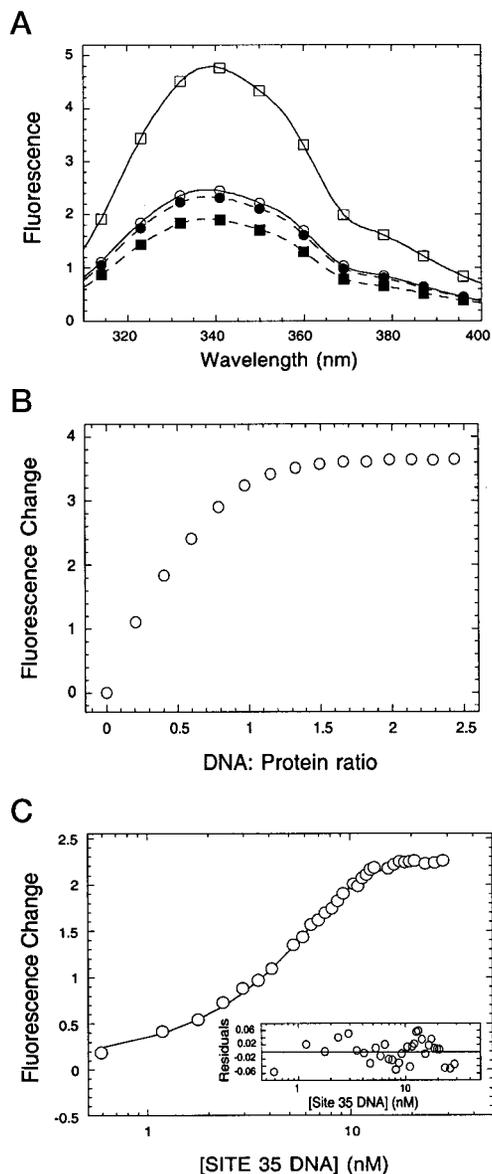


**FIG. 2. Binding of ED-10 and ED-84 to site 35 DNA.** *A*, electrophoretic mobility shift assay. Site 35 DNA at  $3 \mu\text{M}$  (*Free*, first lane) was incubated with 4-fold either ED-10 (*second lane*) or ED-84 Fabs (*third lane*) for 30 min at room temperature as described under 'Materials and Methods,' prior to gel loading. *B*, same as *A*. Site 35 DNA at  $3 \mu\text{M}$  was incubated with increasing amounts of a-DNABs ( $0$ – $12 \mu\text{M}$ ). ED-10 was shown as an example. Running conditions and gel staining are explained under 'Materials and Methods.' *C*, quantitative ELISA binding experiment. The binding of the a-DNAB IgGs ( $1.6 \text{ nm}$  per site) to the DNA-coated plate was prevented by incubation with increasing amounts of free site 35 DNA, and the resulting optical density decrease is a measure of the binding (*inset*). The data were transformed and plotted as  $1/\nu$  against  $1/\text{Ag}$  (Klotz), and the  $K_D$  was obtained from the slope, after linear regression analysis (see 'Materials and Methods').

ELISA in solution (19) and yield dissociation constants of  $1.85 \pm 0.05$  and  $1.69 \pm 0.18 \text{ nm}$  for ED-10 and ED-84, respectively (Fig. 2C). This is the first strong indication that the affinity of both antibodies for the E2 site 35 DNA is very high. Nevertheless, we wanted to test a spectroscopic method that would confirm the tight binding and provide the highest accuracy in solution.

The tryptophan fluorescence of the purified Fabs is quenched by 30–40% upon binding to the DNA oligonucleotide, depending on the antibody (Fig. 3A). The DNA does not quench the spectrum of a control Fab that recognizes the E2C protein (Fig. 3A). We carried out equilibrium titration experiments in solution monitored by tryptophan fluorescence. Titration of site 35 double-stranded DNA with ED-10 and ED-84 Fabs confirms high affinity-saturable binding and a clear 1:1 stoichiometry, as expected from univalent Fabs (Fig. 3B).

For an accurate determination of the dissociation constants ( $K_D$ ) for DNA binding, we carried out tryptophan fluorescence binding experiments at near-dissociation conditions for both antibodies. A typical DNA binding experiment for ED-10 is shown in Fig. 3C. Such analysis yielded dissociation constants of  $0.73 \pm 0.07$  and  $2.5 \pm 0.4 \text{ nm}$  for ED-10 and ED-84, respectively (Table I). The affinity of the same site 35 DNA oligonucleotide for the E2C domain, the natural partner, is  $0.2 \text{ nm}$  (18).



**FIG. 3. DNA binding by fluorescence spectroscopy.** *A*, fluorescence spectral change upon binding of DNA. Proteins were incubated at  $2 \mu\text{M}$  concentration in TBS buffer, pH 7.4, and the spectra were recorded prior and after addition of  $2.5 \mu\text{M}$  site 35 DNA. The spectra of ED-84 Fab were shown as an example, no addition (*open squares*), plus DNA (*closed squares*). As a control, we used an anti-E2C Fab, ED-5: *open circles*, no addition; *closed circles*, plus DNA. *B*, a stoichiometric titration was carried out for both a-DNABs (ED-10 shown in the figure), at  $200 \text{ nm}$  protein concentration, monitoring fluorescence upon addition of site 35 DNA, as described under 'Materials and Methods.' *C*, the dissociation constants,  $K_D$ , were determined in titration experiments at near-dissociation conditions. The figure shows the titration of ED-10 Fab, at  $5 \text{ nm}$  concentration. The data were fitted as described under 'Materials and Methods,' and the residuals are shown (*inset*). The binding data of both antibodies to all the sequences tested are shown in Table I.

**Sequence Discrimination**—The untranslated regulatory region of the HPV16 genome contains three similar, but not identical, high affinity E2 binding sites. To test whether our anti-site 35 DNA antibodies were capable of fine specificity tuning as the E2C domain, we analyzed the binding of the a-DNABs to another natural E2 binding site, site 7450 (Fig. 4A). Both antibodies are able to discriminate HPV-16 E2 site 7450 from site 35 with 2–3-fold lower affinity (Table I).

In fluorescence binding experiments, phosphorylation of site 35-18 bp produced an increase in  $K_D$  of 20- and 50-fold for ED-10 and ED-84 Fabs, respectively (Table I), in agreement

TABLE I  
Sequence discrimination and affinity of a-DNABs ED-10 and ED-84

The dissociation constants were determined as described in the legend to Fig. 3C.

DNA	ED-10			ED-84		
	$K_D$	$D^a$	$\Delta\Delta G^b$	$K_D$	$D$	$\Delta\Delta G$
	<i>nM</i>		<i>kcal mol<sup>-1</sup></i>	<i>nM</i>		<i>kcal mol<sup>-1</sup></i>
dsDNA <sup>c</sup>						
Site 35–18 bp	0.73 ± 0.07	1	0	2.52 ± 0.38	1	0
Site 35–26 bp	6.7 ± 0.7	9.1	1.3	4.9 ± 0.7	2.0	0.4
Site 35–18 bp-P	13.7 ± 1.1	19	1.7	122 ± 11	48	2.3
Site 7450–18 bp	2.1 ± 1.1	2.9	0.6	5.6 ± 1.6	2.2	0.5
7450–36 bp-FITC	5.7 ± 1.6	7.8	1.2	3.31 ± 1.8	1.3	0.2
ARC	158 ± 0.3	217	3.1	344 ± 0.6	136	2.9
CRE	208 ± 0.2	285	3.2	316 ± 0.3	125	2.8
EBNA	973 ± 150	1333	4.2	1,200 ± 153	476	3.6
V <sub>H</sub> mouse	<sup>d</sup>			1,526 ± 244	605	3.8
HPV-16 2300	265 ± 0.3	362	3.4	444 ± 0.4	176	2.5
ssDNA						
Site 35	15,100 ± 8757	20561	5.8	7,570 ± 2327	3004	4.7
ARC	895 ± 109	1226	4.2	2,957 ± 757	1173	4.2
HPV-16 2300	579 ± 0.7	794	3.9	1,074 ± 145	426	3.6

<sup>a</sup> The discrimination factor is the ratio  $K_{D, \text{non-specific}}/K_{D, \text{site 35-18}}$ .

<sup>b</sup>  $\Delta G = -RT \ln K$ ;  $\Delta\Delta G = \Delta G_{\text{site 35-18}} - \Delta G_{\text{DNAB}}$ .

<sup>c</sup> See legend of Figure 4A for details.

<sup>d</sup> This DNA displayed a very low affinity interaction, too difficult to fit to a single binding event.

## A

site 35-18bp:	5'-GTAACCGAAATCGGTTGA-3'
site 35-26bp:	5'-AGCTTCAACCGAAATCGGTTGCATGC-3'
site 7450-18bp:	5'-GTAACCGAAATCGGTTGA-3'
site 7450: 36bp-FITC	5'-TTGTAGCTTCAACCGAAATCGGTTGCATGCTTTT-3'
ARC:	5'-ATGATAGAAGCACTCTACTAT-3'
CRE:	5'-AAATGACGTCATGGTAA-3'
EBNA:	5'-GGGTAGCATATGCTACCC-3'
VH mouse:	5'-GCTACTGGCTACACATTC-3'
HPV-16 2300:	5'-GCTAACACAGGTAATCA-3'

## B

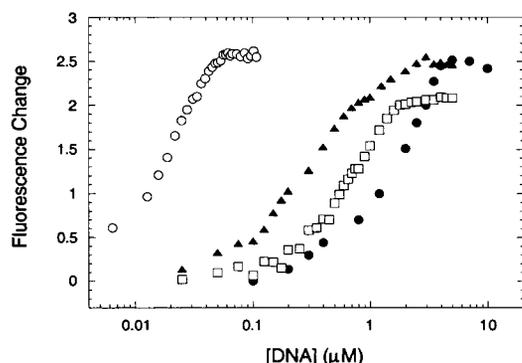


FIG. 4. Sequence discrimination by the a-DNABs. A, DNA sequences of various oligonucleotides tested for binding. Site 35, HPV-16 E2 site; site 7450, HPV-16 E2 site; ARC, the specific operator sequence for the arc repressor; CRE, cyclic AMP-responsive element; EBNA, Epstein-Barr nuclear antigen binding site; V<sub>H</sub> mouse, a randomly selected mouse V<sub>H</sub> sequence; HPV-16 2300, an internal DNA sequence of the HPV-16 genome. B, logarithmic plot of the binding of ED-84 Fab to site 35 (open circles), single strand site 35 (closed circles), cyclic AMP-responsive element (CRE) (closed triangles), V<sub>H</sub> mouse (open squares).

with preliminary EMSA experiments using radioactively phosphorylated site 35. Since the phosphorylation at the 5'-OH affects the binding of the a-DNABs to the 18-bp site, we tested a longer double-stranded oligonucleotide containing the E2 site 35. Table I shows that a 26-mer (site 35-26 bp) still binds with high affinity, albeit lower than the 18-bp site by 1.3 and 0.4

$\text{kcal mol}^{-1}$ , respectively, for each a-DNAB. The binding appears to be weakened to a lesser extent when compared with phosphorylated site 35-18 bp. In addition, we analyzed the binding of the a-DNABs to a 36-bp site containing the other natural E2 site (7450), with both 5'-OH ends modified with fluorescein (FITC). Using tryptophan fluorescence quenching, we determined that the binding affinities are marginally modified with respect to site 7450, marking a difference with the site 35 DNA, which was used as immunogen. FITC fluorescence intensity and anisotropy of site 7450-36 bp-FITC remain completely unchanged after addition of a-DNABs (not shown), suggesting that in longer DNA stretches, the 5'-OH is not involved in recognition.

We determined the binding properties of purified Fab fragments corresponding to both a-DNABs to a set of different palindromic and nonpalindromic double-stranded DNA oligonucleotides of similar length (Fig. 4A). The affinities for most sequences tested were markedly lower than the target sequence, with discrimination factors ( $K_{D, \text{non-specific}}/K_{D, \text{site 35}}$ ) ranging from 125 to 20,000 (Fig. 4B and Table I). ED-84 binds with 3-fold less affinity for specific DNA, but binds to most nonspecific double- and single-stranded DNAs with higher affinity than ED-10. This suggests that the slightly lower affinity of ED-84 for the specific DNA could eventually be linked to a lower capacity of sequence discrimination ranging from 2- to 7-fold depending on the DNA sequence, evidenced in the ratio of discrimination factors ( $D_{\text{ED-10}}/D_{\text{ED-84}}$ , see legend for Table I).

The discrimination factor for single-stranded site 35 DNA was 20,500 and 3,000 for ED-10 and ED-84, respectively. Binding affinity for two nonspecific single-stranded DNAs was noticeably higher, but since it is essentially a nonspecific interaction, we shall await detailed structural information to fully understand the molecular basis of this phenomenon. In any case, it should be pointed out that discrimination between single- and double-stranded DNA in natural antibodies found in autoimmune disease appears to be at the level of backbone recognition (22), *i.e.* conformational, while the type of interaction we are now describing is sequence-specific.

## DISCUSSION

We have shown that it is possible to generate antibodies against a desired and predetermined double-stranded DNA sequence. The strong anti-DNA response generated could be

the result of the high stability of the protein-DNA complex conferring a longer lifetime to the DNA, possibly enhanced by the high immunogenicity of the E2C protein. Further analysis of the immune response to the E2C-DNA complex will be required to fully understand the process.

The fluorescence spectroscopic method developed allowed us to evaluate the binding affinities with high accuracy and is therefore promising when aiming at a full characterization of the binding mechanism. Although the structural basis for this change cannot be unequivocally assigned, the HCDR3s contain a high proportion of aromatic residues as potential targets for fluorescence quenching. We have shown that a classical phosphorylation-based EMSA missed the binding event, as it was described for some protein-DNA interactions using filter assays (23). The binding was physically confirmed by ELISA and ethidium bromide-stained EMSA, which underestimate the binding by at least 1 order of magnitude in our simplified assay conditions (Fig. 2B and see "Materials and Methods").

The monoclonal antibodies obtained display high specificity and affinity approaching that of the natural DNA binding domain E2C from the human infecting high risk strain, HPV-16, determined in our laboratory by spectroscopic methods to be 0.2 nM (18). The affinity of the highly related bovine (BPV-1) E2C domain for the site 35 DNA oligonucleotide was recently determined in our laboratory in similar buffer conditions and was shown to be over 300-fold lower than the specific anti-site 35 DNA antibodies we now describe (18). On the other hand, the a-DNABs discriminate the HPV16 E2 binding site, 7450, from site 35, used to raise the antibodies. The two sites are almost identical, except for a single base change in a flexible noncontact region. These two results are a clear indication of the high affinity and sequence discrimination of the specific a-DNABs we describe, even for a minimal change in sequence.

The free 5'-OH in the specific oligonucleotide appears to participate in high affinity binding, but the difference in binding energies of phosphorylated species is well below the 3–5 kcal mol<sup>-1</sup> difference observed for nonspecific sequences. Moreover, the a-DNABs bind tightly to the specific sequence contained within longer DNA oligonucleotides and even to oligonucleotides with 5'-OH of both strands modified with FITC. Binding of a site 7450 oligonucleotide of 36 bp is as tight as the 18-bp site 7450, suggesting that modifications in the 5'-OH of shorter oligonucleotides would interfere with the binding. Without structural data, at this stage we can only speculate about the origin of the effect of 5' phosphorylation, which could in part be that it is recognized by the a-DNAB, but possible effects on the DNA conformation or stability, particularly in an 18-bp palindrome, cannot be ruled out. This could also lead to slow conformational changes that might affect the binding at equilibrium. All the evidence accumulated so far strongly suggests that the major determinant for binding is still the DNA base sequence.

The fact that the a-DNABs specifically bind to the E2 site 35 DNA oligonucleotide does not imply that they recognize the E2 consensus sequence, *i.e.* ACCGN<sub>4</sub>CGGT, in the same manner as the viral domains. The a-DNABs could well recognize part of the conserved consensus sequence and part of the flexible non-

contact region, as the lower binding affinity for a related site suggests. Alternatively, they may recognize bases outside the consensus sequence of the synthetic site 35 DNA oligonucleotide. The precise sequence binding requirements will be mapped using a variety of synthetic oligonucleotides or hopefully uncovered by detailed structural analysis. The palindromic nature of this oligonucleotide suggests a possible duplex-hairpin equilibrium in solution, but more evidence is needed to ascertain the precise recognition motif.

It is hardly possible that the DNA recognition mechanism by the a-DNABs is similar to the natural DNA binding domain. The latter binds the DNA at the surface, conferring a substantial bent to it (11), while antibody binding sites frequently display large flat and extended surfaces or deep cavities (24). Furthermore, given the evident lack of positive charges at the combining site of the antibodies, an electrostatic component as high as that found in natural DNA-binding proteins is also unlikely. Detailed atomic structures and thermodynamic binding analysis will shed light into this puzzling novel protein-DNA recognition interface.

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